Proenkephalin Mediates the Enduring Effects of Adolescent Cannabis Exposure Associated with Adult Opiate Vulnerability

Hilarie C. Tomasiewicz, Michelle M. Jacobs, Matthew B. Wilkinson, Steven P. Wilson, Eric J. Nestler, and Yasmin L. Hurd

Background: Marijuana use by teenagers often predates the use of harder drugs, but the neurobiological underpinnings of such vulnerability are unknown. Animal studies suggest enhanced heroin self-administration (SA) and dysregulation of the endogenous opioid system in the nucleus accumbens shell (NAcsh) of adults following adolescent Δ^9-tetrahydrocannabinol (THC) exposure. However, a causal link between proenkephalin (*Penk*) expression and vulnerability to heroin has yet to be established.

Methods: To investigate the functional significance of NAcsh *Penk* tone, selective viral-mediated knockdown and overexpression of *Penk* was performed, followed by analysis of subsequent heroin SA behavior. To determine whether adolescent THC exposure was associated with chromatin alteration, we analyzed levels of histone H3 methylation in the NAcsh via chromatin immunoprecipitation at five sites flanking the *Penk* gene transcription start site.

Results: Here we show that regulation of the *Penk* opioid neuropeptide gene in NAcsh directly regulates heroin SA behavior. Selective viral-mediated knockdown of *Penk* in striatopallidal neurons attenuates heroin SA in adolescent THC-exposed rats, whereas *Penk* overexpression potentiates heroin SA in THC-naïve rats. Furthermore, we report that adolescent THC exposure mediates *Penk* upregulation through reduction of histone H3 lysine 9 (H3K9) methylation in the NAcsh, thereby disrupting the normal developmental pattern of H3K9 methylation.

Conclusions: These data establish a direct association between THC-induced NAcsh *Penk* upregulation and heroin SA and indicate that epigenetic dysregulation of *Penk* underlies the long-term effects of THC.

Key Words: Drug addiction, epigenetics, marijuana, nucleus accumbens, rat, striatopallidal

Drug addiction is a chronic and relapsing disease that often begins during adolescence. Epidemiologic evidence documents an association between marijuana use during adolescence and subsequent abuse of drugs such as heroin and cocaine (1,2). Although many factors including societal pressures, family, culture, and drug availability can contribute to this apparent "gateway" association, little is known about the neurobiological basis underlying such potential vulnerability. Of the neural substrates that have been investigated, the enkephalinergic opioid system is consistently altered by developmental marijuana exposure (3–5), perhaps reflecting neuroanatomic interactions between cannabinoid receptor type 1 and the enkephalinergic system (6,7). Debates exist, however, regarding the relationship between proenkephalin (*Penk*) dysregulation and opiate susceptibility. We previously reported that adult rats exposed to Δ^9-tetrahydrocannabinol (THC; the primary psychoactive component of marijuana) during adolescence exhibit increased heroin self-administration (SA) as well as increased expression of *Penk*, the gene encoding the opioid neuropeptide enkephalin, in the nucleus accumbens shell (NAcsh), a mesolimbic structure critically involved in reward-related behaviors (3). Although these data suggest that increased NAcsh *Penk* expression and heroin SA behavior are independent consequences of adolescent THC exposure, they do not address a possible causal relationship between THC-induced *Penk* upregulation in NAcsh and enhanced behavioral susceptibility to opiates. Moreover, insights regarding the neurobiological mechanisms through which adolescent THC exposure maintains upregulation of *Penk* into adulthood remain unknown.

Here we take advantage of viral-mediated gene transfer strategies to show that adulthood addiction-like behaviors induced by adolescent THC exposure are dependent on discrete regulation of NAcsh *Penk* gene expression. A number of recent studies have demonstrated an important role for histone methylation in the regulation of drug-induced behaviors and transcriptional plasticity, particularly alteration of repressive histone H3 lysine 9 (H3K9) methylation at NAc gene promotors (8,9). We report here that one mechanism through which adolescent THC exposure may mediate *Penk* upregulation in adult NAcsh is through reduction of H3K9 di- and trimethylation, a functional consequence of which may be decreased transcriptional repression of *Penk*.

Methods and Materials

Animals and THC Treatment

Male 21-day-old Long Evans rats (Taconic, Hudson, New York) were used; procedures conducted in accordance with approved protocols. Rats received intraperitoneal injections of THC (1.5 mg/kg; RTI International, Durham, North Carolina) or vehicle (.9% sodium chloride with .3% Tween 80) every third day (eight injections) during adolescence (postnatal days 28–49) (3). For SA experiments, *Penk*- and green fluorescent protein (GFP)-infused rats were treated with vehicle during adolescence and GFP-, microRNA (miR) control

From the Fishburg Department of Neuroscience (HCT, MBW, EJN, YLH); Department of Psychiatry (HCT, MMJ, EJN, YLH); Department of Pharmacology and Systems Therapeutics (MMJ, EJN, YLH); Friedman Brain Institute (HCT, MMJ, MBW, EJN, YLH); Mount Sinai School of Medicine; James J. Peters VA Medical Center (YLH), New York, New York; and Department of Pharmacology (SPW), Physiology and Neuroscience, University of South Carolina School of Medicine, Columbia, South Carolina.

Address correspondence to Yasmin Hurd, Ph.D., Departments of Psychiatry, Pharmacology and Systems Therapeutics, and Neuroscience, Mount Sinai School of Medicine, New York, NY; E-mail: Yasmin.hurd@mssm.edu.

Received Jan 26, 2012; revised Apr 12, 2012; accepted Apr 12, 2012.

0006-3223/$36.00
http://dx.doi.org/10.1016/j.biopsych.2012.04.026

(ctrl)-, and miR *Penk*-infused rats were treated with THC during adolescence.

Lentiviral Vectors

Lentiviral vectors were constructed as described (Supplement 1). In all cases, in vivo transgene expression was validated and NAcsh-specific expression confirmed via in situ hybridization histochemistry.

Surgeries

Two weeks after final THC treatment, rats were anesthetized with isoflurane/oxygen (2.5%–4.5%) and bilaterally stereotaxically infused with .5 μL virus (*Penk* or GFP) or 1.0 μL virus (GFP, miR ctrl, miR *Penk*) into NAcsh (from bregma: anteroposterior +1.7 mm; mediolateral +2.3 mm; dorsoventral −6.8 mm [from dura], 10° from midline) at .1 μL/min. Two weeks subsequently, rats underwent surgery for jugular catheters (Brian Fromant, Cambridge, United Kingdom) for future SA experiments.

