Archival Report

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Sex-Specific Regulation of Fear Memory by Targeted Epigenetic Editing of *Cdk5*

Ajinkya S. Sase, Sonia I. Lombroso, Brandon A. Santhumayor, Rozalyn R. Wood, Carissa J. Lim, Rachael L. Neve, and Elizabeth A. Heller

ABSTRACT

BACKGROUND: Sex differences in the expression and prevalence of trauma- and stress-related disorders have led to a growing interest in the sex-specific molecular and epigenetic mechanisms underlying these diseases. Cyclin-dependent kinase 5 (CDK5) is known to underlie both fear memory and stress behavior in male mice. Given our recent finding that targeted histone acetylation of *Cdk5* regulates stress responsivity in male mice, we hypothesized that such a mechanism may be functionally relevant in female mice as well.

METHODS: We applied epigenetic editing of *Cdk5* in the hippocampus and examined the regulation of fear memory retrieval in male and female mice. Viral expression of zinc finger proteins targeting histone acetylation to the *Cdk5* promoter was paired with a quantification of learning and memory of contextual fear conditioning, expression of CDK5, and enrichment of histone modifications of the *Cdk5* gene.

RESULTS: We found that male mice exhibit stronger long-term memory retrieval than do female mice, and this finding was associated with male-specific epigenetic activation of hippocampal *Cdk5* expression. Sex differences in behavior and epigenetic regulation of *Cdk5* occurred after long-term, but not short-term, fear memory retrieval. Finally, targeted histone acetylation of hippocampal *Cdk5* promoter attenuated fear memory retrieval and increased tau phosphorylation in female but not male mice.

CONCLUSIONS: Epigenetic editing uncovered a female-specific role of *Cdk5* activation in attenuating fear memory retrieval. This finding may be attributed to CDK5 mediated hyperphosphorylation of tau only in the female hippocampus. Sex-specific epigenetic regulation of *Cdk5* may reflect differences in the effect of CDK5 on downstream target proteins that regulate memory.

Keywords: CDK5, Epigenetics, Memory, PTSD, Sexual dimorphism, Zinc fingers

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A prominent mechanism by which cells respond to environmental stimuli is regulation of histone posttranslational modifications (hPTMs) (1,2). Evidence of such epigenetic modifications in the context of fear learning and memory is widely documented (3–5). Sexually dimorphic epigenetic gene regulation (6,7) may underlie observed sex differences in pathological memory formation associated with posttraumatic stress disorder (PTSD) (8,9) as well as a host of other neuropathological disorders, including depression (10–12). Many of these disorders include cognitive and anxiety symptoms that are modeled by fear conditioning and memory retrieval in rodents (6,13). However, the precise molecular mechanisms by which sex-specific epigenetic regulation of a given target gene modulates behavior is poorly understood. Targeted epigenetic editing is a novel approach to elucidate the direct causal relevance of epigenetic regulation of a given gene of interest to neuropsychiatric (14,15) and neurodevelopmental (16,17) disease.

One key gene involved in both fear memory formation and stress-related behavior is cyclin-dependent kinase 5 (*Cdk5*), whose involvement has been shown through conditional deletion in the hippocampus (HPC) (18), striatum (19), and forebrain (20) to regulate both the expression and magnitude of fear-related memory and depressive-like phenotypes in male mice. Repeated stress in male mice is accompanied by activation of CDK5, phosphorylation of glucocorticoid receptors, increased expression of histone deacetylase 2, and reduced expression of memory-related genes in the HPC (21). We recently reported that targeted epigenetic activation of *Cdk5* in the nucleus accumbens (NAc) is sufficient to attenuate a depressive phenotype following male social defeat stress (15). Recent studies also point to a role for *Cdk5* gene expression in human depression (22) and a role for *Cdk5* gene expression in sexually dimorphic stress behavior in mice (23). We hypothesized that histone acetylation of the *Cdk5* promoter in the HPC is sufficient to regulate its expression and influence fear memory formation in both male and female mice. We systematically investigated *Cdk5* gene regulation by fear-related memory and retrieval in both sexes. Using the approach of targeted histone acetylation, we identified a novel, sexually dimorphic, epigenetic mechanism that is sufficient to attenuate fear memory retrieval specifically in female mice.

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METHODS AND MATERIALS

Animals and Behavioral Paradigms

Experiments used male and female C57BL/6J mice that were 8 to 10 weeks of age. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Fear conditioning was performed as described previously (24) with three 2-second foot shocks (0.4 mA), separated by a 60-second interval. Memory retrieval test was measured for 5 minutes after 1 hour (short-term memory [STM]) or 24 hours (long-term memory [LTM]). Cocaine-induced conditioned place preference (CPP) was carried out as described previously (15) for 2 days, using 15 mg/kg cocaine. Additional details are presented in the Supplement.

Tissue Collection, RNA Extraction, and Quantitative Real-Time Polymerase Chain Reaction

Mice were euthanized by cervical dislocation. Hippocampal punches were dissected and processed as described previously (15). Additional details, including quantitative real-time polymerase chain reaction primers and analysis method, are provided in the Supplement.

Quantitative Chromatin Immunoprecipitation

Quantitative chromatin immunoprecipitation was performed as described previously (14) on bilateral, 1-mm-diameter punches pooled from CA1 of two mice. A detailed protocol is provided in the Supplement.

Viral-Mediated Gene Transfer

Herpes simplex virus (HSV) expressing zinc finger proteins (ZFPs) fused to p65 were prepared as previously described (15) and as detailed in the Supplement.

Protein Extraction and Western Blotting

Protein extraction and Western blotting was carried out as described previously (14). Complete details of electrophoresis conditions and antibodies used are provided in the Supplement.

Statistics

Statistical analysis was performed using GraphPad Prism version 7 (GraphPad, San Diego, CA). Data were analyzed using two-way analysis of variance with conditioning, virus, and/or sex as factors followed by Bonferroni post hoc analysis for multiple comparisons. All data are expressed as mean ± SEM. Results were considered statistically significant when $p < .05$. Outliers were removed using Grubbs' test ($n = 1$ outlier per cohort). Sample size was determined empirically and based on published literature. Sample size is included in the figure legends.

