Published in final edited form as:
Biol Psychiatry. 2012 November 15; 72(10): 803–810. doi:10.1016/j.biopsych.2012.04.026.

PROENKEPHALIN MEDIATES THE ENDURING EFFECTS OF ADOLESCENT CANNABIS EXPOSURE ASSOCIATED WITH ADULT OPIATE VULNERABILITY

Hilarie C. Tomasiewicz[1,2,4], **Michelle M. Jacobs**[2,3,4], **Matthew B. Wilkinson**[1,4], **Steven P. Wilson**[5], **Eric J. Nestler**[1,2,3,4], and **Yasmin L. Hurd**[1,2,3,4]

[1]Fishburg Department of Neuroscience

[2]Department of Psychiatry

[3]Department of Pharmacology & Systems Therapeutics

[4]Friedman Brain Institute Mount Sinai School of Medicine, New York, NY 10029, USA

[5]Department of Pharmacology, Physiology and Neuroscience, University of South Carolina School of Medicine, Columbia, SC 29208, USA

Abstract

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 *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 ChIP at five sites flanking the *Penk* gene transcription start site.

Results—Here, we show that regulation of the proenkephalin (*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.

Corresponding author: Dr. Yasmin Hurd, Mount Sinai School of Medicine, Departments of Psychiatry, Pharmacology and Systems Therapeutics, and Neuroscience, New York, NY, USA. Tel: 212-241-4007. Fax: 212-996-7214. Yasmin.hurd@mssm.edu..

DISCLOSURE OF BIOMEDICAL FINANCIAL INTERESTS AND POTENTIAL CONFLICTS OF INTEREST The authors report no biomedical financial interest or potential conflicts of interest.

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.

Keywords

drug addiction; marijuana; rat; nucleus accumbens; striatopallidal; epigenetics

INTRODUCTION

Drug addiction is a chronic and relapsing disease that often begins during adolescence. Epidemiological evidence documents an association between marijuana use during adolescence and subsequent abuse of drugs such as heroin and cocaine (1, 2). While 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 neuroanatomical 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; 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 by 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 by 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*.

MATERIALS AND METHODS

Animals and THC Treatment

Male 21-day old Long Evans rats (Taconic) were used; procedures conducted in accordance with approved protocols. Rats received intraperitoneal injections of THC (1.5mg/kg) (RTI International, USA) or vehicle (0.9% NaCl with 0.3% Tween 80) every 3rd day (8 injections) during adolescence (PND 28–49)(3). For SA experiments, *Penk*- and GFP-infused rats were treated with vehicle during adolescence and GFP-, miR ctrl-, and miR *Penk*-infused rats were treated with THC during adolescence.

Lentiviral Vectors

Lentiviral vectors were constructed as described. (Supplemental Information). 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/O_2 (2.5–4.5%), and bilaterally stereotaxically infused with 0.5μl virus (*Penk* or GFP) or 1.0μl virus (GFP, miR ctrl, miR *Penk*) into NAcsh (from bregma: AP +1.7mm; ML +2.3mm; DV −6.8mm (from dura), 10° from midline) at 0.1μl/min. Two weeks subsequently, rats underwent surgery for jugular catheters (Brian Fromant, Cambridge, UK) for future SA experiments.

Heroin Self-Administration and Locomotor Activity

Heroin SA was conducted according to published protocols (3, 5)(Supplemental Information). Briefly, rats self-administered heroin (30μg/kg/injection) 3hr daily under a fixed-ratio 1 (FR1) 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 3wk abstinence period, cue-induced drug-seeking behavior was evaluated (depression of the drug-paired lever had no programmed consequence) followed 1wk later by mild stress (24hr food deprivation)-induced drug-seeking. Both tests were 1hr. Activity was measured by infrared beams during the SA sessions.