Heroin Self-Administration and Locomotor Activity

Heroin SA was conducted according to published protocols (3,5) (Supplement 1). Briefly, rats self-administered heroin (30 μg/kg/injection) 3-hour daily under a fixed-ratio 1 reinforcement schedule until stable baseline responding was established. Following stabilization, a between-session dose response was conducted (30/7.5/100/15/60 μg/kg/injection; 1 dose/day). After a 3-week abstinence period, cue-induced drug-seeking behavior was evaluated (depression of the drug-paired lever had no programmed consequence) followed 1 week later by mild stress (24-hour food deprivation)-induced drug seeking. Both tests were 1 hour. Activity was measured by infrared beams during the SA sessions.

In Situ Hybridization Histochemistry

In situ hybridization histochemistry was conducted according to published protocols (3,10) (Supplement 1). [^{35}S]-labeled *Penk* riboprobe (generated from a polymerase chain reaction [PCR]-derived complementary DNA (cDNA) fragment: bases 585–1140; Genbank accession: NM_017139) was applied to duplicate brain sections (2×10^3 cpm/mm^2), overnight 55°C hybridization, and sections exposed (imaging plate; Fujifilm, Tokyo, Japan) with ^{14}C standards for 28hr. Disintegrations-per-minute (dpm/mg) autoradiographic measurements were obtained for the NAc and averaged/animal.

Gene Expression Analysis

RNA was prepared from fresh-frozen bilateral NAcsh punches. cDNA was obtained using a first-strand synthesis kit (Quanta Biosciences, Gaithersburg, Maryland). Quantitative real-time PCR analysis was performed using Taqman-based probes (Applied Biosystems, Carlsbad, California; *Penk,* Rn00567566_m1; 18 S, 4319413E; *Pdyn*, Rn00571351_m1.); reactions run in triplicate, each gene run separately. Data normalized to eukaryotic 18S ribosomal RNA and analyzed via the ΔΔCT method (11).

Chromatin Immunoprecipitation

Fresh tissue was prepared for chromatin immunoprecipitation as previously described (8, 12) with minor modifications (Supplement 1). Briefly, two bilateral NAcsh punches/rat (three rats pooled per sample) were collected and processed. Immunoprecipitated samples (antibodies: H3K9me2, ab1220; H3K9me3, ab8898; H3K36me3, ab9050; H3K4me3, ab8580; abcam, Cambridge, Massachusetts) were subject to quantitative PCR (SYBR Green; Roche, Basel, Switzerland) and normalized to their non-immunoprecipitated input controls. Each reaction was run in triplicate and analyzed via the ΔΔCT method (11).

Statistics

We used two-tailed unpaired Student's *t* tests (for comparison of two groups), one-way analyses of variance (ANOVAs) followed by followed by Tukey's honest significant difference test or two-tailed Student's *t* tests when appropriate (for three groups), and two-way repeated-measures ANOVAs followed by one-way ANOVAs (to examine significant repeated-measure effects). All values represent mean ± SEM. Statistical calculations performed using JMP software (SAS, Cary, North Carolina).

Results

Penk Overexpression in NAcsh Increases Heroin Self-Administration

To investigate the direct role of NAcsh *Penk* tone in the regulation of heroin SA behavior, we first verified the effects of local overexpression of *Penk* by infusing a lentiviral vector encoding *Penk* and GFP into NAcsh of adult rats (Figure 1A, B). Viral spread was 1 mm^3 from the needle tip.

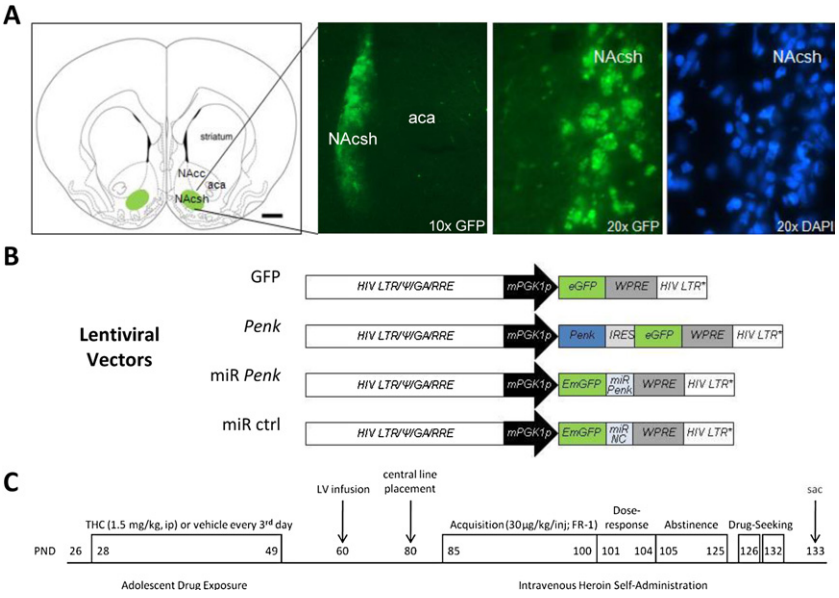

Figure 1. Lentivirus-mediated *Penk* gene manipulation and heroin self-administration. **(A)** Green fluorescent protein (GFP) expression is restricted to nucleus accumbens shell (NAcsh; reprinted from Paxinos and Watson [34] with permission from Elsevier, copyright 2007). **(B)** Lentiviral vectors. **(C)** Behavioral research design. Ψ, encapsidation signal including the 5′ portion of the gag gene (GA); aca, anterior commissure; DAPI, 4′,6-diamidino-2-phenylindole; eGFP, enhanced green fluorescent protein; EmGFP, emerald green fluorescent protein; HIV LTR, human immunodeficiency virus long terminal repeat; IRES, encephalomyocarditis virus internal ribosome entry site; LTR*, LTR with deletion in the U3 region; LTR, long terminal repeat; LV, lentiviral vector; miR NC, microRNA targeting nonvertebrate gene (Invitrogen, Carlsbad, California); miR ctrl, microRNA control; miR *Penk,* miRNA targeting nucleotides 709-729 of rat *Penk* coding region; mPGK1p, mouse phosphoglycerate kinase-1 promoter; NAcc, nucleus accumbens core; *Penk,* 956 nucleotide fragment containing the coding region of the rat *Penk* cDNA; PND, postnatal day; RRE, Rev-responsive element; sac, sacrifice; WPRE, woodchuck post-transcriptional regulatory element. See also Table S1 and Figure S2 in Supplement 1.