RESULTS

Long-term Fear Memory Retrieval Differs Between Male and Female Mice

To expand understanding of the well-established role of *Cdk5* in both learning- and stress-related behavior in male mice, we focused our attention on fear-related memory in both sexes. We quantified STM retrieval as 1 hour and LTM retrieval as 24 hours after fear conditioning (Figure 1A). Percentage time spent freezing was quantified in fear-conditioned and non-shocked, context-only control mice (Figure 1B). In both sexes, freezing behavior increased over the course of the three-shock acquisition phase (Figure 1B), freezing was greatest during LTM retrieval, and overall freezing behavior was greater in shocked mice than in nonshocked mice. Interestingly, we found that female mice displayed a reduced magnitude of LTM retrieval compared with that of male mice. There was no sex difference in acquisition or STM retrieval.

Sex-Specific Activation of CDK5 Expression Following Long-term Fear Memory Retrieval

Given the observed sex differences in LTM retrieval and the known role of CDK5 in fear memory (18,25) and stress (15) in male mice, we next examined expression of CDK5 following acquisition and short- and long-term memory retrieval (see Figure 1A). We examined CDK5 expression CA1 of the HPC, as this region is functionally relevant to fear memory formation

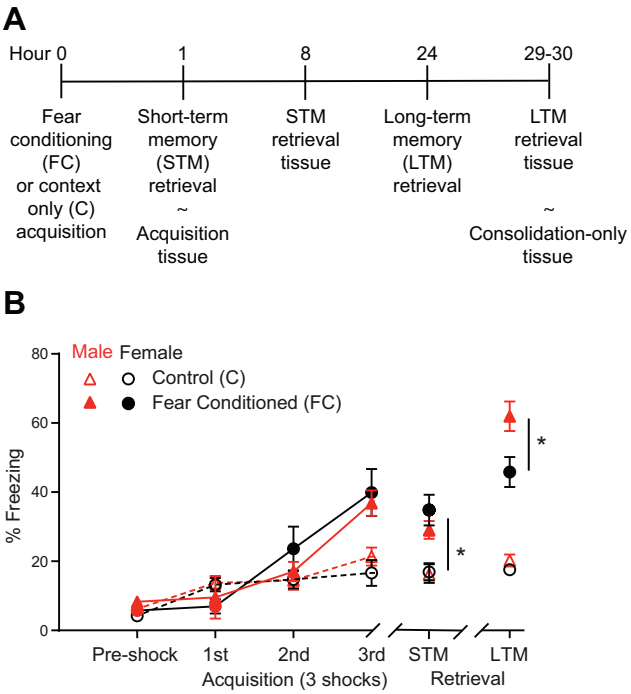


Figure 1. Long-term memory (LTM) retrieval differs between male and female mice. **(A)** Experimental timeline, in hours, depicting phases of fear conditioning and tissue collection. **(B)** Male and female mice respond differently to contextual fear conditioning, measured as percentage of time freezing (two-way analysis of variance: main effects of sex [$F_{3,35} = 9.833$, $p < .0001$], fear conditioning [$F_{4,140} = 67.67$, $p < .0001$], interaction [$F_{12,140} = 10.45$, $p < .0001$]). There was no significant difference between freezing in fear-conditioned male and female mice during acquisition (preshock, $p > .9999$; first shock, $p > .9999$; second shock, $p = .4593$; third shock, $p = .7521$) and short-term memory (STM) retrieval ($p = .2410$), but there was a lower percentage of time freezing during LTM retrieval ($p = .0377$) in female mice compared with that of male mice. In all cases, *Cdk5* expression in fear-conditioned (FC) animals was compared with that in their nonshocked, context-only control counterparts (C). All data are presented as mean ± SEM. $n = 8$–10, $^*p < .05$.

(26–28). In order to distinguish regulation of *Cdk5* by learning and memory from that of re-exposure to the unconditioned stimuli (context), we included a consolidation-only group that underwent fear conditioning and LTM consolidation but no retrieval test.

We found that expression of *Cdk5* messenger RNA (mRNA) was increased in CA1 of male, but not female, mice after LTM retrieval, compared with its expression in the respective non-shocked, context-only control groups (Figure 2A). The male-specific increase in *Cdk5* expression was limited to the LTM retrieval phase, as there was no difference in *Cdk5* mRNA expression in CA1 of mice of either sex after acquisition or consolidation phases, compared with that found in control mice (Figure 2A). We further validated the mRNA result by Western blotting and confirmed that CDK5 protein expression in CA1 was increased after LTM retrieval in male but not female mice (Figure 2B; Supplemental Figure S1).

To determine whether fear memory regulation of CDK5 expression was specific to CA1, we next examined expression of *Cdk5* in the NAc, a reward-related brain area in which CDK5 expression is known to regulate affective behavior in male mice (15,29) There was no significant change in *Cdk5* mRNA expression after LTM retrieval in the NAc of mice of either sex (Figure 2C). We also examined mRNA expression of brain-derived neurotrophic factor from alternative promoter IV, which is implicated in LTM retrieval (4). We found a significant increase in *Bdnf* mRNA expression following LTM retrieval in CA1 region of both sexes (Figure 2D), indicating that male-specific fear-induced gene expression is not universal.

To ensure that hormonal regulation of *Cdk5* was not responsible for the observed effects on LTM retrieval and CDK5 expression, we analyzed *Cdk5* mRNA expression in a separate cohort of naïve estrous-tracked females. We selected to compare proestrous and estrous females with males because, although proestrus lasts for short time, it shows maximum hormonal changes (30,31). No significant differences in *Cdk5* expression were measured between these groups (Supplemental Figure S2).