In situ Hybridization Histochemistry (ISHH)

ISHH was conducted according to published protocols (3, 10) (Supplemental Information). [^{35}S]-labeled *Penk* riboprobe (generated from a PCR-derived 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) with ^{14}C standards for 28hr. Disintegrations-per-minute (dpm/mg) autogradiographic 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). Quantitative real-time PCR analysis was performed using Taqman-based probes (Applied Biosystems; *Penk*, Rn00567566_m1; 18 S, 4319413E; *Pdyn*, Rn00571351_m1.); reactions run in triplicate, each gene run separately. Data normalized to eukaryotic 18S rRNA and analyzed via the ΔΔCT method (11).

Chromatin Immunoprecipitation (ChIP)

Fresh tissue was prepared for ChIP as previously described (8, 12) with minor modifications (Supplemental Information). Briefly, two bilateral NAcsh punches/rat (three rats pooled per sample) were collected and processed. Immunoprecipitated (IP'd) samples (antibodies: H3K9me2, ab1220; H3K9me3, ab8898; H3K36me3, ab9050; H3K4me3, ab8580) abcam, MA, USA) were subject to qPCR (SYBR Green (Roche)) and normalized to their non-IP'd 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 ANOVAs followed by followed by Tukey's HSD 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 (*p<0.05; **p<0.01; ***p<0.001). Statistical calculations performed using JMP software (SAS, NC, USA).

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 green fluorescent protein (GFP) into NAcsh of adult rats (Fig. 1a,b). Viral spread was 1 mm^3 from the needle tip.

To investigate the functional significance of enhanced NAcsh *Penk* tone, we tested the involvement of increased NAcsh *Penk* expression on a FR1 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 (Fig. 1c; Table S1 in the Supplement). Bilateral NAcsh infusions of *Penk* enhanced responding for heroin (treatment by session interaction, $F_{22,213}$=2.036, P<0.01, Fig. 2a) and mean heroin intake (treatment by session interaction, $F_{22,213}$=2.589, P<0.001, Fig. 2b), without modifying inactive lever pressing or locomotor activity (Fig. S1A in the Supplement), 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 (Fig. 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 (Fig. S2 in the Supplement). 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<0.001, Fig. 2c) and higher drug intake across the range of doses studied (Fig. 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 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<0.05, Fig. 2e) when compared to 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 if 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 24hr food deprivation. NAcsh *Penk* overexpression, but not adolescent THC-exposure, potentiated stress-induced heroin-seeking compared to GFP-infused controls ($F_{2,19}$=4.829, P<0.05; Fig. 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 ISHH