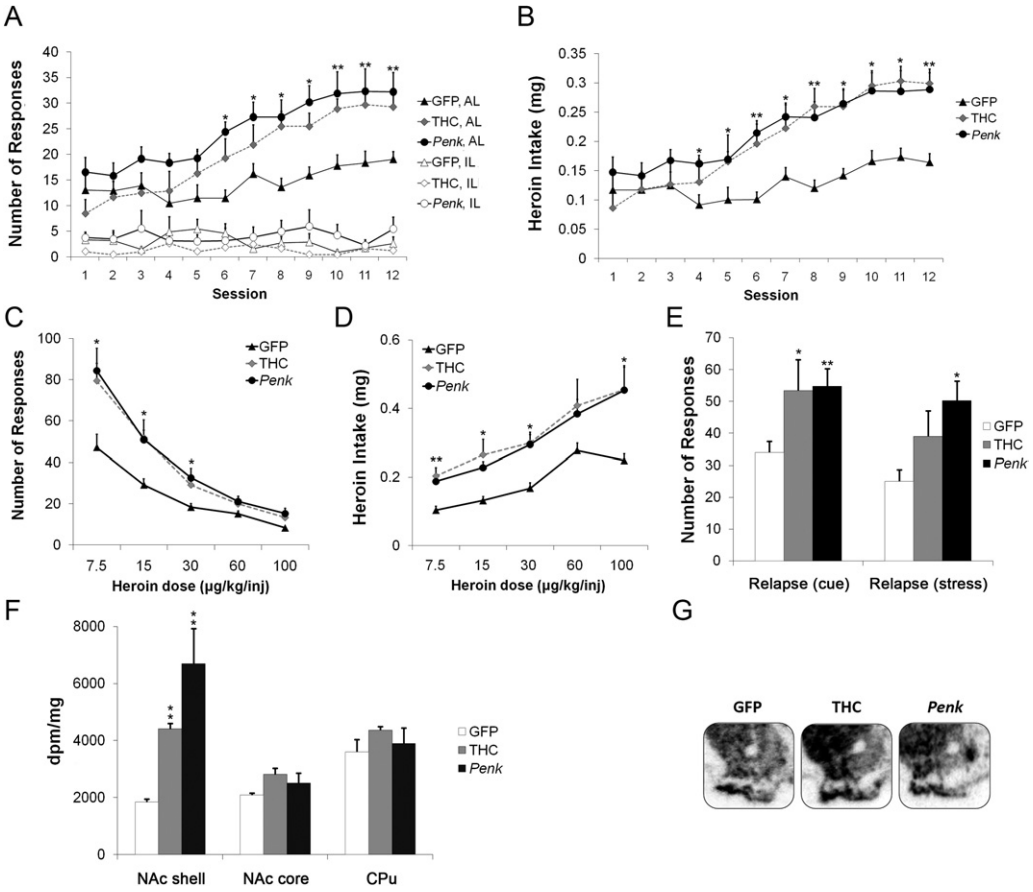

Figure 2. *Penk* overexpression in nucleus accumbens shell (NAcsh) potentiates heroin self-administration. **(A)** Acquisition of heroin self-administration (SA; fixed-ratio 1, 30 μg/kg/injection). **(B)** Mean heroin intake. **(C)** Between-session dose-response (7.5, 15, 30, 60, 100 μg/kg/infusion; randomized order). **(D)** Mean heroin intake. **(E)** Heroin-seeking behavior (cue- and stress-induced) in *Penk*-infused, green fluorescent protein (GFP)-infused, or Δ^9-tetrahydrocannabinol (THC)-exposed rats. **(F)** *Penk* mRNA levels in NAcsh, nucleus accumbens (NAc) core, and caudate-putamen (CPu) following heroin SA. **(G)** Representative in situ hybridization autoradiograms demonstrating striatal *Penk* mRNA expression following heroin SA. For all figures, $n = 5$–9 per group. Data shown as mean ± SEM. $^*p < .05$; $^{**}p < .01$ compared with GFP-expressing controls for each session. AL, active lever; IL, inactive lever, dpm, disintegrations per minute. See also Figure S1A in Supplement 1.

To investigate the functional significance of enhanced NAcsh *Penk* tone, we tested the involvement of increased NAcsh *Penk* expression on a fixed-ratio 1 schedule of heroin SA. Two cohorts of animals were treated with vehicle during adolescence and then given bilateral NAcsh infusions of lentivirus vectors expressing *Penk* or GFP in young adulthood. A third cohort of rats was treated with THC during adolescence (Figure 1C; Table S1 in Supplement 1). Bilateral NAcsh infusions of *Penk* enhanced responding for heroin [treatment by session interaction, $F(22,213) = 2.036$, $p < .01$, Figure 2A] and mean heroin intake [treatment by session interaction, $F(22,213) = 2.589$, $p < .001$, Figure 2B], without modifying inactive lever pressing or locomotor activity (Figure S1A in Supplement 1), compared with GFP-infused controls. Consistent with previous data (3), animals treated with THC during adolescence also exhibited increased heroin SA compared to control conditions (GFP-infusion), an effect similar in magnitude to that resulting from NAcsh *Penk* overexpression (Figure 2A, B). Animals treated with THC during adolescence also exhibited increased heroin SA compared with vehicle-exposed "sham" animals that had undergone bilateral NAcsh infusion with saline (Figure S2 in Supplement 1). Following stabilization of heroin SA behavior, we next examined whether NAcsh *Penk* overexpression affected dose-dependent responding for heroin in a between-session dose response test. *Penk* overexpression and adolescent THC treatment led to upward vertical shifts in the heroin dose-response function, including higher peak SA rates on the descending limb of the dose-response curve [treatment by dose interaction, $F(8,74) = 3.859$, $p < .001$, Figure 2C] and higher drug intake across the range of doses studied (Figure 2D).