Sex-Specific Epigenetic Regulation of *Cdk5* Expression Following Long-term Fear Memory Retrieval

Histone acetylation and methylation of the *Cdk5* promoter activate and repress *Cdk5* expression, respectively, in brain reward regions to affect stress and depression (15,32), yet epigenetic regulation of *Cdk5* in the HPC has not yet been examined. Thus, we tested the hypothesis that histone acetylation of the *Cdk5* promoter underlies male-specific mRNA expression following fear conditioning. We used

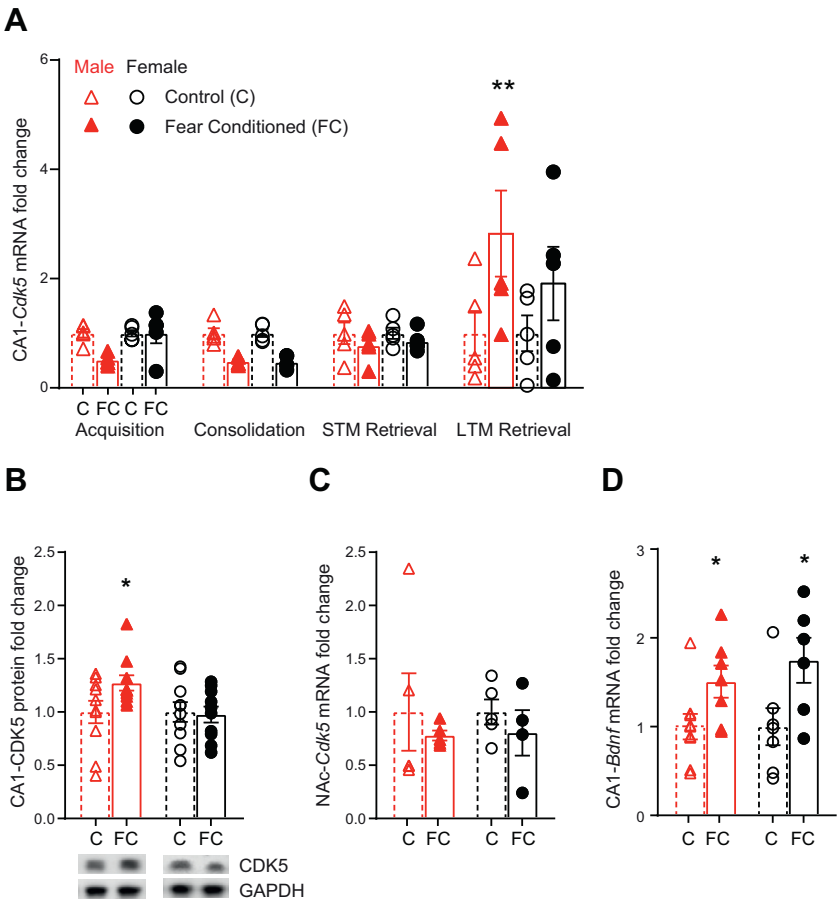


Figure 2. Sex-specific activation of cyclin-dependent kinase 5 (CDK5) expression following long-term memory (LTM) retrieval. **(A)** *Cdk5* messenger RNA (mRNA) expression was quantified by quantitative real-time polymerase chain reaction after each phase of fear conditioning (FC) (two-way analysis of variance [ANOVA], main effect of sex [$F_{1,62}$ = 0.04781, p = .8276], and FC [$F_{7,62}$ = 6.666, $p < .0001$] as factors). *Cdk5* expression was increased following LTM retrieval in CA1 of male (p = .0096) but not female ($p > .9999$) mice compared with that in respective behavioral control mice (C) (n = 10). **(B)** CDK5 protein expression in CA1 after LTM retrieval (two-way ANOVA, main effect of sex [$F_{1,36}$ = 5.861, p = .0206], FC [$F_{1,36}$ = 6.266, p = .0170], interaction [$F_{1,36}$ = 4.197, p = .0478]). CDK5 protein expression was increased after LTM retrieval in male (p = .0470) but not in female ($p > .9999$) mice compared with that in respective behavioral control mice (n = 10). **(C)** *Cdk5* expression in the nucleus accumbens (NAc) did not change after LTM retrieval in mice of either sex (two-way ANOVA, main effect of sex [$F_{1,15}$ = 0.003143, p = .9560], FC [$F_{1,15}$ = 0.8602, p = .3683]) (n = 4–5). **(D)** Brain-derived neurotrophic factor (*Bdnf*) mRNA expression in CA1 was increased following LTM retrieval (two-way ANOVA, main effect of FC [$F_{1,24}$ = 14.33, p = .0009] and sex [$F_{1,24}$ = 0.0006153, p = .9804]) in both male (p = .0424) and female (p = .0421) mice (n = 7–10). All data are presented as mean ± SEM. *$p < .05$, **$p < .01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; STM, short-term memory.

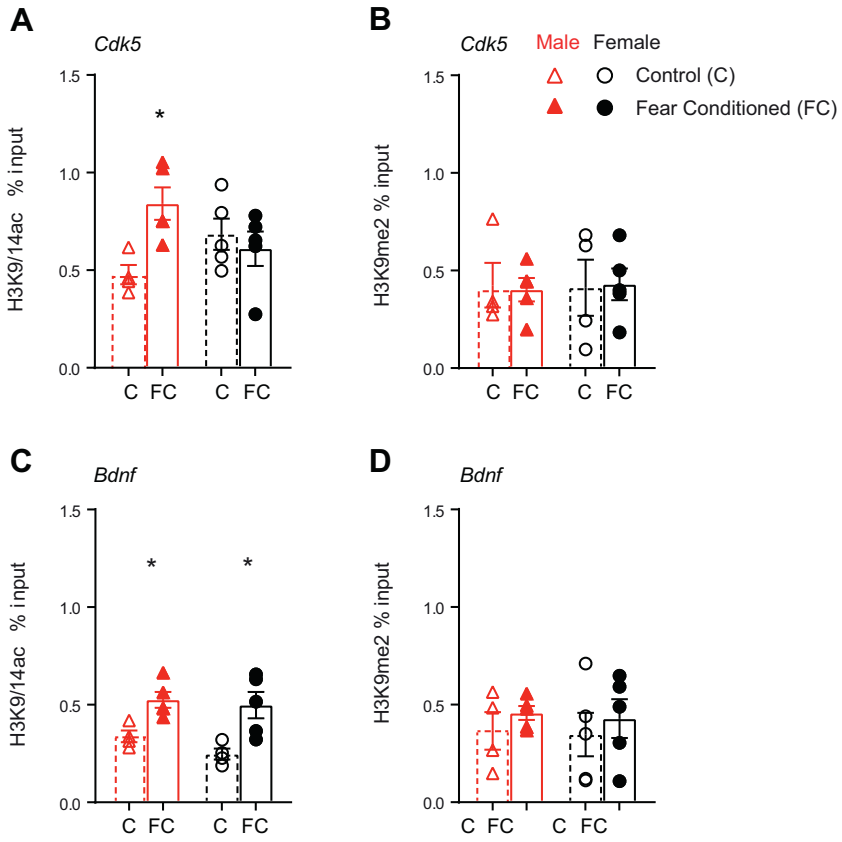
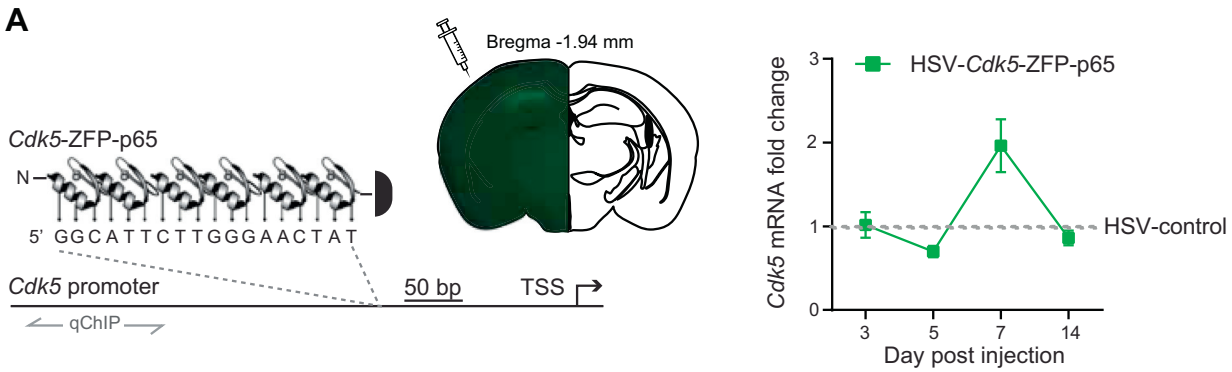
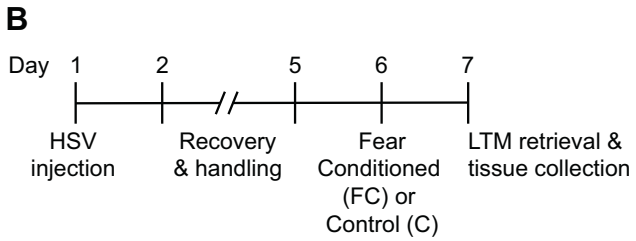