image analysis revealed significant upregulation of *Penk* mRNA expression in NAcsh of *Penk*-infused and adolescent THC-exposed animals compared to GFP-infused control animals ($F_{2,23}$=11.026, P<0.05; Fig. 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 (Fig. S3 in the Supplement). 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 -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* (Fig. S4 in the Supplement), we verified the effects of local overexpression of a lentiviral vector containing the *Penk* miR tagged with GFP (miR *Penk*) into NAcsh (Fig. 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 miRNA sequence (GFP) and the other a miR known to target a sequence not found in vertebrate DNA (miR ctrl), in young adulthood (Fig. 1c; Table S1 in the Supplement). 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<0.05, Fig. 3a) and mean heroin intake (treatment by session interaction, $F_{22,211}$=2.136, P<0.01, Fig. 3b) compared to GFP- and miR ctrl-infused control animals. Interestingly, the heroin-taking behavior exhibited by miR *Penk* animals was similar to the behavior displayed by GFP animals unexposed to THC during adolescence (Fig. 2a,b). NAcsh miR *Penk* also increased locomotor activity ($F_{2,15}$=11.441, P<0.001; Fig. S1B in the Supplement) 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<0.001, Fig. 3c) and heroin intake (Fig. 3d) on the lower end of the dose-response curve compared to GFP and miR ctrl-infused control animals. As 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 to GFP and miR ctrl controls (Fig. 3e). There was a significant downregulation of NAcsh *Penk* mRNA expression in miR *Penk* animals ($F_{2,24}$=222.929, P<0.001; Fig. 3f,g) compared to 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 (Fig. S3 in the Supplement). 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 (Fig. 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<0.05; Fig. 4a) compared to control animals. As a first step towards 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 ChIP followed by qPCR analysis of five sites flanking the *Penk* gene transcription start site (TSS), three spanning regulatory elements in the 5'UTR, and two in the coding region (Figure S5 and Table S2 in the Supplement). 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<0.05; −0.9-t_{11}=−2.738, P<0.05; point-wise comparison Fig. 4b) compared to 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 (Fig. 4c). No change was observed in H3K36me3 (Fig. 4d). H3K4me3 levels were increased at each region evaluated (−1.5-t_{10}=2.545, P<0.05; −0.9-t_9=7.109, P<0.0001; −0.6-t_{10}=5.621, P<0.001; +0.2-t_{10}=3.550, P<0.01; +0.4-t_{10}=8.144, P<0.0001; Fig. 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 0.9 kb and 0.6 kb upstream of the *Penk* TSS (−0.9-t_9=−2.260, P<0.05; −0.6-t_8=−2.480, P<0.05; Fig. 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<0.001; −0.9-t_{10}=−7.172, P<0.0001; −0.6-t_{10}=−6.959, P<0.0001; +0.2-t_{10}=−5.681, P<0.001; +0.4-t_{10}=−6.451, P<0.0001; Fig. 4c), a finding consistent with the increased *Penk* gene expression (Fig. 4a) in these animals. No alterations were observed in H3K36me3 or H3K4me3 in adult animals (Fig. 4d,e). 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 (Fig. 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<0.0001; −0.9-t_9=8.589, P<0.0001; −0.6-t_{10}=7.160, P<0.0001; +0.2-t_{10}=5.473, P<0.0001; +0.4-t_{10}=6.249, P<0.0001; Fig. 5b). H3K4me3 was also elevated in adulthood compared to the adolescent period at all regions (−1.5-t_9=4.211, P<0.01; −0.9-t_9=5.172, P<0.001; −0.6-t_9=5.586, P<0.001; +0.2-t_9=4.179, P<0.01; +0.4-t_9=5.434, P<0.001; Fig. 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.

DISCUSSION

The present 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). While 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 the present 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 impact 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 non-drug 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. While 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, as 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). While increased H3K9me2 binding has been demonstrated at promoters of repressed eukaryotic genes, the present findings confirm that reduced H3K9me2 binding plays a role at promoters of *activated* eukaryotic genes. Of the histone marks quantified in the present 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 peri-centromeric 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. Though presently 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 as compared to 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. While 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* as there was no difference in *Penk* mRNA levels in adolescent *vs.* 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. Though 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 neurobiologic effects of THC exposure in adolescence versus adulthood, but mounting evidence documents differential responsivity to drugs of abuse in

the adolescent as compared to 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 which 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 upon 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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

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

REFERENCES

1. Kandel D. Stages in adolescent involvement in drug use. Science. 1975; 190:912–914. [PubMed: 1188374]
2. Hall WD, Lynskey M. Is cannabis a gateway drug? Testing hypotheses about the relationship between cannabis use and the use of other illicit drugs. Drug Alcohol Rev. 2005; 24:39–48. [PubMed: 16191720]
3. Ellgren M, Spano SM, Hurd YL. Adolescent cannabis exposure alters opiate intake and opioid limbic neuronal populations in adult rats. Neuropsychopharmacology. 2007; 32:607–615. [PubMed: 16823391]
4. Spano MS, Ellgren M, Wang X, Hurd YL. Prenatal cannabis exposure increases heroin seeking with allostatic changes in limbic enkephalin systems in adulthood. Biol Psychiatry. 2007; 61:554–563. [PubMed: 16876136]
5. Ellgren M, Artmann A, Tkalych O, Gupta A, Hansen HS, Hansen SH, et al. Dynamic changes of the endogenous cannabinoid and opioid mesocorticolimbic systems during adolescence: THC effects. Eur Neuropsychopharmacol. 2008
6. Rodriguez JJ, Mackie K, Pickel VM. Ultrastructural localization of the CB1 cannabinoid receptor in mu-opioid receptor patches of the rat Caudate putamen nucleus. J Neurosci. 2001; 21:823–833. [PubMed: 11157068]
7. Pickel VM, Chan J, Kash TL, Rodriguez JJ, MacKie K. Compartment-specific localization of cannabinoid 1 (CB1) and mu-opioid receptors in rat nucleus accumbens. Neuroscience. 2004; 127:101–112. [PubMed: 15219673]
8. Renthal W, Kumar A, Xiao G, Wilkinson M, Covington HE 3rd, Maze I, et al. Genome-wide analysis of chromatin regulation by cocaine reveals a role for sirtuins. Neuron. 2009; 62:335–348. [PubMed: 19447090]
9. Maze I, Covington HE 3rd, Dietz DM, LaPlant Q, Renthal W, Russo SJ, et al. Essential role of the histone methyltransferase G9a in cocaine-induced plasticity. Science. 2010; 327:213–216. [PubMed: 20056891]

