

Role of Dorsal Striatum Histone Deacetylase 5 in Incubation of Methamphetamine Craving

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ABSTRACT

BACKGROUND: Methamphetamine (meth) seeking progressively increases after withdrawal (incubation of meth craving). We previously demonstrated an association between histone deacetylase 5 (HDAC5) gene expression in the rat dorsal striatum and incubation of meth craving. Here we used viral constructs to study the causal role of dorsal striatum HDAC5 in this incubation.

METHODS: In experiment 1 (overexpression), we injected an adeno-associated virus bilaterally into dorsal striatum to express either green fluorescent protein (control) or a mutant form of HDAC5, which strongly localized to the nucleus. After training rats to self-administer meth (10 days, 9 hours/day), we tested the rats for relapse to meth seeking on withdrawal days 2 and 30. In experiment 2 (knockdown), we injected an adeno-associated virus bilaterally into the dorsal striatum to express a short hairpin RNA either against luciferase (control) or against HDAC5. After training rats to self-administer meth, we tested the rats for relapse on withdrawal days 2 and 30. We also measured gene expression of other HDACs and potential HDAC5 downstream targets.

RESULTS: We found that HDAC5 overexpression in dorsal striatum increased meth seeking on withdrawal day 30 but not day 2. In contrast, HDAC5 knockdown in the dorsal striatum decreased meth seeking on withdrawal day 30 but not on day 2; this manipulation also altered other HDACs (*Hdac1* and *Hdac4*) and potential HDAC5 targets (*Gnb4* and *Suv39h1*).

CONCLUSIONS: Results demonstrate a novel role of dorsal striatum HDAC5 in incubation of meth craving. These findings also set up future work to identify HDAC5 targets that mediate this incubation.

Keywords: Dorsal striatum, Epigenetics, HDAC5, Incubation, Methamphetamine, Relapse

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A key feature of drug addiction is high relapse rates during abstinence (1,2). In rats, drug seeking progressively increases or incubates after withdrawal from drug self-administration (3–5). This incubation phenomenon was observed in rats across several drug classes (6–10), including methamphetamine (meth) (11). Incubation of meth craving was also observed in meth-dependent patients (12). Recently, we and others have begun to explore mechanisms of incubation of meth craving (13–17), extending previous studies that primarily focused on incubation of cocaine craving (4,5,18). In these studies, we found that reversible inactivation of either the central amygdala (16) or the dorsal striatum (DS) (15) decreased “incubated” meth seeking after prolonged withdrawal. The Wolf group demonstrated a critical role of calcium-permeable alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors in the nucleus accumbens (NAc) in this incubation (17). We also used a choice-based procedure to achieve voluntary abstinence and found that either systemic injections of a positive regulator of metabotropic glutamate receptor 2 (AZD8529) (13) or inactivation of

relapse test-activated Fos neurons in the dorsomedial striatum (DMS) (14) decreases incubated meth seeking after voluntary abstinence.

In the current study, we examined the role of the epigenetic enzyme histone deacetylase 5 (HDAC5) in DS in incubation of meth craving. HDAC5 is a member of class IIa HDACs that also includes HDAC4, 7, and 9 (19). Like other HDACs, HDAC5 generally suppresses gene expression by deacetylating histones (20). HDAC5 regulates gene expression and other signaling pathways in an activity-dependent manner by shuttling between the nucleus and cytoplasm, depending on its phosphorylation state (21,22). Previous studies have shown that systemic injections of nonspecific HDAC inhibitors decrease reinstatement of cocaine-induced conditioned place preference (CPP) in mice (23) and decrease reinstatement of cocaine (24) and nicotine (25) seeking in rats, but enhance heroin seeking in rats (26). The Wood group demonstrated in mice that systemic injections of an HDAC3 (a class I HDAC) inhibitor facilitate cocaine CPP extinction (27) while genetic deletion of HDAC3 in the NAc enhances cocaine CPP

acquisition (28). Kennedy *et al.* (29) found in mice that genetic deletion of HDAC1 (another class I HDAC) in the NAc or NAc injections of a class I HDAC inhibitor decrease cocaine locomotor sensitization.

Here, we focused on HDAC5 in DS based on both earlier studies with cocaine and our recent study with meth. For cocaine, previous studies showed a role of NAc HDAC5 in cocaine locomotor sensitization and CPP (30,31); a recent study also showed a role of NAc HDAC5 in cocaine CPP and reinstatement induced by exposure to cocaine cues and cocaine priming injections (32). For meth, we found that *Hdac5* messenger RNA (mRNA) expression is increased in both DS homogenates and relapse test-activated DS neurons after prolonged withdrawal, when meth seeking is high (15). Based on the latter evidence, we hypothesized that HDAC5 positively regulates incubation of meth craving. There are no specific pharmacological compounds to manipulate HDAC5 function (33). Therefore, we used viral approaches to either overexpress or knockdown DS HDAC5 expression and determined whether these manipulations would increase or decrease, respectively, incubation of meth craving. To overexpress HDAC5, we used an adeno-associated virus (AAV) expressing a mutant form of HDAC5 that increases its nuclear localization (32). To knock down HDAC5 expression, we used an AAV expressing a short hairpin against *Hdac5* mRNA (34). After HDAC5 knockdown, we also measured mRNA expression of other DS HDACs (to determine whether HDAC5 knockdown leads to compensatory changes of other HDACs) and potential HDAC5 downstream targets identified in the previous study with HDAC5 knockout mice (30). Finally, we determined the effect of HDAC5 knockdown within either the DMS or the dorsolateral striatum (DLS) on incubation of meth craving to determine the role of HDAC5 in the two major DS anatomical subregions in this incubation.