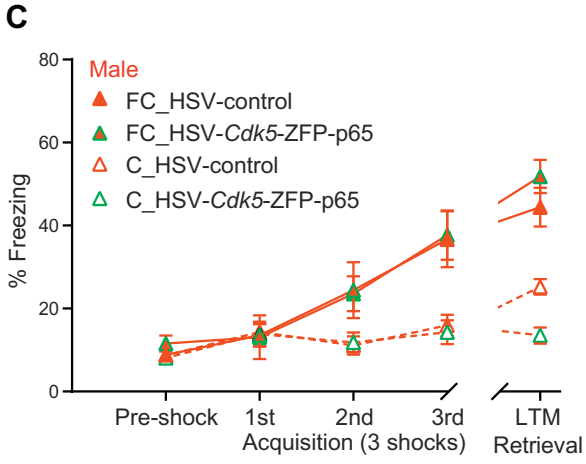
Figure 3. Sex-specific epigenetic regulation of cyclin-dependent kinase 5 (CDK5) expression following long-term memory retrieval. **(A)** Histone H3 lysine 9/14 acetylation (H3K9/14ac) enrichment of the *Cdk5* promoter in CA1 was quantified by quantitative chromatin immunoprecipitation after long-term memory retrieval (two-way analysis of variance, main effect of sex [$F_{1,15}$ = 0.02164, p = .8850], fear conditioning (FC) [$F_{1,15}$ = 3.282, p = .0901], interaction [$F_{1,15}$ = 7.504, p = .0152]). FC enriched H3K9/14ac at the *Cdk5* promoter in CA1 of male (p = .0415) but not female (p > .9999) mice, compared with that of the respective behavioral control mice (C). **(B)** FC had no effect on enrichment of histone H3 lysine 9 di-methylation (H3K9me2) at the *Cdk5* promoter in CA1 of either male (p > .9999) or female (p > .9999) mice (two-way analysis of variance, main effect of sex [$F_{1,14}$ = 0.006133, p = .9387], FC [$F_{1,14}$ = 0.0007834, p = .9781], interaction [$F_{1,14}$ = 0.04226, p = .8401]). **(C)** H3K9/14ac enrichment was greater at the brain-derived neurotrophic factor (*Bdnf*) IV promoter following FC (two-way analysis of variance, main effect of FC [$F_{1,14}$ = 23.93, p = .0002], sex [$F_{1,14}$ = 2.094, p = .1699], interaction [$F_{1,14}$ = 0.2557, p = .6210]) in both male (p = .0469) and female (p = .0113) mice compared with that in respective behavioral control mice. **(D)** There was no difference in H3K9me2 enrichment at the *Bdnf* factor IV promoter following FC (two-way analysis of variance, main effect of sex [$F_{1,15}$ = 0.07074, p = .7939], FC [$F_{1,15}$ = 0.9145, p = .3541], interaction [$F_{1,15}$ = 0.003047, p = .9567]). Quantitative chromatin immunoprecipitation data are normalized to the input of the corresponding sample. n = 4 or 5 chromatin immunoprecipitation samples per behavioral group. All data are presented as mean ± SEM. *p < .05.

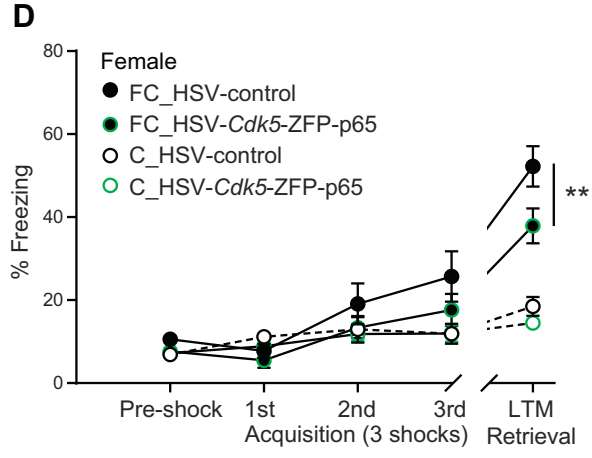
quantitative chromatin immunoprecipitation to measure enrichment of histone H3 lysine 9/14 acetylation (H3K9/14ac) and histone H3 lysine 9 di-methylation (H3K9me2), which are hPTMs associated with gene activation and repression, respectively. We examined these epigenetic changes in CA1 following LTM retrieval because of the male-specific increase in *Cdk5* mRNA expression at this time point (see Figure 2A). H3K9/14ac was enriched at the *Cdk5* promoter in male CA1 following fear conditioning compared with that of non-shocked, context-only control mice (Figure 3A). There was no difference in H3K9/14ac at *Cdk5* in female CA1 (Figure 3A). There was no difference in *Cdk5* enrichment of