10. Hurd YL. In situ hybridization with isotopic riboprobes for detection of striatal neuropeptide mRNA expression after dopamine stimulant administration. Methods Mol Med. 2003; 79:119–135. [PubMed: 12506693]
11. Tsankova NM, Berton O, Renthal W, Kumar A, Neve RL, Nestler EJ. Sustained hippocampal chromatin regulation in a mouse model of depression and antidepressant action. Nat Neurosci. 2006; 9:519–525. [PubMed: 16501568]
12. Kumar A, Choi KH, Renthal W, Tsankova NM, Theobald DE, Truong HT, et al. Chromatin remodeling is a key mechanism underlying cocaine-induced plasticity in striatum. Neuron. 2005; 48:303–314. [PubMed: 16242410]
13. Jaffe JH, Cascella NG, Kumor KM, Sherer MA. Cocaine-induced cocaine craving. Psychopharmacology (Berl). 1989; 97:59–64. [PubMed: 2496428]
14. O'Brien CP, Childress AR, McLellan AT, Ehrman R. Classical conditioning in drug-dependent humans. Ann N Y Acad Sci. 1992; 654:400–415. [PubMed: 1632593]
15. Kreek MJ, Koob GF. Drug dependence: stress and dysregulation of brain reward pathways. Drug Alcohol Depend. 1998; 51:23–47. [PubMed: 9716928]
16. Li B, Carey M, Workman JL. The role of chromatin during transcription. Cell. 2007; 128:707–719. [PubMed: 17320508]
17. Shilatifard A. Molecular implementation and physiological roles for histone H3 lysine 4 (H3K4) methylation. Curr Opin Cell Biol. 2008; 20:341–348. [PubMed: 18508253]
18. Kelley AE, Bakshi VP, Haber SN, Steininger TL, Will MJ, Zhang M. Opioid modulation of taste hedonics within the ventral striatum. Physiol Behav. 2002; 76:365–377. [PubMed: 12117573]
19. Skoubis PD, Lam HA, Shoblock J, Narayanan S, Maidment NT. Endogenous enkephalins, not endorphins, modulate basal hedonic state in mice. Eur J Neurosci. 2005; 21:1379–1384. [PubMed: 15813947]
20. Zhou L, Furuta T, Kaneko T. Chemical organization of projection neurons in the rat accumbens nucleus and olfactory tubercle. Neuroscience. 2003; 120:783–798. [PubMed: 12895518]
21. Rice JC, Allis CD. Histone methylation versus histone acetylation: new insights into epigenetic regulation. Curr Opin Cell Biol. 2001; 13:263–273. [PubMed: 11343896]
22. Martin C, Zhang Y. The diverse functions of histone lysine methylation. Nat Rev Mol Cell Biol. 2005; 6:838–849. [PubMed: 16261189]
23. Cao R, Wang L, Wang H, Xia L, Erdjument-Bromage H, Tempst P, et al. Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science. 2002; 298:1039–1043. [PubMed: 12351676]
24. Rice JC, Briggs SD, Ueberheide B, Barber CM, Shabanowitz J, Hunt DF, et al. Histone methyltransferases direct different degrees of methylation to define distinct chromatin domains. Mol Cell. 2003; 12:1591–1598. [PubMed: 14690610]
25. Schotta G, Lachner M, Sarma K, Ebert A, Sengupta R, Reuter G, et al. A silencing pathway to induce H3-K9 and H4-K20 trimethylation at constitutive heterochromatin. Genes Dev. 2004; 18:1251–1262. [PubMed: 15145825]
26. Maze I, Feng J, Wilkinson MB, Sun H, Shen L, Nestler EJ. Cocaine dynamically regulates heterochromatin and repetitive element unsilencing in nucleus accumbens. Proc Natl Acad Sci U S A. 2011; 108:3035–3040. [PubMed: 21300862]
27. Brinkman AB, Roelofsen T, Pennings SW, Martens JH, Jenuwein T, Stunnenberg HG. Histone modification patterns associated with the human X chromosome. EMBO Rep. 2006; 7:628–634. [PubMed: 16648823]
28. Rougeulle C, Chaumeil J, Sarma K, Allis CD, Reinberg D, Avner P, et al. Differential histone H3 Lys-9 and Lys-27 methylation profiles on the X chromosome. Mol Cell Biol. 2004; 24:5475–5484. [PubMed: 15169908]
29. Squazzo SL, O'Geen H, Komashko VM, Krig SR, Jin VX, Jang SW, et al. Suz12 binds to silenced regions of the genome in a cell-type-specific manner. Genome Res. 2006; 16:890–900. [PubMed: 16751344]
30. Vakoc CR, Mandat SA, Olenchock BA, Blobel GA. Histone H3 lysine 9 methylation and HP1gamma are associated with transcription elongation through mammalian chromatin. Mol Cell. 2005; 19:381–391. [PubMed: 16061184]