Penk Overexpression in NAcsh Promotes Enhanced Heroin Seeking

To determine whether NAcsh *Penk* infusion facilitated subsequent behavioral susceptibility to relapse, we measured heroin-seeking behavior following abstinence. In light of self-reports by drug-dependent individuals indicating that exposure to drug-associated stimuli and stress precipitate drug craving and relapse (13–15), we first assessed heroin-seeking behavior under cue-induced reinstatement conditions. The magnitude of heroin-seeking behavior in the absence of reinforcement was assessed by the amount of responding at the previously drug-paired lever. Adolescent THC exposure and NAcsh *Penk* overexpression led to an increase in drug-paired lever responding [$F(2,20) = 3.859$, $p < .05$, Figure 2E] compared with GFP-infused controls, indicating that prior NAcsh *Penk* infusion enhanced the ability of the drug-associated environment to elicit drug-seeking behavior. We next examined whether NAcsh *Penk* infusion enhanced drug-seeking behavior triggered by

exposure to a stressor previously shown to increase heroin seeking (4). One week after the first drug-seeking test, we evaluated drug-seeking behavior following 24-hour food deprivation. NAcsh *Penk* overexpression, but not adolescent THC-exposure, potentiated stress-induced heroin-seeking compared with GFP-infused controls [$F(2,19) = 4.829$, $p < .05$; Figure 2E]. Thus, increased *Penk* tone in NAcsh increased heroin-seeking behavior triggered by a mildly stressful event, indicating an enhanced propensity for relapse in these animals. Importantly, autoradiographic in situ hybridization histochemistry image analysis revealed significant upregulation of *Penk* messenger (m)RNA expression in NAcsh of *Penk*-infused and adolescent THC-exposed animals compared to GFP-infused control animals [$F(2,23) = 11.026$, $p < .05$; Figure 2F, G) following the completion of behavioral experiments. In contrast, *Pdyn* mRNA levels in NAcsh were unchanged in vehicle-exposed, GFP-infused, and *Penk*-infused animals (Figure S3 in Supplement 1). These data demonstrate that specific *Penk* upregulation in NAcsh promotes drug seeking after prolonged abstinence from heroin, indicating an important role for increased NAcsh *Penk* tone in the propensity for both cue- and stress- induced heroin-seeking behavior.

NAcsh *Penk* Knockdown Attenuates Adolescent THC-Induced Heroin Self-Administration

The pronounced increase in heroin-taking and heroin-seeking behavior resulting from NAcsh *Penk* overexpression prompted us to assess whether *Penk*-mediated regulation of these behaviors were selective to striatopallidal neurons that preferentially express the *Penk* gene. To this end, we used a microRNA (miR) targeting the *Penk* mRNA, allowing for miR-mediated mRNA cleavage specific to *Penk*-expressing neurons. After confirming the specific activity of the *Penk* miR in vitro (Figure S4 in Supplement 1), we verified the effects of local overexpression of a lentiviral vector containing the *Penk* miR tagged with GFP (miR *Penk*) into NAcsh (Figure 1B).

To investigate whether miR-mediated *Penk* knockdown could reverse the behavioral phenotype that was induced by adolescent THC exposure and NAcsh *Penk* infusion, three cohorts of animals were treated with THC during adolescence and then given bilateral NAcsh infusions of the miR *Penk* lentivirus or one of two lentiviral control vectors, one of which contained no targeting miR sequence (GFP) and the other a miR known to target a sequence not found in vertebrate DNA (miR ctrl), in young adulthood (Figure 1C; Table S1 in Supplement 1). Despite the marked downregulation of *Penk* mRNA expression that resulted from miR *Penk* infusion, animals readily learned the SA paradigm. miR *Penk* in NAcsh reduced both overall responding for heroin [treatment by session interaction, $F(22,213) = 1.759$, $p < .05$, Figure 3A] and mean heroin intake [treatment by session interaction, $F(22,211) = 2.136$, $p < .01$, Figure 3B] compared with GFP- and miR ctrl-infused control animals. Interestingly, the heroin-taking behavior exhibited by miR *Penk* animals

Figure 3. *Penk* knockdown in nucleus accumbens shell (NAcsh) attenuates heroin self-administration. **(A)** Acquisition of heroin self-administration (SA; fixed-ratio 1, 30 μg/kg/injection). **(B)** Mean heroin intake. **(C)** Between-session dose-response (7.5, 15, 30, 60, 100 μg/kg/infusion; randomized order). **(D)** Mean heroin intake. **(E)** Heroin-seeking behavior (cue- and stress-induced) in microRNA (miR) *Penk*-infused, miR c-infused, or green fluorescent protein (GFP)-infused rats exposed to adolescent Δ^9-tetrahydrocannabinol. **(F)** *Penk* mRNA levels in NAcsh, nucleus accumbens (NAc) core, and caudate-putamen (CPu) following heroin SA. **(G)** Representative in situ hybridization autoradiograms demonstrating striatal *Penk* mRNA expression following heroin SA. For all figures, $n = 5$–9 per group. Data shown as mean ± SEM. *$p < .05$; **$p < .01$ compared with GFP-expressing controls for each session; ***$p < .001$ compared with GFP and miR controls. AL, active lever; IL, inactive lever, dpm, disintegrations per minute. See also Figure S1B in Supplement 1.