Figure 4. Epigenetic editing of the cyclin-dependent kinase 5 (*Cdk5*) promoter regulates long-term memory (LTM) retrieval in female mice only. **(A)** Schematic of zinc finger protein (ZFP)–mediated epigenetic editing and representative image of CA1-expressing herpes simplex virus (HSV)–*Cdk5*-ZFP-p65; green fluorescent protein. Time-course analysis found that maximum activation of *Cdk5* in CA1 occurred 7 days after HSV injection. *Cdk5* messenger RNA (mRNA) was analyzed by quantitative real-time polymerase chain reaction and normalized to a nonfunctional control virus, HSV-control (n = 8 or 9). **(B)** Timeline of mice injected with either HSV-control or HSV-*Cdk5*-ZFP-p65 and subjected to fear conditioning (FC). **(C)** Male and **(D)** female FC mice injected with either HSV-*Cdk5*-ZFP-p65 or HSV-control showed a gradual increase in freezing during acquisition (two-way analysis of variance [ANOVA], main effect of FC [$F_{4,36}$ = 67.92, p < .0001], sex [$F_{3,27}$ = 3.946, p < .0187], interaction [$F_{12,108}$ = 0.9452, p = .4969]). Male FC mice injected with HSV-*Cdk5*-ZFP-p65 did not differ in acquisition or LTM retrieval freezing compared with that of FC mice injected with HSV-control (preshock p > .9999, first shock p > .9999, second shock p > .9999, third shock p > .9999, and LTM p > .9999). Female FC mice injected with HSV-*Cdk5*-ZFP-p65 displayed comparable freezing during acquisition and reduced freezing in LTM retrieval compared with that of HSV-control–injected mice (preshock p > .9999, first shock p > .9999, second shock p > .9999, third shock p > .9999, and LTM p = .0365). HSV-*Cdk5*-ZFP-p65–injected female mice froze less than males during LTM retrieval (p = .0285). Nonshocked control mice injected with HSV-*Cdk5*-ZFP-p65 did not differ from those injected with HSV-control in terms of freezing (preshock p > .9999, first shock p > .9999, second shock p > .9999, third shock p > .9999, and LTM p > .9597). **(E)** Male and **(F)** female Western blot analysis of CDK5 expression after LTM retrieval confirmed increased CDK5 levels in HSV-*Cdk5*-ZFP-p65–injected mice (two-way ANOVA, main effect of virus [$F_{1,5}$ = 9.939, p = .0253], sex [$F_{1,5}$ = 1.159, p = .2213], interaction [$F_{1,5}$ = 7.321, p = .0458]). CDK5 expression in mice injected with HSV-*Cdk5*-ZFP-p65 was greater than that of HSV-control–injected mice (male p = .0026, female p = .0417). **(G, H)** Western blot analyses of phosphorylated tau protein (p-Tau) expression after LTM retrieval in HSV-*Cdk5*-ZFP-p65–injected **(G)** male and **(H)** female mice (two-way ANOVA, main effect of virus [$F_{1,5}$ = 16.13, p = .0102], sex [$F_{1,5}$ = 10.73, p = .0221], interaction [$F_{1,5}$ = 13.28, p = .0148]). p-Tau levels in mice injected with HSV-*Cdk5*-ZFP-p65 were greater in female (p = .0276) but not male (p > .9999) mice compared with those in HSV-control–injected mice. There was no effect on p-tau levels in HSV-*Cdk5*-ZFP-p65–injected, nonshocked control mice (two-way ANOVA, main effect of virus [$F_{1,5}$ = 1.965, p = .2199], sex [$F_{1,5}$ = 2.649, p = .1646], interaction [$F_{1,5}$ = 1.351, p = .2976]). All data are presented as mean ± SEM. n = 6–10. *p < .05, **p < .01. bp, base pair; C, control; qChIP, quantitative chromatin immunoprecipitation; TSS, transcription start site.


A
Bregma -1.94 mm
Cdk5-ZFP-p65
N
5' GGCATTCTTGGGAACTAT
Cdk5 promoter
50 bp
TSS
qChIP
HSV-Cdk5-ZFP-p65
HSV-control
Cdk5 mRNA fold change
Day post injection


B
Day 1 2 5 6 7
HSV injection
Recovery & handling
Fear Conditioned (FC) or Control (C)
LTM retrieval & tissue collection


C
Male
FC_HSV-control
FC_HSV-Cdk5-ZFP-p65
C_HSV-control
C_HSV-Cdk5-ZFP-p65
% Freezing
Pre-shock
1st
2nd
3rd
Acquisition (3 shocks)
LTM Retrieval


D
Female
FC_HSV-control
FC_HSV-Cdk5-ZFP-p65
C_HSV-control
C_HSV-Cdk5-ZFP-p65
% Freezing
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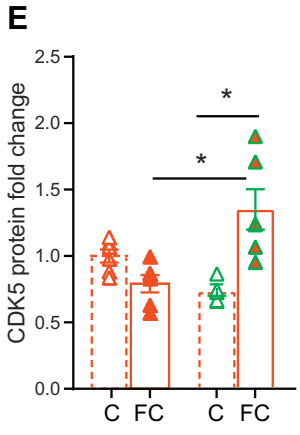
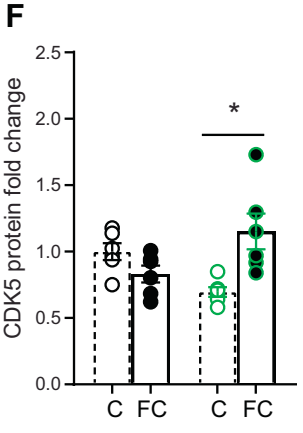
Pre-shock
1st
2nd
3rd
Acquisition (3 shocks)
LTM Retrieval