31. Vakoc CR, Sachdeva MM, Wang H, Blobel GA. Profile of histone lysine methylation across transcribed mammalian chromatin. Mol Cell Biol. 2006; 26:9185–9195. [PubMed: 17030614]

32. Schramm-Sapyta NL, Cha YM, Chaudhry S, Wilson WA, Swartzwelder HS, Kuhn CM. Differential anxiogenic, aversive, and locomotor effects of THC in adolescent and adult rats. Psychopharmacology (Berl). 2007; 191:867–877. [PubMed: 17211649]

33. Izenwasser S. Differential effects of psychoactive drugs in adolescents and adults. Crit Rev Neurobiol. 2005; 17:51–67. [PubMed: 16808727]

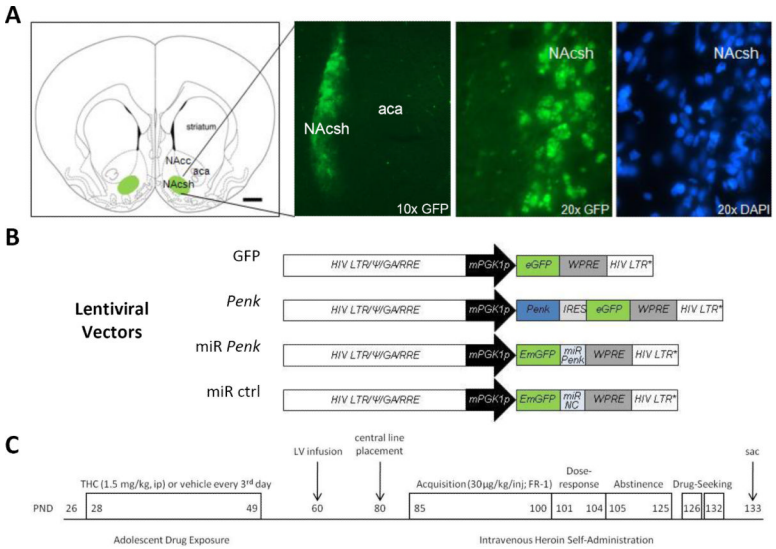

Figure 1. Lentivirus-mediated *Penk* gene manipulation and heroin self-administration