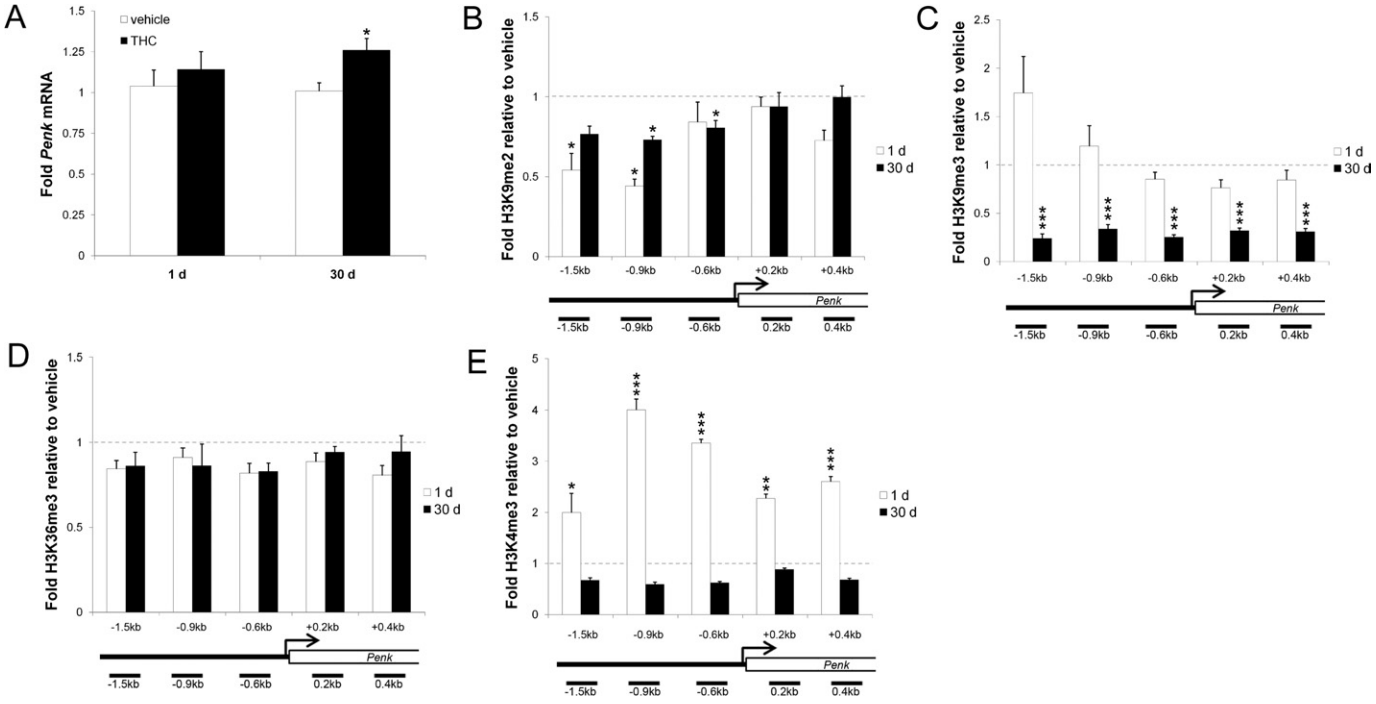

Figure 4. Adolescent Δ^9-tetrahydrocannabinol (THC) regulates *Penk* gene expression and histone H3 methylation in nucleus accumbens shell (NAcsh). **(A)** NAcsh *Penk* messenger RNA (mRNA) levels 1 day (adolescent) and 30 days (adult) after adolescent THC or vehicle ($n = 9–10$/group). **(B–E)** NAcsh histone H3 methylation fold changes at the *Penk* gene 1 day and 30 days after the last adolescent exposure to THC relative to vehicle treated animals ($n = 6–8$/group; 3 animals pooled/*n*). **(B)** H3K9me2. **(C)** H3K9me3. **(D)** H3K36me3. **(E)** H3K4me3. Data shown as mean ± SEM. *$p < .05$; **$p < .01$; ***$p < .001$ compared with vehicle-exposed animals at the same time point. kb, kilobases; TSS, transcription start site. See also Figure S3 and Table S2 in Supplement 1.

was similar to the behavior displayed by GFP animals unexposed to THC during adolescence (Figure 2A and 2B). NAcsh miR *Penk* also increased locomotor activity [$F(2,15) = 11.441$, $p < .001$; Figure S1B in Supplement 1], in line with the inhibitory role of the striatopallidal pathway in regulating motor behavior. Overall, these data provide evidence that NAcsh miR *Penk* blocks the behavioral phenotype induced by adolescent THC and further implicate a role for NAcsh *Penk* as a key mediator of heroin susceptibility.

In contrast to *Penk* overexpression, miR *Penk* in NAcsh led to a downward shift in the dose–response function, including both lower maximal SA rates [treatment by dose interaction, $F(8,77) = 4.120$, $p < .001$, Figure 3C] and heroin intake (Figure 3D) on the lower end of the dose–response curve compared with GFP and miR ctrl-infused control animals. Because a downward shift in the dose–response curve opposes alterations thought to be associated with the transition to more addicted states, these data indicate that reduced shell *Penk* tone decreases apparent behavioral susceptibility to heroin reinforcement. Given that NAcsh miR *Penk* attenuated the behavioral phenotype induced by adolescent THC exposure, we next investigated whether NAcsh miR *Penk* affected behavioral susceptibility to drug seeking. Mir *Penk* did not affect cue- or stress-induced heroin seeking when compared with GFP and miR ctrl controls (Figure 3E). There was a significant downregulation of NAcsh *Penk* mRNA expression in miR *Penk* animals [$F(2,24) = 222.929$, $p < .001$; Figure 3F, G] compared with GFP and miR ctrl control animals following the completion of behavioral experiments. The viral manipulation was specific to the *Penk* gene as *Pdyn* mRNA levels in NAcsh were unchanged as a result of miR *Penk* infusion (Figure S3 in Supplement 1). Taken together, these data establish a causal link between adolescent THC-mediated *Penk* dysregulation and the subsequent expression of behavioral susceptibility to heroin.

Adolescent THC Regulates Repressive Histone H3 Methylation at the *Penk* Gene in NAcsh

Given the protracted behavioral consequences of THC exposure during adolescence (3), we aimed to identify whether alterations at the level of chromatin regulation were associated with the transcriptional dysregulation of *Penk* that follows adolescent THC exposure. One day after the last THC treatment, NAcsh *Penk* mRNA levels were not significantly altered (Figure 4A); however, consistent with previous data, *Penk* mRNA expression was significantly increased in NAcsh 30 days after cessation of adolescent THC administration [$t(15) = 2.78$, $p < .05$; Figure 4A] compared with control animals. As a first step toward characterizing the potential epigenetic regulation of *Penk*, we investigated whether adolescent THC exposure was associated with altered levels of histone H3 methylation. In light of recent reports describing cocaine-induced alteration of repressive histone H3 lysine 9 (H3K9) methylation at gene promotors in the NAc (8,9), we studied di- and trimethylation of H3K9 (H3K9me2, H3K9me3) at the *Penk* gene, as well as trimethylation of histone H3 lysine 36 (H3K36me3) and lysine 4 (H3K4me3), marks that have been associated with transcriptional activation (16,17). Animals were treated with THC during adolescence and levels of H3K9me2, H3K9me3, H3K4me3, and H3K36me3 were analyzed in NAcsh via chromatin immunoprecipitation followed by quantitative PCR analysis of five sites flanking the *Penk* gene transcription start site, three spanning regulatory elements in the 5′ untranslated region and two in the coding region (Figure S5 and Table S2 in Supplement 1). One day following the final THC treatment, H3K9me2 was decreased at two sites in the *Penk* promoter region in the most upstream regions evaluated [$-1.5 - t(11) = -2.417$, $p < .05$; $-.9 - t(11) = -2.738$, $p < .05$; point-wise comparison Figure 4B] compared with vehicle-treated adolescent control animals. H3K9me3 did not differ statistically between the groups but did