E
CDK5 protein fold change
C FC C FC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CDK5 protein fold change
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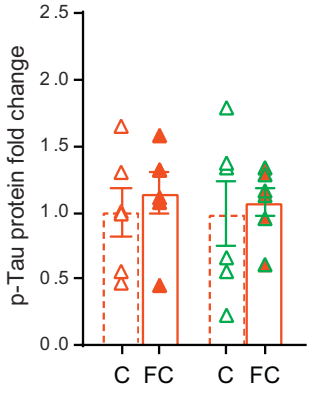
G
p-Tau protein fold change
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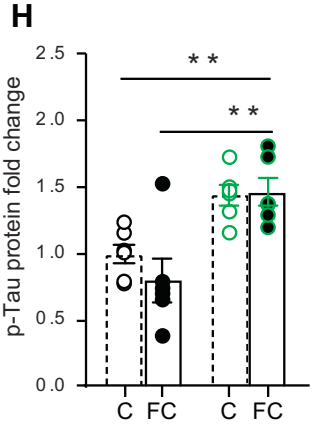
H
p-Tau protein fold change
C FC C FC

the repressive modification, H3K9me2, following LTM retrieval in mice of either sex (Figure 3B). As a positive control, we also examined histone modifications at the *Bdnf* exon IV promoter and found that H3K9/14ac was enriched in CA1 of both sexes (Figure 3C). There was no difference in the enrichment of H3K9me2 in mice of either sex at the *Bdnf* promoter (Figure 3D). These results are consistent with a male-specific increase in *Cdk5* expression and an increase in *Bdnf* expression in both sexes following fear conditioning.

Targeted Epigenetic Editing of *Cdk5* Attenuates Long-term Fear Memory Retrieval in Female Mice Only

To directly test the causal relevance of male-specific acetylation and expression of *Cdk5* in long-term fear memory consolidation, we applied targeted epigenetic editing to acetylate H3K9/14 at the *Cdk5* promoter in CA1. Engineered ZFPs were composed of six zinc fingers that uniquely bind an 18–base pair motif in the *Cdk5* promoter region (15) and regulate acetylation via fusion to the p65 transcriptional activation domain (Figure 4A). This approach mimics experience-driven transcriptional regulation of *Cdk5* in both magnitude (29,33,34) and mechanism (35,36).

Cdk5-ZFP-p65 constructs were packaged into HSV and stereotactically delivered to the CA1 region of the HPC (Figure 4A). Prior studies have found that epigenetic editing of the *Cdk5* locus regulates behaviors learned over 4 to 10 days (15). Because fear conditioning is a single-trial learning paradigm, we performed an initial time-course study and determined that the maximum activation of *Cdk5* in CA1 by HSV-*Cdk5*-ZFP-p65 occurs 7 days after HSV injection (Figure 4A). To recapitulate the timing of endogenous *Cdk5* acetylation and expression following fear conditioning (34) (see Figure 3A), we injected CA1 with HSV-*Cdk5*-ZFP-p65 and subjected mice to fear conditioning on day 6, followed by LTM retrieval on day 7 (Figure 4B). We confirmed targeting and expression of HSV-*Cdk5*-ZFP-p65 in CA1 using a fluorescent stereoscope (see representative image in Figure 4A); non–HPC-targeted or nonexpressing animals were removed from the study. We compared the effects of HSV-*Cdk5*-ZFP-p65 with those of a nonfunctional control virus, HSV-control, which expresses the p65 subunit alone (14,15).

All fear-conditioned mice increased freezing during LTM retrieval relative to that of context-only control mice (Figure 4C, D), indicating that viral injection did not interfere overall with the formation of fear memory in mice of either sex. Surprisingly, targeted acetylation of *Cdk5* had no effect on LTM in male mice (Figure 4C), while it decreased LTM in female mice, relative to respective HSV-control–injected mice (Figure 4D). Further, LTM retrieval in female HSV-*Cdk5*-ZFP-p65–injected mice was lower than that in males (compare Figure 4C, D), which is consistent with reduced freezing levels in virus-naïve female mice relative to those in male mice (see Figure 1B). There was no effect of HSV-*Cdk5*-ZFP-p65 on acquisition in mice of either sex, nor on freezing levels in nonshocked, context-only control mice (Figure 4C, D). In mice of both sexes, HSV-*Cdk5*-ZFP-p65 injection increased CDK5 expression compared with that in HSV-control–injected mice (Figure 4E, F; Supplemental Figure S3A).

We noted that in male mice injected with HSV, fear conditioning did not cause an increase in CDK5 expression (Figure 4E, F; Supplemental Figure S3) as expected based on results in virus-naïve male mice (Figure 2A). We hypothesized that surgery and anesthesia may repress *Cdk5* expression, masking the effect of fear-conditioning–activated expression in this context. This hypothesis is supported by the fact that in the rat HPC, general anesthesia decreases histone H3 acetylation and histone acetyltransferase activity of cyclic adenosine monophosphate response element binding protein-binding protein, leading to repression of brain-derived neurotrophic factor and c-Fos expression (37). To determine the effect on *Cdk5* expression of two commonly used anesthetics, ketamine and isoflurane, with and without intracranial surgery, and to recapitulate the observed effect in virus-injected fear conditioning experiments (Figure 4), we collected CA1 tissue 7 days after surgery and analyzed *Cdk5* expression by quantitative real-time polymerase chain reaction. Indeed, we found that ketamine and isoflurane anesthesia, coupled with intracranial surgery, repressed *Cdk5* expression compared with that in surgery-naïve male and female mice (Supplemental Figure S4A). We also found that HSV-*Cdk5*-ZFP-p65 expression overcomes the repression caused by anesthesia and surgery, while HSV-control does not (Supplemental Figure S4B).

CDK5 Activation Led to Hyperphosphorylation of Tau Protein in CA1 of Female but Not Male Mice

To elucidate a potential mechanism for CDK5-mediated attenuation of LTM retrieval, we measured phosphorylation of tau protein, a direct and well-characterized downstream target of CDK5. Tau protein phosphorylation has been implicated in memory deficits, including those of working and reference memory (38,39) and spatial memory (40–42). CDK5 phosphorylates tau protein at serine 396 (43), and female-specific neurological effects of hyperphosphorylated tau protein are well documented (44–48). To determine the role of CDK5-mediated hyperphosphorylation of tau protein in memory deficits, we measured serine 396 phosphorylated tau protein relative to total tau protein in male and female mice injected with HSV-*Cdk5*-ZFP-p65 and subjected to fear conditioning. In male mice, HSV-*Cdk5*-ZFP-p65 and LTM retrieval did not change phosphorylated tau protein levels compared with those of HSV-control–injected and nonshocked, context-only male mice (Figure 4G and Supplemental Figure S3A). Conversely, in female mice, HSV-*Cdk5*-ZFP-p65 and LTM retrieval increased phosphorylated tau protein levels (Figure 4H and Supplemental Figure S3B). The female-specific phosphorylation of tau protein is consistent with female-specific attenuation of LTM retrieval following activation of *Cdk5* expression.