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

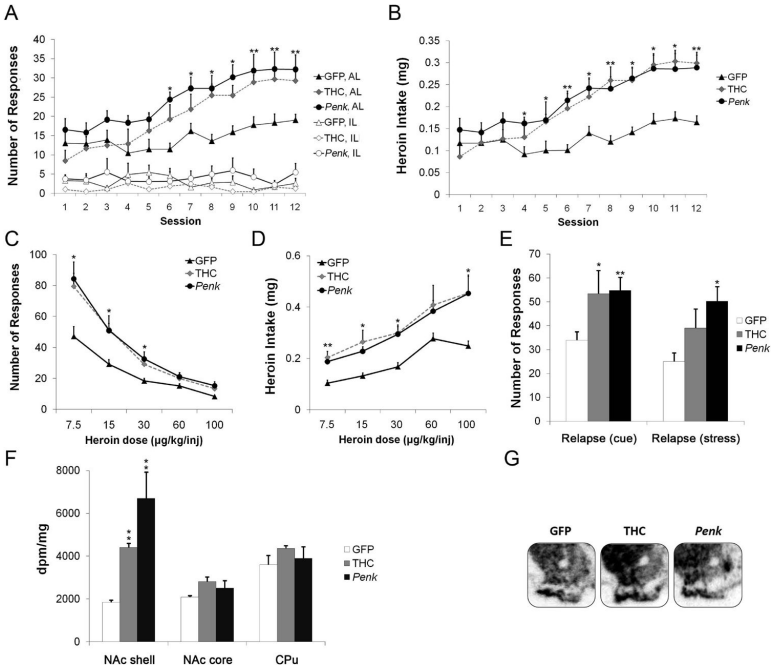

Figure 2. *Penk* overexpression in NAcsh potentiates heroin self-administration

(A) Acquisition of heroin SA (FR-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, GFP-infused, or THC-exposed rats. (F) *Penk* mRNA levels in NAcsh, 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/group. Data shown as mean±SEM. **P*<0.05; ***P*<0.01 compared to GFP-expressing controls for each session. AL, active lever; IL, inactive lever, dpm, disintegrations per minute. See also Fig. S1A in the Supplement.