tend to be increased at the same promoter regions where H3K9me2 was decreased (Figure 4C). No change was observed in H3K36me3 (Figure 4D). H3K4me3 levels were increased at each region evaluated [$-1.5-t(10) = 2.545$, $p < .05$; $-.9-t(9) = 7.109$, $p < .0001$; $-.6-t(10) = 5.621$, $p < .001$; $+.2-t(10) = 3.550$, $p < .01$; $+.4-t(10) = 8.144$, $p < .0001$; Figure 4E].

One month following cessation of adolescent THC treatment, H3K9me2 remained decreased at *Penk* in adult NAcsh, but significant effects were observed at promoter sites .9 kb and .6 kb upstream of the *Penk* transcription start site [$-.9-t(9) = -2.260$, $p < .05$; $-.6-t(8) = -2.480$, $p < .05$; Figure 4B]. In contrast to the pattern of H3K9me3 observed in adolescent NAcsh, however, H3K9me3 was decreased at all regions of the *Penk* gene in adult NAcsh [$-1.5-t(10) = -4.698$, $p < .001$; $-.9-t(10) = -7.172$, $p < .0001$; $-.6-t(10) = -6.959$, $p < .0001$; $+.2-t(10) = -5.681$, $p < .001$; $+.4-t(10) = -6.451$, $p < .0001$; Figure 4C], a finding consistent with the increased *Penk* gene expression (Figure 4A) in these animals. No alterations were observed in H3K36me3 or H3K4me3 in adult animals (Figure 4D and 4E). Taken together, these data suggest that decreases in H3K9me2 and H3K9me3 binding at the *Penk* promoter in adult NAcsh may mediate the upregulation of *Penk* transcription characteristic of adult animals with adolescent THC exposure.

Developmental Regulation of Histone H3 Methylation at the *Penk* Gene in NAcsh

Given that few studies have investigated the ontogeny of the enkephalinergic system, we were interested to study potential developmental differences in the regulation of *Penk* gene expression and histone H3 methylation at the *Penk* gene. Evaluation of NAcsh *Penk* mRNA levels in THC-naïve adolescent and adult animals revealed no significant difference between developmental periods (Figure 5A). We next examined H3K9me2, H3K9me3, H3K36me3, and H3K4me3 levels at the *Penk* gene of THC-naïve adolescent and adult animals. While H3K9me2 and H3K36me3 levels were similar between adolescent and adult animals, levels of H3K9me3 were increased in adult NAcsh at all regions studied [$-1.5-t(9) = 4.941$, $p < .0001$; $-.9-t(9) = 8.589$, $p < .0001$; $-.6-t(10) = 7.160$, $p < .0001$; $+0.2-t(10) = 5.473$, $p < .0001$; $+0.4-t(10) = 6.249$, $p < .0001$; Figure 5B]. H3K4me3 was also elevated in adulthood compared with the adolescent period at all regions [$-1.5-t(9) = 4.211$, $p < .01$; $-.9-t(9) = 5.172$, $p < .001$; $-.6-t(9) = 5.586$, $p < .001$; $+.2-t(9) = 4.179$, $p < .01$; $+.4-t(9) = 5.434$, $p < .001$; Figure 5B]. The concomitant enrichment of both H3K9me3 and H3K4me3 at the *Penk* gene could account for the lack of significant difference observed in *Penk* mRNA levels between adolescence and adulthood. These data provide evidence that specific histone H3 methyl marks in NAcsh are developmentally regulated.

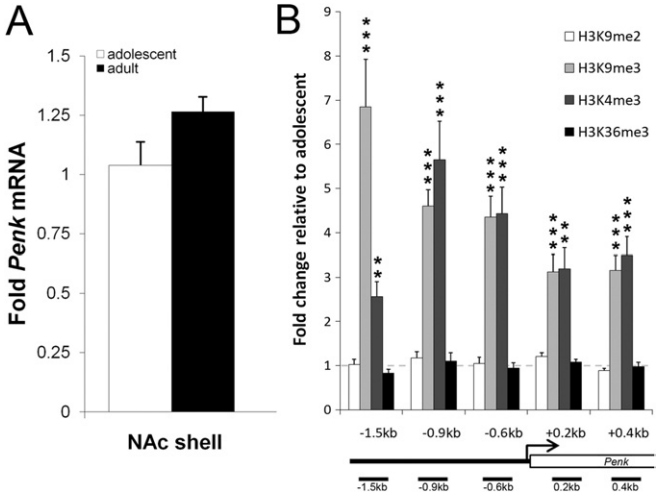

Figure 5. Histone H3 methylation at the Penk gene in nucleus accumbens shell (NAcsh) is dynamic during normal development. **(A)** Adolescent and adult NAcsh *Penk* messenger RNA (mRNA) levels (n = 9–10/group). **(B)** NAcsh histone H3 methylation fold changes at the *Penk* gene (n = 6–8/group; 3 animals pooled/n). Data shown as mean ± SEM. *$p < .05$; **$p < .01$; ***$p < .001$ compared with adolescent animals. kb, kilobases.

Discussion

This studies reveal a direct link between NAcsh *Penk* gene expression and enhanced behavioral susceptibility to heroin SA that mimics that seen in adult animals exposed to THC during adolescence. Such findings lend strong support to the hypothesis that adolescent THC exposure contributes to an opiate-vulnerable phenotype in adulthood. Here, we show that overexpression of NAcsh *Penk* in THC-naïve animals potentiates heroin SA, a behavioral effect that is attenuated by striatopallidal *Penk* knockdown in THC-exposed animals. Together, these data indicate a direct relationship between adolescent THC-induced *Penk* upregulation and heightened heroin taking in adulthood. Furthermore, we suggest that adolescent THC exposure may mediate adult NAcsh *Penk* upregulation through regulation of repressive histone H3K9 methylation, an epigenetic effect that represents a profound pathologic departure from the distinct developmental pattern of histone H3 methylation that normally occurs at *Penk* in NAcsh across the transition from adolescence to adulthood.