METHODS AND MATERIALS

For information on subjects, apparatus, intravenous surgery, meth self-administration (Figure 1), withdrawal phase, and relapse tests, see the Supplement.

For information on AAV preparation and injections, immunohistochemistry, image acquisition and HDAC5 immunofluorescence quantification, RNA extraction, complementary DNA synthesis and quantitative polymerase chain reaction (qPCR), and immunoblotting, see the Supplement.

Experiment 1: Effect of Overexpressing Nuclear HDAC5 in DS on Incubation of Meth Craving

We performed intravenous surgery on two groups of rats (total $n = 23$) and injected AAV2-green fluorescent protein (GFP) ($n = 10$) or AAV2-mHDAC5 ($n = 13$) bilaterally into the DS (see Supplement and Figure 2A, B). A week after surgery, we trained both groups of rats for meth self-administration. On withdrawal day 2, we tested the rats for relapse to meth seeking in a 30-minute session. On withdrawal day 30, we tested the rats for relapse in a 3-hour session. Active lever presses during testing [the operational measure of drug seeking in incubation of craving studies (3,4)] resulted in contingent presentations of the tone-light cue, previously paired with meth infusions, but not the drug. After the final relapse test, we perfused the rats and processed their brains for immunohistochemistry (both GFP and HDAC5; see the Supplement). The duration of the test session on day 2 was 30 minutes to minimize carryover effect of extinction learning, which may subsequently decrease drug seeking on day 30 testing (35,36).

We also validated HDAC5 knockdown at the protein levels in drug-naïve rats. We injected AAV-mHDAC5 into one DS hemisphere and AAV-mHDAC5 into the other hemisphere. Three weeks later, when AAV expression was

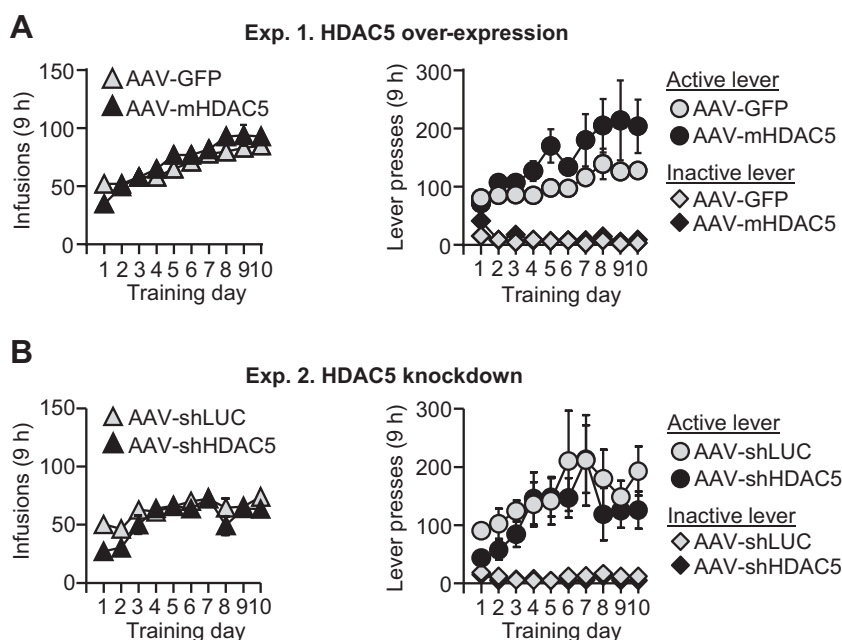


Figure 1. Methamphetamine (meth) self-administration training. Data are mean \pm SEM number of meth infusions (0.1 mg/kg/infusion) and active and inactive lever presses during the ten 9-hour daily self-administration sessions for (A) experiment (Exp.) 1 (total $n = 23$) and (B) experiment 2 (total $n = 22$). During training, active lever presses were reinforced on a fixed ratio 1/20-second timeout reinforcement schedule and meth infusions were paired with a 5-second tone-light cue. AAV, adeno-associated virus; GFP, green fluorescent protein; HDAC5, histone deacetylase 5; mHDAC5, mutant histone deacetylase 5; shHDAC5, short hairpin RNA against histone deacetylase 5; shLUC, short hairpin RNA against luciferase.

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maximal (37), we collected DS tissue for subsequent immunoblotting assays (see the Supplement and Supplemental Figure S1A).

Experiment 2: Effect of Knocking Down HDAC5 Expression in the DS on Incubation of Meth Craving

We performed intravenous surgery on two groups of rats (total $n = 22$) and injected AAV-short hairpin RNA against luciferase (shLUC) ($n = 11$) or AAV-short hairpin RNA against HDAC5 (shHDAC5) ($n = 11$) bilaterally into the DS (see the Supplement and Figure 3A). A week after surgery, we trained both groups of rats for meth self-administration. On withdrawal day 2, we tested the rats for relapse in a 30-minute session. On withdrawal day 30, we tested the rats for relapse in a 3-hour session. After the final relapse tests, we collected DS tissue for subsequent qPCR analysis.