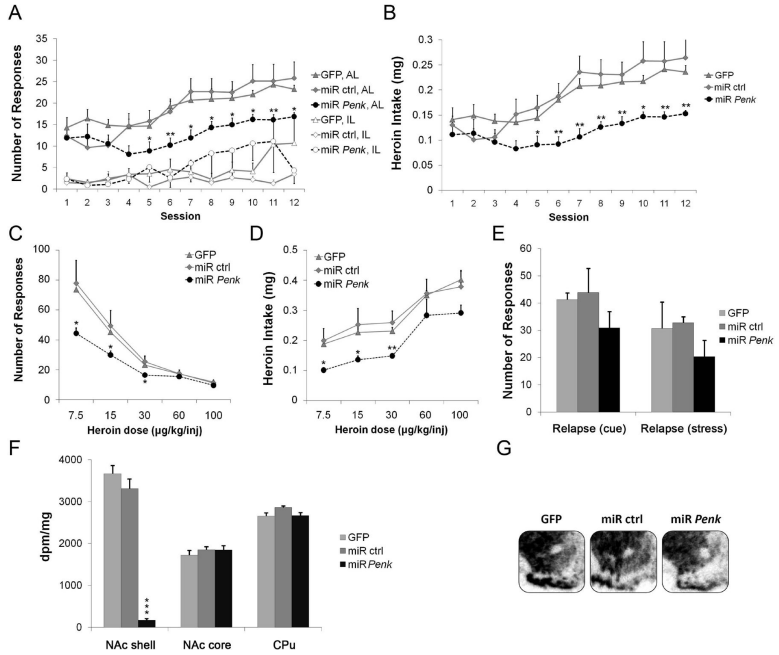

Figure 3. *Penk* knockdown in NAcsh attenuates heroin self-administration

(A) Acquisition of heroin SA (FR-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 miR *Penk*-infused, miR ctrl-infused, or GFP-infused rats exposed to adolescent THC. (F) *Penk* mRNA levels in NAcsh, 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/group. Data shown as mean±SEM. **P*<0.05; ***P*<0.01 compared to GFP-expressing controls for each session. AL, active lever; IL, inactive lever, dpm, disintegrations per minute. See also Fig. S1B in the Supplement.

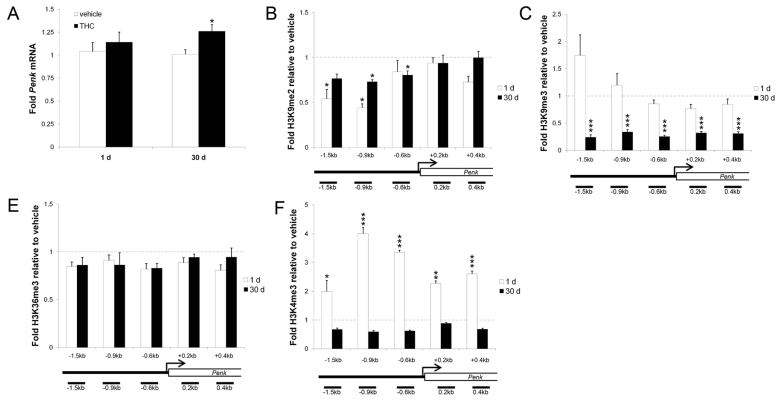

Figure 4. Adolescent THC regulates *Penk* gene expression and histone H3 methylation in NAcsh

(A) NAcsh *Penk* 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.* $P<0.05$; ** $P<0.01$; *** $P<0.001$ compared to vehicle-exposed animals at the same time point. kb, kilobases; TSS, transcription start site. See also Fig. S3 and Table S2 in the Supplement.

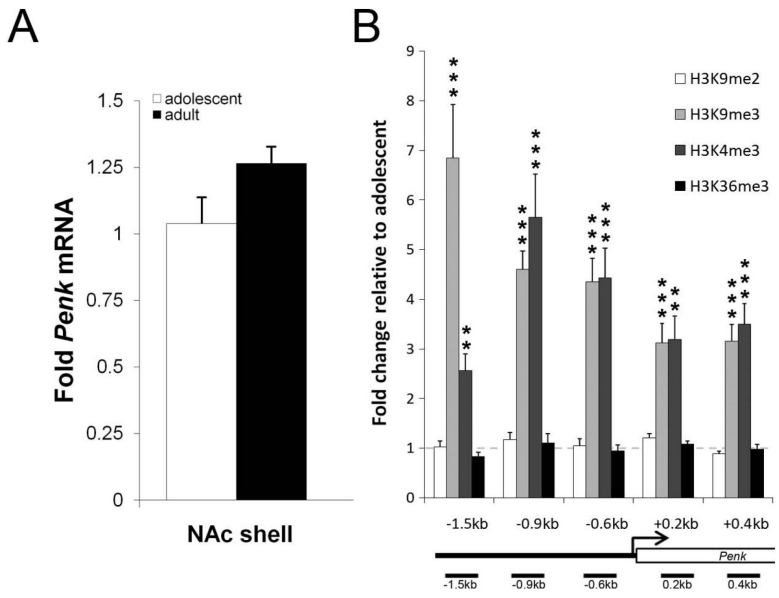

Figure 5. Histone H3 methylation at the Penk gene in NAcsh is dynamic during normal development

(A) Adolescent and adult NAcsh *Penk* 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*<0.05; ***P*<0.01; ****P*<0.001 compared to adolescent animals. kb, kilobases; TSS, transcription start site.