Of the opioid neuropeptides, enkephalin is consistently associated with regulating hedonic state (18,19). Although our SA paradigm was not designed to dissociate between reward and incentive motivational state, heroin SA behavior did not differ between groups during the early stages of acquisition, arguing against a *Penk*-mediated generalized impairment of basal hedonic tone. Moreover, although selective knockdown of *Penk* expression reduced overall heroin intake over time, it did not affect acquisition of SA behavior. Instead, *Penk*-overexpressing and THC-exposed animals continued to increase their heroin intake, ultimately stabilizing at a higher drug intake level during the maintenance phase, suggesting that these animals have different hedonic set points compared to controls (3). Additionally, the present data demonstrate that animals with elevated NAcsh *Penk* expression exhibit potentiated drug-seeking behavior induced by drug-associated environmental cues and mild stress. Interestingly, stress-induced sensitivity to heroin drug seeking was also apparent in adults following prenatal THC exposure (4). Although the animals' affective state underlying sensitivity to heroin is not yet fully understood, the present experiments implicate a direct role for NAcsh *Penk* in the opiate-susceptible behavioral phenotype similar to the consequence of adolescent THC exposure.

In the NAc, *Penk* is predominantly expressed in striatopallidal medium spiny neurons that project to ventral pallidum (20). Viral overexpression of *Penk* was not localized to a specific striatal subpopulation in our study, but it nevertheless resulted in the same behavioral pattern of heroin SA demonstrated by rats exposed to adolescent THC, suggesting that an increase in NAcsh enkephalinergic tone may be sufficient to affect opioid susceptibility. In contrast, miR knockdown of *Penk* is inherently specific to striatopallidal cells, and such manipulations attenuated the enhancement of heroin SA induced by adolescent THC exposure. Importantly, NAcsh *Pdyn* levels were unaffected by any of the manipulations, indicating

specificity of the behavioral alterations to selective NAcsh *Penk* alteration. Together, these findings emphasize the important role of *Penk* in mediating long-term effects of THC that contribute to opiate susceptibility. How regulation of *Penk* striatopallidal regulation contributes to specific components of addiction-related behavior in the nondrug state remains to be established.

Given the protracted behavioral effects of adolescent THC exposure, alterations at the level of chromatin regulation are prime candidates for investigation. Although several studies have suggested an important role for transient histone modifications in the regulation of drug-induced behaviors, only recently has histone methylation, a more stable modification, been demonstrated as a potential mediator of drug-induced transcriptional plasticity (8,9). Histone methylation is highly complex; N-terminal histone lysine residues can be mono, di, or trimethylated, with each valence state differentially regulating the recruitment of proteins that activate or repress transcription (21,22). Although increased H3K9me2 binding has been demonstrated at promoters of repressed eukaryotic genes, our findings confirm that reduced H3K9me2 binding plays a role at promoters of *activated* eukaryotic genes. Of the histone marks quantified in this study, dimethylation of H3K9 at upstream regions of the *Penk* gene in NAcsh was reduced both 1 day and 30 days after THC administration. In contrast, the pronounced enrichment across the *Penk* promoter of the activating mark H3K4me3 seen 1 day after THC exposure was normalized by adulthood.

In addition to modulation of H3K9me2, adolescent THC exposure also had significant effects on H3K9me3, an unexpected finding given that H3K9me3 is typically enriched at pericentromeric heterochromatin and sites of repressed chromatin (23–26). However, several groups have reported the presence of H3K9me3 in transcribed regions of active mammalian genes (27–30). The current finding that H3K9me3 was decreased long term (30 days) in the transcribed regions of *Penk* in the adult NAcsh as a consequence of adolescent THC exposure raises the possibility that reduced H3K9me3 in the coding regions of active genes may also contribute to transcriptional plasticity. Current technologies cannot establish causal regulation of histone methylation at a single gene level, but accumulating evidence suggests that H3K9me3 may play a significant role in regulating active genes (30,31). Given the low levels of H3K9me3 at most expressed genes (26), however, the magnitude of the fold changes seen with adolescent THC in the adult may be artificially enhanced. Although it is currently impossible to know the absolute neurobiological consequences of small relative changes in histone marks, the differential profile of H3K9me3 at the *Penk* gene 1 day compared with 30 days after THC exposure, coupled with the potentiated SA behavior evident in adult animals, support a functional role for even small perturbations in H3K9me3 at the *Penk* gene and thus requires further investigation.

To date, no studies have examined histone methylation during normal development. Adolescence is a critical phase of brain maturation, and the current results demonstrate distinct development-specific patterns of histone H3 modifications at the NAcsh *Penk* gene. Although stable levels of H3K9me2 and H3K36me3 were observed in NAcsh of adolescent and adult animals, the profiles of H3K9 and H3K4 trimethylation varied across this developmental period. The chromatin landscape is highly complex, but trimethylation of H3K4 (transcriptional activation) concomitant with trimethylation of H3K9 (transcriptional repression) may account for the developmental transcriptional stability of NAcsh *Penk* because there was no difference in *Penk* mRNA levels in adolescent versus adult. Furthermore, that H3K9me3 and H3K4me3 displayed similar magnitudes of induction and distribution across the *Penk* gene in adolescent NAcsh suggests that trimethylation of these marks may be coordinated (30) during normal development. Although the functional consequences on NAcsh *Penk* gene expression did not differ between adolescence and adulthood, the distinct epigenetic profiles during these ontogenetically disparate periods may allow the *Penk* gene to be "primed" to respond differentially to similar environmental cues. Limited studies have investigated the differential neurobiological effects of THC exposure in adolescence versus adulthood, but mounting evidence documents differential responsivity to drugs of abuse in the adolescent compared with the adult brain (32,33). Overall, our study emphasizes that adolescent THC exposure leads to a departure of the normal trajectory of the transcriptional and epigenetic state of the *Penk* gene, a disruption that may mediate the expression of enhanced behavioral vulnerability to opiates in adulthood.

In conclusion, our findings indicate that marijuana exposure in and of itself can serve as a risk factor that acts "above the genome" and can imprint on the existing epigenetic landscape of adolescent neurodevelopment. Thus, the epigenetic effects of adolescent THC exposure may act in concert to augment future behavioral responses to drugs of abuse via stable and long-term regulation of genes at the transcriptional level. The results also support a novel role for the *Penk* gene as an emergent endogenous risk factor resulting from adolescent THC exposure, the dysregulation of repressive histone H3 methylation of which may underlie the long-term behavioral consequences of adolescent THC.