We also independently validated HDAC5 knockdown at both the mRNA ($n = 4$) and protein ($n = 4$) levels in drug-naïve rats. We injected AAV-shLUC into the DS of one hemisphere and AAV-shHDAC5 into the other hemisphere. We collected DS tissue at 3 weeks for subsequent qPCR and immunoblotting assays (Supplemental Figure S1B).

Experiment 3: Effect of Knocking Down HDAC5 Expression in the DMS or DLS on Incubation of Meth Craving

We performed intravenous surgery on four groups of rats (total $n = 47$). We injected AAV-shLUC or AAV-shHDAC5 into the DMS (AAV-shLUC, $n = 11$; AAV-shHDAC5, $n = 13$) or the DLS (AAV-shLUC, $n = 10$; AAV-shHDAC5, $n = 13$; Supplemental Figures S2 and S3). A week after surgery, we trained the rats for meth self-administration. On withdrawal day 2, we tested the rats for relapse in a 30-minute session. On withdrawal day 30, we tested the rats for relapse in a 3-hour session. After the final relapse tests, we collected DMS and DLS tissue for subsequent qPCR analysis.

For the statistical analysis, see the Supplement.

RESULTS

Experiment 1: Effect of Overexpressing Nuclear HDAC5 in the DS on Incubation of Meth Craving

The goal of experiment 1 was to examine whether DS HDAC5 plays a sufficient role in incubation of meth craving. For this purpose, we delivered an AAV into the DS to overexpress a nuclear-localized HDAC5 (AAV-mHDAC5; Figure 2B, C) and examined whether this overexpression would increase incubation of meth craving.

Self-administration Training. For information on self-administration training, see Figure 1A and the Supplement.

HDAC5 Protein Expression. The AAV-mHDAC5 group showed ~2-fold increase of HDAC5 immunofluorescence intensity in the DS compared with the AAV-GFP group ($t_{21} = 4.5$, $p < .001$; Figure 2C). We also validated AAV-mHDAC5 using immunoblotting in drug-naïve rats and obtained similar results (Supplemental Figure S1A).

Relapse Tests. DS HDAC5 overexpression increased meth seeking on withdrawal day 30, but not on day 2. As we tested the rats for relapse in a 30-minute session on day 2 and in a 3-hour session on day 30, we performed two analyses (Figure 2D, E). The first analysis of the relapse tests on days 2 and 30 included the between-subjects factor of virus condition (AAV-GFP, AAV-mHDAC5), the within-subjects factor of withdrawal day (2,30), and inactive lever as a covariate; for this analysis, we used the data from the 30-minute relapse test on day 2 and the first 30 minutes of the relapse test on day 30. The analysis showed significant main effects of withdrawal day ($F_{1,19} = 42.9$, $p < .001$) and virus condition ($F_{1,19} = 5.6$, $p = .029$), and an approaching significant interaction between the two factors ($F_{1,19} = 3.5$, $p = .075$). The second analysis of day 30 only included the between-subjects factor of virus condition, the within-subjects factor of session minutes (30-minute intervals), and inactive lever as a covariate; for this analysis, we used the data from the 3-hour relapse test. The analysis showed main effects of virus condition ($F_{1,21} = 5.4$, $p = .030$) and session minutes ($F_{5,105} = 34.6$, $p < .001$), but no interaction between the two factors ($p > .1$); the lack of interaction suggests that HDAC5 overexpression had no effect on within-session extinction learning.

In summary, the data in experiment 1 demonstrated that DS HDAC5 overexpression modestly potentiated incubated meth seeking, and establish that HDAC5 in this region plays a sufficient role in this incubation.

Experiment 2: Effect of Knocking Down HDAC5 Expression in the DS on Incubation of Meth Craving

The goal of experiment 2 was to examine whether HDAC5 plays a necessary role in incubation of meth craving. For this purpose, we delivered an AAV into the DS to express an shHDAC5 (AAV-shHDAC5), which decreased *Hdac5* expression at both the mRNA and protein levels (Supplemental Figure S1B). We examined whether this downregulation would decrease incubation of meth craving. We used an AAV expressing an shLUC, not expressed in mammals, as the control AAV (AAV-shLUC).

Self-administration Training. For information on self-administration training, see Figure 1B and the Supplement.

Gene Expression of HDACs. We first validated HDAC5 knockdown in the DS of drug-naïve rats (Figure 3C, Supplemental Figure S1B). HDAC5 of the AAV-shHDAC5-injected hemisphere decreased to ~50% of the AAV-shLUC-injected hemispheres at both the mRNA ($t_3 = 6.8$, $p = .006$) and protein ($t_3 = 4.3$, $p = .023$) levels. HDAC5 knockdown (Figure 3C; $t_{19} = 12.7$, $p < .001$) also led to a compensatory increase in expression of *Hdac1* and *Hdac4* mRNA levels (*Hdac1* [$t_{19} = 2.7$, $p = .015$], *Hdac4* [$t_{19} = 2.6$, $p = .016$]).