Targeted Epigenetic Editing of *Cdk5* Attenuates Short-term Fear Memory Retrieval in Female Mice Only and Has No Effect on Acquisition or CPP

Although *Cdk5* mRNA expression was not regulated during fear memory acquisition or STM retrieval in mice of either sex (Figure 2A), we considered that exogenous acetylation might impact these phases of learning and memory. To

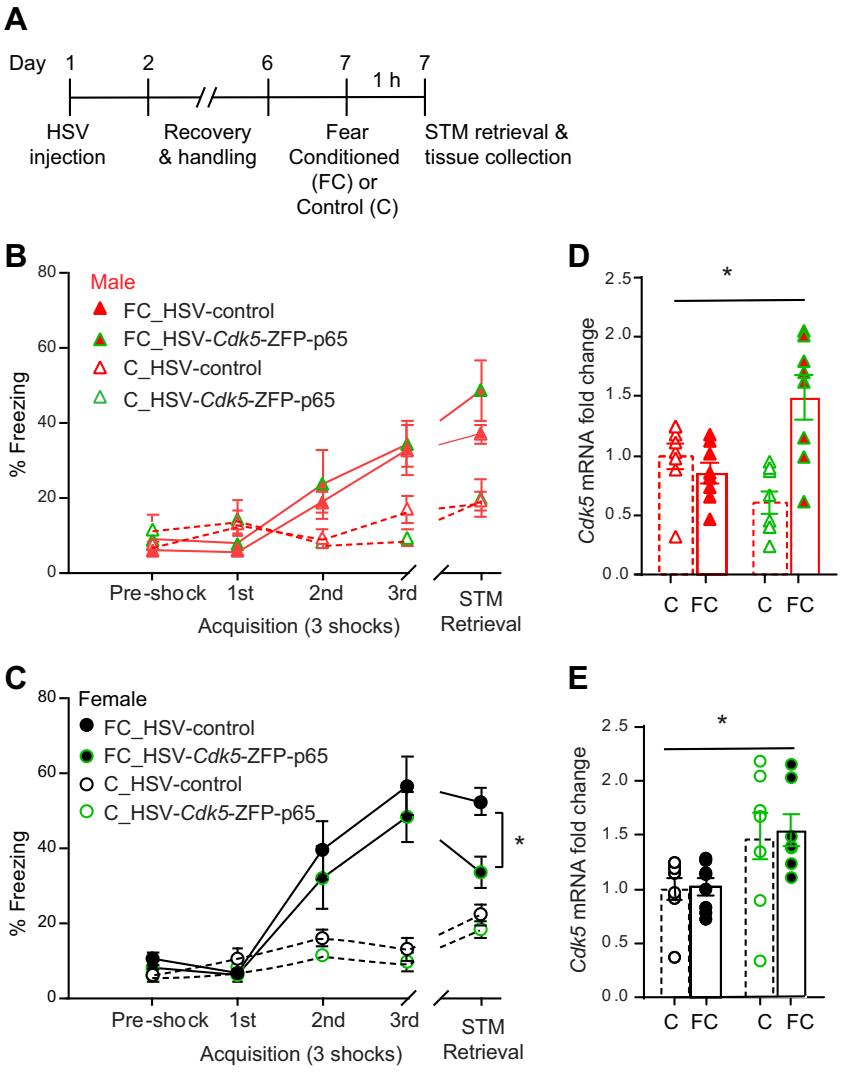

Figure 5. Epigenetic editing of the cyclin-dependent kinase 5 (*Cdk5*) promoter regulates short-term memory (STM) retrieval in female mice only. **(A)** Mice were injected with either the nonfunctional control herpes simplex virus (HSV) (HSV-control) or HSV-*Cdk5*–zinc finger protein (ZFP)–p65 and subjected to fear conditioning (FC) followed by STM retrieval. **(B)** Male and **(C)** female FC mice injected with either HSV-*Cdk5*-ZFP-p65 or HSV-control showed gradual increase in freezing during acquisition (two-way analysis of variance, main effect of FC [$F_{4,24}$ = 110.8, $p < .0001$], sex [$F_{3,18}$ = 1.303, p = .3042], interaction [$F_{12,72}$ = 1.985, p = .0380]). Male FC mice injected with HSV-*Cdk5*-ZFP-p65 did not differ in acquisition or short-term memory freezing from FC mice injected with HSV-control (preshock $p > .9999$, first shock $p > .9999$, second shock $p > .9999$, third shock $p > .9999$, and STM $p > .1188$). Female FC mice injected with HSV-*Cdk5*-ZFP-p65 displayed comparable freezing during acquisition and reduced freezing during STM retrieval (preshock $p > .9999$, first shock $p > .9999$, second shock $p > .9999$, third shock $p > .9999$, and STM p = .0270), compared with freezing of mice injected with HSV-control. Nonshocked, male control mice (C) injected with HSV-*Cdk5*-ZFP-p65 did not differ in freezing from control mice injected with HSV-control (preshock $p > .9999$, first shock $p > .9999$, second shock $p > .9999$, third shock $p > .9999$, and STM $p > .9999$). HSV-*Cdk5*-ZFP-p65–injected female mice froze less than male mice in STM retrieval (p = .0068). **(D, E)** Quantitative real-time polymerase chain reaction analysis of *Cdk5* after STM retrieval showed increased *Cdk5* expression in HSV-*Cdk5*-ZFP-p65–injected **(D)** male and **(E)** female mice (two-way analysis of variance, main effect of virus [$F_{1,7}$ = 6.795, p = .0351], sex [$F_{1,7}$ = 0.3431, p = .5764], interaction [$F_{1,7}$ = 2.263, p = .1762]). *Cdk5* expression in mice injected with HSV-*Cdk5*-ZFP-p65 was greater than that of HSV-control–injected mice (male p = .0011, female p = .0125). *Cdk5* messenger RNA (mRNA) expression was increased in HSV-*Cdk5*-ZFP-p65–injected, nonshocked control mice after STM retrieval (two-way analysis of variance, main effect of virus [$F_{1,7}$ = 7.888, p = .0262], sex [$F_{1,7}$ = 0.0004, p = .9839], interaction [$F_{1,7}$ = 0.0003, p = .9850]). *Cdk5* expression in mice injected with HSV-*Cdk5*-ZFP-p65 showed a trend toward increased expression compared with that of HSV-control–injected mice (male p = .0696, female p = .0724). All data are presented as mean ± SEM. n = 7 or 8. *$p < .05$.