This work was funded by the National Institute on Drug Abuse, Grant Nos. DA024929 (HCT), T32 DA007135 (MMJ), DA08227 (EJN), DA030359 (YLH), and DA19350 (YLH).

We thank Dr. Ian Maze for comments on the manuscript and Dr. Yanhua Ren for technical advice.

The authors report no biomedical financial interest or potential conflicts of interest.

Supplementary material cited in this article is available online.

1. Kandel D (1975): Stages in adolescent involvement in drug use. *Science* 190:912–914.
2. Hall WD, Lynskey M (2005): Is cannabis a gateway drug? Testing hypotheses about the relationship between cannabis use and the use of other illicit drugs. *Drug Alcohol Rev* 24:39–48.
3. Ellgren M, Spano SM, Hurd YL (2007): Adolescent cannabis exposure alters opiate intake and opioid limbic neuronal populations in adult rats. *Neuropsychopharmacology* 32:607–615.
4. Spano MS, Ellgren M, Wang X, Hurd YL (2007): Prenatal cannabis exposure increases heroin seeking with allostatic changes in limbic enkephalin systems in adulthood. *Biol Psychiatry* 61:554–563.
5. Ellgren M, Artmann A, Tkalych O, Gupta A, Hansen HS, Hansen SH, *et al.* (2008): Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. *Eur Neuropsychopharmacol* 18:826–834.
6. Rodriguez JJ, Mackie K, Pickel VM (2001): Ultrastructural localization of the CB1 cannabinoid receptor in mu-opioid receptor patches of the rat caudate putamen nucleus. *J Neurosci* 21:823–833.
7. Pickel VM, Chan J, Kash TL, Rodriguez JJ, MacKie K (2004): Compartment-specific localization of cannabinoid 1 (CB1) and mu-opioid receptors in rat nucleus accumbens. *Neuroscience* 127:101–112.
8. Renthal W, Kumar A, Xiao G, Wilkinson M, Covington HE 3rd, Maze I, *et al.* (2009): Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. *Neuron* 62:335–348.
9. Maze I, Covington HE 3rd, Dietz DM, LaPlant Q, Renthal W, Russo SJ, *et al.* (2010): Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. *Science* 327:213–216.
10. Hurd YL (2003): In situ hybridization with isotopic riboprobes for detection of striatal neuropeptide mRNA expression after dopamine stimulant administration. *Methods Mol Med* 79:119–135.

11. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ (2006): Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. *Nat Neurosci* 9:519–525.
12. Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, *et al.* (2005): Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. *Neuron* 48:303–314.
13. Jaffe JH, Cascella NG, Kumor KM, Sherer MA (1989): Cocaine-induced cocaine craving. *Psychopharmacology (Berl)* 97:59–64.
14. O'Brien CP, Childress AR, McLellan AT, Ehrman R (1992): Classical conditioning in drug-dependent humans. *Ann N Y Acad Sci* 654:400–415.
15. Kreek MJ, Koob GF (1998): Drug dependence: Stress and dysregulation of brain reward pathways. *Drug Alcohol Depend* 51:23–47.
16. Li B, Carey M, Workman JL (2007): The role of chromatin during transcription. *Cell* 128:707–719.
17. Shilatifard A (2008): Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. *Curr Opin Cell Biol* 20:341–348.
18. Kelley AE, Bakshi VP, Haber SN, Steininger TL, Will MJ, Zhang M (2002): Opioid modulation of taste hedonics within the ventral striatum. *Physiol Behav* 76:365–377.
19. Skoubis PD, Lam HA, Shoblock J, Narayanan S, Maidment NT (2005): Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. *Eur J Neurosci* 21:1379–1384.
20. Zhou L, Furuta T, Kaneko T (2003): Chemical organization of projection neurons in the rat accumbens nucleus and olfactory tubercle. *Neuroscience* 120:783–798.
21. Rice JC, Allis CD (2001): Histone methylation versus histone acetylation: New insights into epigenetic regulation. *Curr Opin Cell Biol* 13:263–273.
22. Martin C, Zhang Y (2005): The diverse functions of histone lysine methylation. *Nat Rev Mol Cell Biol* 6:838–849.
23. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, *et al.* (2002): Role of histone H3 lysine 27 methylation in Polycomb-group silencing. *Science* 298:1039–1043.
24. Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, *et al.* (2003): Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. *Mol Cell* 12:1591–1598.
25. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, *et al.* (2004): A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. *Genes Dev* 18:1251–1262.
26. Maze I, Feng J, Wilkinson MB, Sun H, Shen L, Nestler EJ (2011): Cocaine dynamically regulates heterochromatin and repetitive element unsilencing in nucleus accumbens. *Proc Natl Acad Sci U S A* 108:3035–3040.
27. Brinkman AB, Roelofsen T, Pennings SW, Martens JH, Jenuwein T, Stunnenberg HG (2006): Histone modification patterns associated with the human X chromosome. *EMBO Rep* 7:628–634.
28. Rougeulle C, Chaumeil J, Sarma K, Allis CD, Reinberg D, Avner P, *et al.* (2004): Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. *Mol Cell Biol* 24:5475–5484.
29. Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX, Jang SW, *et al.* (2006): Suz12 binds to silenced regions of the genome in a cell-type-specific manner. *Genome Res* 16:890–900.
30. Vakoc CR, Mandat SA, Olenchock BA, Blobel GA (2005): Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. *Mol Cell* 19:381–391.
31. Vakoc CR, Sachdeva MM, Wang H, Blobel GA (2006): Profile of histone lysine methylation across transcribed mammalian chromatin. *Mol Cell Biol* 26:9185–9195.
32. Schramm-Sapyta NL, Cha YM, Chaudhry S, Wilson WA, Swartzwelder HS, Kuhn CM (2007): Differential anxiogenic, aversive, and locomotor effects of THC in adolescent and adult rats. *Psychopharmacology (Berl)* 191:867–877.
33. Izenwasser S (2005): Differential effects of psychoactive drugs in adolescents and adults. *Crit Rev Neurobiol* 17:51–67.
34. Paxinos G, Watson C (2007): *The Rat Brain in Stereotaxic Coordinates, 6th ed*. New York: Academic Press.