Relapse Tests. HDAC5 knockdown decreased meth seeking on withdrawal day 30, but not on day 2 (Figure 3D, E). We performed two analyses identical to those described in experiment 1. The first analysis of days 2 and 30 included the between-subjects factor of virus condition (AAV-shLUC, AAV-shHDAC5), the within-subjects factor of withdrawal day, and inactive lever as a covariate; for this analysis, we used the data from the 30-minute relapse test on day 2 and the first 30 minutes of the

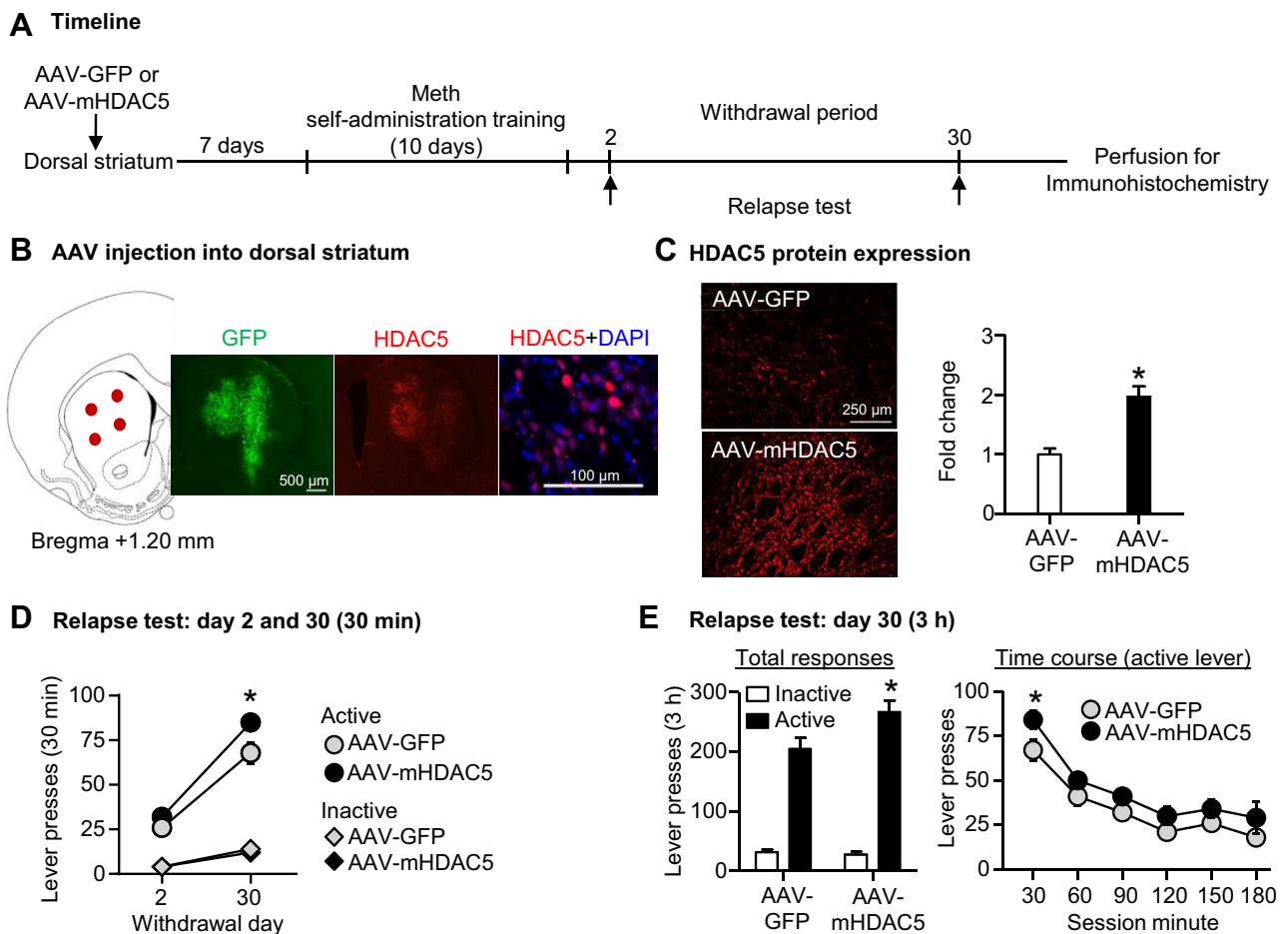


Figure 2. Overexpressing nuclear histone deacetylase 5 (HDAC5) in the dorsal striatum (DS) potentiated incubation of methamphetamine craving. **(A)** Timeline of the experiment. **(B)** The left image shows representative anatomical locations of adeno-associated virus (AAV) injections (red dots) into the DS [millimeters from Bregma (97)]. The right images are representative of green fluorescent protein (GFP) immunostaining from the AAV-GFP group, HDAC5 immunostaining from the AAV-mutant HDAC5 (mHDAC5) group, and HDAC5 (red) + DAPI (blue) double staining from the AAV-mHDAC5 group. **(C)** The left images are representative of HDAC5 immunostaining from the DS of the AAV-GFP and AAV-mHDAC5 groups. The right image is the relative HDAC5 fluorescent intensity in the DS. Data are presented as fold change of mean values of the AAV-GFP group. * $p < .001$; $n = 10-13$ per group. Error bars indicate SEM. **(D)** Relapse test on withdrawal days 2 and 30: data are mean \pm SEM of responses on the previously active lever and on the inactive lever during the 30-minute relapse test on withdrawal day 2 and the first 30 minutes of the 3-hour relapse test on withdrawal day 30. *Different from day 2, $p < .05$; $n = 10-13$ per group. **(E)** Relapse test on withdrawal day 30: data are mean \pm SEM of responses on the previously active lever and on the inactive lever during the 3-hour relapse test. During testing, lever presses led to contingent presentations of the tone-light cue previously paired with meth infusions during training, but not meth. *Different from AAV-GFP, $p < .01$; $n = 10-13$ per group.

relapse test on day 30. The analysis showed a significant interaction between withdrawal day and virus condition ($F_{1,18} = 12.0$, $p = .003$). The second analysis of day 30 included the between-subjects factor of virus condition, the within-subjects factor of session minutes, and inactive lever as a covariate; for this analysis, we used the data from the 3-hour relapse test. This analysis showed a significant interaction between virus condition and session minutes ($F_{5,100} = 5.1$, $p < .001$).

Gene Expression of Potential HDAC5 Targets. Based on previous study in HDAC5 knockout mice (30), we measured mRNA expression of 10 HDAC5 targets (Figure 3F). Consistent with previous findings, *Gnb4* and *Suv39h1* in the DS of the AAV-shHDAC5 group increased compared with the AAV-shLUC control group (*Gnb4* [$t_{19} = 3.0$, $p = .007$], *Suv39h1*

[$t_{19} = 3.1$, $p = .006$]). In contrast, other genes either did not change (*Grin2a*, *Kcnk4*, *Kcnq5*, *Rgs20*, *Rapgef6*, *Abca5*, *Cuedc1*; $p > .05$) or decreased (*Tacr1* [$t_{19} = 2.7$, $p = .015$]).

In summary, the data in experiment 2 demonstrated that DS HDAC5 knockdown decreased incubated meth seeking, indicating that HDAC5 in this brain region plays an important role in incubation of meth craving. Additionally, downregulation of HDAC5 led to increased expression of other HDACs and some HDAC5 targets.

Experiment 3: Effect of Knocking Down HDAC5 Expression in the DMS or DLS on Incubation of Meth Craving

The goal of experiment 3 was to examine whether the role of DS HDAC5 in incubation of meth craving is subregion specific. For

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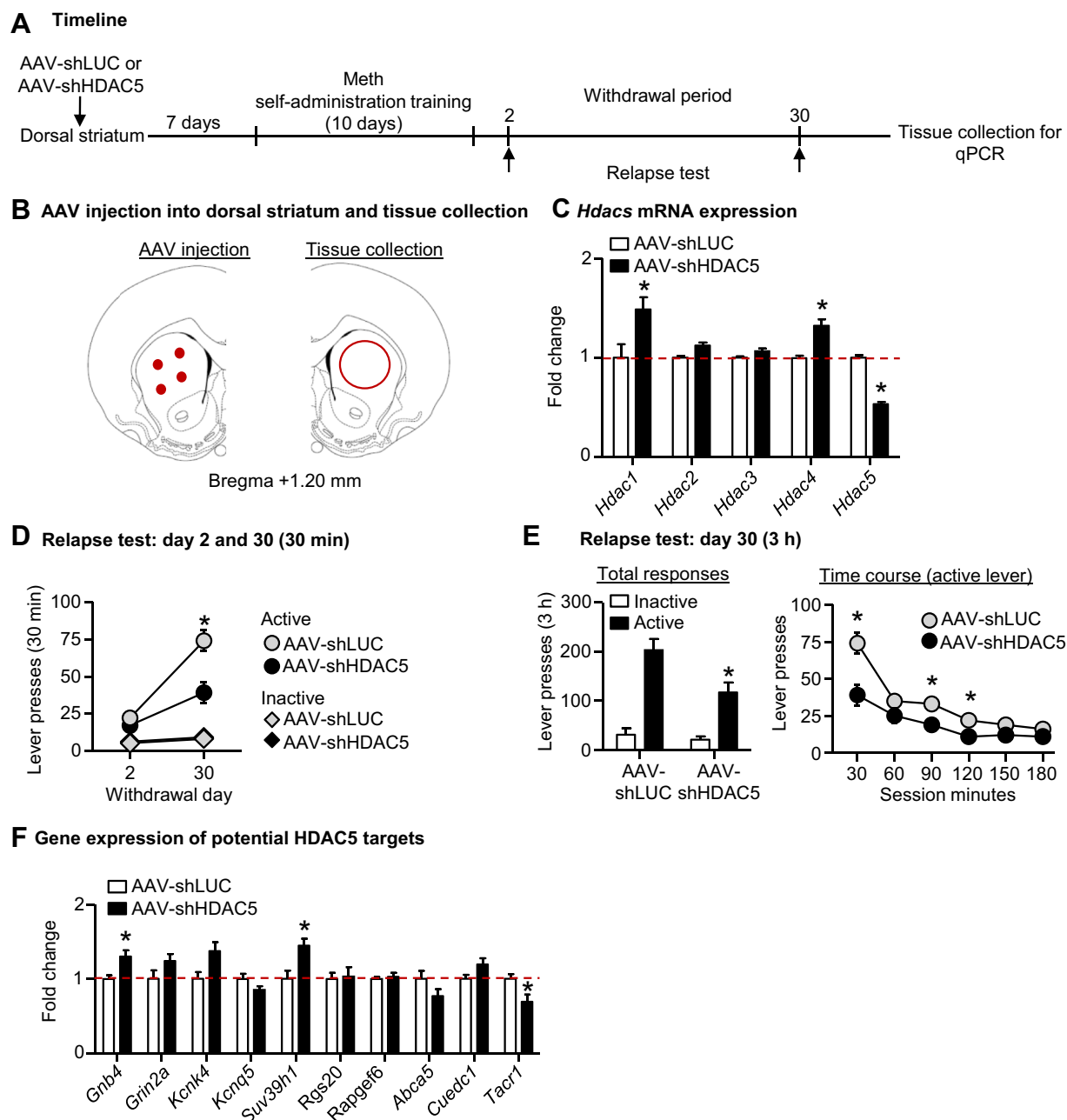


Figure 3. Knocking down histone deacetylase 5 (HDAC5) expression in the dorsal striatum (DS) decreased incubation of methamphetamine craving. **(A)** Timeline of the experiment. **(B)** The left image shows the representative anatomical location of adeno-associated virus (AAV) injections (red dots) to the DS [millimeters from Bregma (97)]. The right image shows a representative schematic of DS tissue collection (red circle). **(C)** *Hdac* messenger RNA (mRNA) expression. Data are presented as fold change of mean values in the AAV-short hairpin RNA against luciferase (shLUC) group. Error bars indicate SEM. * $p < .05$; $n = 9-11$ per group. **(D)** Relapse test on withdrawal days 2 and 30: data are mean \pm SEM of responses on the previously active lever and on the inactive lever during the 30-minute relapse test on withdrawal day 2 and the first 30 minutes of the 3-hour relapse test on withdrawal day 30. *Different from day 2, $p < .05$, $n = 11$ per group. **(E)** Relapse test on withdrawal day 30: data are mean \pm SEM of responses on the previously active lever and on the inactive lever during the 3-hour relapse test. *Different from AAV-short hairpin RNA against HDAC5 (shHDAC5), $p < .01$; $n = 11$ per group. **(F)** Gene expression of potential HDAC5 targets. Data are presented as fold change of mean values in the AAV-shLUC group. Error bars indicate SEM. * $p < .05$; $n = 10-11$ per group. qPCR, quantitative polymerase chain reaction.

this purpose, we delivered an AAV-shHDAC5 into either the DMS or the DLS and examined whether downregulation of HDAC5 in either region would decrease incubation of meth craving.

Self-administration Training. For information on self-administration training, see [Supplemental Figure S2](#) and the [Supplement](#).

Gene Expression of HDAC5. We validated HDAC5 knockdown in the DMS and DLS. HDAC5 expression in the DMS or DLS of the AAV-shHDAC5 group decreased to ~50% of its respective AAV-shLUC group (DMS [$t_{20} = 7.8, p < .001$], DLS [$t_{20} = 8.0, p < .001$]; [Supplemental Figure S3C](#)).

Relapse Tests. HDAC5 knockdown in the DMS or DLS had no significant effect on meth seeking on either withdrawal day 2 or 30 ([Supplemental Figure S3D–F](#)). We analyzed the DMS and DLS HDAC5 knockdown experiments separately. We performed two analyses in each experiment like those described in experiment 1, except that we did not use inactive lever presses as a covariate (see the [Supplement](#)). The first analysis included the between-subjects factor of virus condition and the within-subjects factor of withdrawal day; for this analysis, we used the data from the 30-minute relapse test on day 2 and the first 30 minutes of the relapse test on day 30. The analysis showed significant main effects of withdrawal day (DMS [$F_{1,22} = 20.2, p < .001$], DLS [$F_{1,21} = 62.4, p < .001$]), but not virus condition (DMS [$F_{1,22} = 0.6, p = .811$], DLS [$F_{1,21} = 3.2, p = .087$]). For the DMS, the interaction between the two factors was not significant ($p > .05$), whereas for the DLS the interaction was approaching statistical significance ($F_{1,21} = 3.5, p = .074$). The second analysis of day 30 included the between-subjects factor of virus condition and the within-subjects factor of session minutes; for this analysis, we used the data from the 3-hour relapse test. This analysis showed a main effect of session minutes (DMS [$F_{5,110} = 18.3, p < .001$], DLS [$F_{5,105} = 29.5, p < .001$]), but again no interaction between virus condition and session minutes (p values $> .05$).

In summary, the data in experiment 3 demonstrated that HDAC5 knockdown in the DMS or DLS alone had no significant effect on incubated meth seeking.

DISCUSSION

We used AAVs to study the role of HDAC5 in the DS in incubation of meth craving. The main finding of our study is that HDAC5 overexpression or knockdown in the DS increased or decreased, respectively, incubated meth seeking during late withdrawal. In contrast, neither HDAC5 overexpression nor knockdown in the DS influenced meth seeking during early withdrawal. Additionally, HDAC5 knockdown in the DS changed the transcriptional profiles of some of its putative downstream targets and increased the expression of other *Hdacs* (*Hdac1* and *4*). Finally, HDAC5 knockdown in either the DMS or the DLS alone had no effect on incubation of meth craving. Together, our results demonstrated that HDAC5 function in the entire DS, but not a specific subregion, plays an important role in incubation of meth craving.

Role of Epigenetic Mechanisms in DS in Animal Models of Addiction

Previous studies on epigenetic mechanisms assessed cocaine-induced psychomotor stimulation and CPP ([28–31, 38–44](#)), cocaine self-administration ([40,45–50](#)), extinction of cocaine or morphine CPP ([23,27,51–53](#)), and cocaine cue- and cocaine priming-induced reinstatement ([24,44,54](#)). Recent epigenetic studies also examined extinction and reinstatement

of nicotine seeking ([25](#)), heroin priming-induced reinstatement ([26](#)), and alcohol consumption ([55–59](#)). However, only one published study has examined the causal role of persistent epigenetic adaptations in drug seeking after prolonged withdrawal from extended-access drug self-administration. Massart *et al.* ([60](#)) reported that incubation of cocaine craving is associated with time-dependent changes of DNA methylation in the NAc and that DNA methylation positively regulates this incubation. Our current study extends this previous work and provides new evidence that histone modification plays a causal role in persistent drug seeking after prolonged withdrawal.

Our results on the role of epigenetic mechanisms in DS in relapse extend previous studies on the role of epigenetic changes in the NAc ([41,42](#)), medial prefrontal cortex ([46,48,57](#)), amygdala ([56](#)), and ventral tegmental area ([51,61](#)) in the behavioral effects of addictive drugs. Our results are also in agreement with previous studies on the role of the DS (either the DMS or the DLS, or both) in cue- or context-induced cocaine ([62–65](#)), meth ([15,66](#)), and heroin ([67](#)) seeking. Recent evidence also indicates a role of the DS in alcohol taking and seeking ([68–74](#)).

Regarding epigenetic mechanisms in DS, the only evidence available comes from the Kenny group, who demonstrated critical roles of local microRNA-212 ([75](#)) and methyl CpG binding protein 2 (a transcriptional repressor) ([50](#)) in escalation of cocaine self-administration. Taken together with our data, these findings highlight the role of epigenetic mechanisms in DS in drug reward and relapse.

Role of Striatal HDAC5 in the Behavioral Effects of Psychostimulant Drugs

Our focus on HDAC5 was initially inspired by two publications on the role of HDAC5 in cocaine's effects. At the molecular level, Renthal *et al.* ([30](#)) and Taniguchi *et al.* ([31](#)) demonstrated that repeated cocaine exposure induces transient changes in striatal HDAC5 activity. At the behavioral level, Renthal *et al.* ([30](#)) reported that HDAC5 knockout mice show increased cocaine CPP and that viral overexpression of HDAC5 in the NAc decreases drug CPP. Taniguchi *et al.* ([31](#)) reported that viral overexpression of a nuclear-localized HDAC5 in the NAc decreases cocaine CPP. Very recently, Taniguchi *et al.* ([32](#)) extended their previous findings and reported that viral overexpression of the nuclear-localized HDAC5 decreases cue-induced and cocaine priming-induced reinstatement of cocaine seeking. Overall, these data indicate that cocaine alters HDAC5 activity in the NAc and that HDAC5 in the NAc negatively regulates cocaine reward and relapse.

In contrast, we found a time-dependent increase of *Hdac5* mRNA expression in the rat DS (but not the NAc; Xuan Li, Ph.D., unpublished data, May 2015) when meth seeking is high (incubated) ([15](#)). Together with our current study, these data indicate that HDAC5 in the DS positively regulates incubation of meth craving. What might account for the different roles of striatal HDAC5?

One factor is the duration and amount of drug exposure, which are significantly lower in CPP or short-access self-administration than in extended-access self-administration studies. Indeed, it is well-established that extended drug

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self-administration causes different physiological and behavioral effects from cocaine CPP or short-access cocaine self-administration (5,76–78). Another factor is the drug itself: cocaine versus meth. Recent evidence suggests that the mechanisms of relapse to cocaine and meth seeking are at least partially dissociable (79). For example, we found that inactivation of the ventromedial prefrontal cortex, which inhibits incubation of cocaine craving (80), has no effect on incubation of meth craving (16). Additionally, Fos expression in the NAc only increased after context-induced reinstatement of cocaine, but not meth seeking (66,81).

As an epigenetic enzyme, HDAC5 can affect many genes (30), which leads to two final questions: 1) What are HDAC5's targets? and 2) Does HDAC5 exert its role in incubation of meth craving through these targets? Comprehensive answers to these questions are beyond the scope of our current study, but we did an initial exploration. Based on previous microarray data in the HDAC5 knockout mouse NAc after cocaine exposure (30), we probed gene expression of ten potential HDAC5 targets after HDAC5 knockdown in the DS (experiment 2). We found four genes that showed a consistent expression pattern with the previous study in mice (increased mRNA expression for *Gnb4* and *Suv39h1*, but no change for *Grin2a* and *Kcnk4*), while several other genes did not change (*Kcnq5*, *Rgs20*, *Rapgef6*, *Abca5*, *Cuedc1*) or exhibited decreased mRNA expression (*Tacr1*). We also probed *Npas4*, an HDAC5 target recently identified by Taniguchi *et al.* (32). However, we found that *Npas4* mRNA expression decreased after HDAC5 knockdown in DS (Xuan Li, Ph.D., unpublished data, May 2017). These inconsistencies can be due to the complexity of epigenetic regulation where multiple epigenetic factors modulate gene expression simultaneously (82).

Another potential factor is the compensatory increase of HDAC1 and HDAC4 after HDAC5 knockdown in DS. Based on previous evidence implicating a role of striatal HDAC1 in cocaine locomotor sensitization (29) and a role of striatal HDAC4 in cocaine CPP (83), it is possible that increased HDAC1 or HDAC4 expression (or both) contributes to the decreased incubated meth seeking after HDAC5 knockdown in DS.

Finally, it should be noted that probing a transcriptional profile after reducing HDAC5 expression can only indirectly identify HDAC5's targets. Direct evidence comes from chromatin immunoprecipitation against HDAC5 followed by qPCR or genome-wide sequencing. Indeed, HDAC5's role in regulating gene expression also depends on its deacetylase activity (84–87) and subcellular localization (21,22). However, elucidating genomic binding, enzymatic activity, or subcellular localization of HDAC5 is beyond the scope of our current article.

Overall, our data suggest that HDAC5 modulates incubation of meth craving by altering the transcriptional regulation of its direct or indirect gene targets. For example, upregulation of *Suv39h1* [a histone methyltransferase acting as a transcription repressor (88,89)] may contribute to this incubation by remodeling connectivity of activated neurons (90). Additionally, upregulation of *Gnb4* [a G protein that modulates calcium and potassium ion channels (91)] may contribute to incubation of meth craving by activating G protein-gated inwardly rectifying potassium currents (92,93).

Methodological and Interpretation Issues

From an anatomical and functional perspective, a main question is whether the role of DS HDAC5 in incubation of meth craving is subregion specific. This is a relevant question because there is evidence showing that the DMS and DLS play different roles in cue- and context-induced drug seeking in rat relapse models (79). To answer this question, we performed subregion-specific HDAC5 knockdown in the DMS or DLS. Our data showed that knocking down HDAC5 expression in the DMS or DLS alone had no significant effect on incubation of meth craving (Supplemental Figure S3), suggesting that HDAC5 function in the entire DS, but not a specific subregion, must be manipulated to appreciably control incubation of meth craving.

Another main question is whether the role of DS HDAC5 in incubation of meth craving is cell-type specific. Endogenous HDAC5 is enriched in neurons (94). For experiment 1, we used the AAV2 serotype (95) to increase the neurotropism of HDAC5 expression. For experiment 2, we used AAV1, which expresses in both neurons and glial cells (95). Therefore, we cannot exclude the possibility that the behavioral effects in our study are mediated by nonneuronal HDAC5 such as glia cells. We believe that this possibility is unlikely, because we previously found that blockade of Toll-like receptor 4, an innate immune receptor expressed in primarily in microglia, contributes to incubation of heroin but not meth craving (36).

Another unresolved issue is whether HDAC5 in D1-expressing, D2-expressing, or both cell types in DS is critical for incubation of meth craving. Based on our recent studies showing that incubation of meth craving is associated with increased activity [assessed by the neuronal activity marker, Fos (96)] in both DS cell types (14,15), we speculate that HDAC5 activity in both D1- and D2-expressing DS neurons is important for this incubation. In future studies, we hope to develop cell-type specific approaches to test whether perturbing HDAC5 expression in different DS cell types would have a similar effect on incubation of meth craving.

Finally, our data suggest that HDAC5 plays a selective role in incubated meth seeking during late withdrawal, but not nonincubated meth seeking during early withdrawal. However, this conclusion should be made with caution for two reasons. First, under our experimental conditions, viral expression had been at its maximal expression for a longer duration during late versus early withdrawal. Second, as drug seeking is substantially lower during the early versus late withdrawal relapse tests, negative findings during the early withdrawal relapse tests after DS HDAC5 overexpression or knockdown can be due to a floor effect of low operant responding during early withdrawal.

Conclusions

We demonstrated that DS HDAC5 plays an important role in incubation of meth craving. This finding is consistent with our previous study showing time-dependent increases of DS HDAC5 mRNA expression during this incubation (15). In contrast, this novel role of HDAC5 in meth seeking after extended-access drug self-administration contrasts with

previous findings on the enzyme's role in cocaine's behavioral effects (assessed by CPP, locomotor sensitization, and short-access self-administration) (30–32), indicating that there are dissociated epigenetic mechanisms across drug classes, striatal regions, and behavioral procedures. Finally, HDAC5 likely exerts its role in incubation of meth craving through its downstream gene targets, which can be further characterized by combining RNA sequencing and chromatin immunoprecipitation sequencing in future studies. Finally, from a clinical perspective, our study suggests that selective HDAC5 inhibitors could be a potential therapeutic target for decreasing meth craving and relapse after prolonged withdrawal.

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XL, CWC, EJM, and YS conceived the project, provided intellectual input, and wrote the article. XL, KRW, TZ, OML, JMB, JZ, and FS carried out experiments. MBC, CTR, and BKH developed and validated the adeno-associated virus constructs. HS provided the plasmids.

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