acetylate the *Cdk5* promoter during acquisition and STM retrieval, we injected CA1 with HSV-*Cdk5*-ZFP-p65 and subjected mice to fear conditioning and STM retrieval 7 days later. There was no effect on acquisition in mice of either sex (Figure 5B, C). Alternatively, while targeted *Cdk5* acetylation had no effect on STM retrieval in male mice (Figure 5B), it decreased STM retrieval in female mice, relative to that in respective HSV-control–injected mice (Figure 5C). Distinct from LTM retrieval, STM retrieval following HSV-*Cdk5*-ZFP-p65 injection was not lower in female mice than that in male mice. We measured *Cdk5* mRNA expression by quantitative real-time polymerase chain reaction 6 hours after STM retrieval. In both male and female mice, HSV-*Cdk5*-ZFP-p65 injection and STM retrieval increased *Cdk5* expression compared with that in HSV-control–injected mice (Figure 5D, E).

To assess whether the reduction in LTM produced by HSV-*Cdk5*-ZFP-p65 in CA1 is specific to contextual fear memory, we analyzed the effect of this treatment on cocaine-induced CPP, another type of hippocampal-dependent learning and memory (49,50). Importantly, in the NAc of male mice, targeted methylation of *Cdk5* attenuates cocaine-induced CPP, while acetylation has no effect (15). We analyzed CPP behavior in male and female mice 7 days after CA1 injection of HSV-control and HSV-*Cdk5*-ZFP-p65 (Figure 6A). This time point corresponds to maximal acetylation during the preference test, which is analogous to maximal acetylation during the LTM retrieval in the fear conditioning paradigm. However, unlike the attenuated LTM retrieval observed in female mice following CA1 *Cdk5* promoter acetylation, we did not observe any effect of this manipulation on cocaine-induced CPP in mice of either sex (Figure 6B).

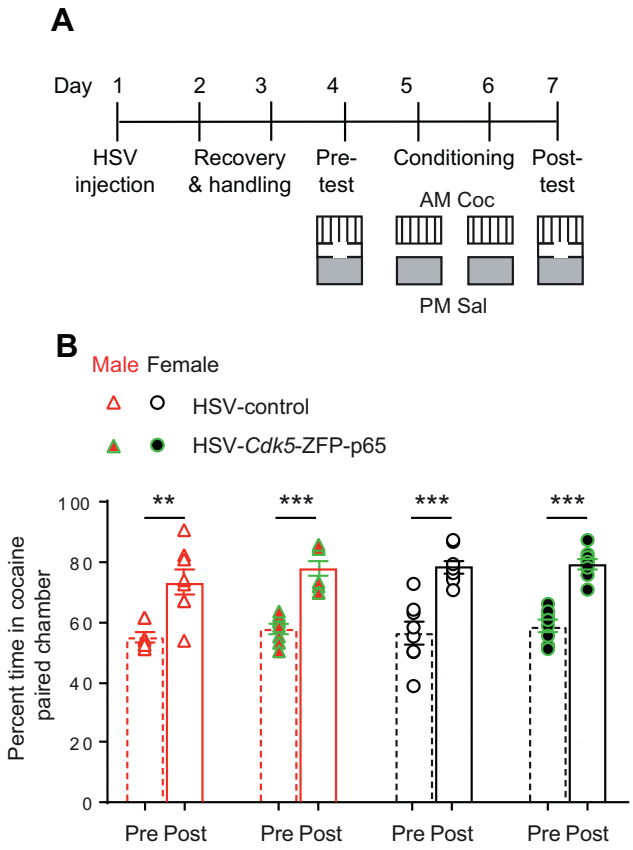

Figure 6. Epigenetic editing of cyclin-dependent kinase 5 (*Cdk5*) promoter in the hippocampus does not affect cocaine-induced conditioned place preference. **(A)** Timeline of herpes simplex virus (HSV) injection and cocaine-induced conditioned place preference. **(B)** Percent time spent in cocaine-paired chamber of virus-injected male and female mice. The male and female mice injected with either the nonfunctional control virus HSV-control or HSV-*Cdk5*–zinc finger protein (ZFP)–p65 spent more time in the cocaine-paired chamber (two-way analysis of variance, main effect of conditioning [$F_{1,7} = 53.17$, $p = .0002$], sex [$F_{3,21} = 2.518$, $p = .0858$]). The time spent in the cocaine-paired chamber was greater in all groups following conditioning (Bonferroni multiple comparison, male HSV-control $p = .0011$, female HSV-control $p = .0001$, male HSV-*Cdk5*-ZFP-p65 $p = .0005$, female HSV-*Cdk5*-ZFP-p65 $p = .0003$). All data are presented as mean ± SEM. $n = 6–8$. $^{**}p < .01$, $^{***}p < .001$. AM Coc, morning cocaine; PM Sal, afternoon saline.

DISCUSSION

Sex differences in the extent and nature of mood disorders have led to a growing interest in the sexual specificity of molecular mechanisms underlying these diseases (51,52). Given our recent finding that histone acetylation and methylation of the *Cdk5* gene promoter regulate stress and reward responsivity in male mice (15), we hypothesized that such mechanisms may be functionally relevant in female mice as well. We focused our attention on the role of *Cdk5* in fear-related memory in order to link the known functions of CDK5 in both learning- and stress-related behavior. Fear conditioning is a robust translational paradigm used in many cases to elucidate fear-related mechanisms of PTSD, such as fear extinction, fear inhibition, and generalization of fear (53–64). We applied the innovative strategy of targeted epigenetic editing to elucidate the precise causal relevance of specific chromatin modifications to *Cdk5* expression and fear memory.

We first observed that female mice showed lower fear memory retrieval than did male mice, suggesting a female-specific mechanism for fear memory protection. While the difference is subtle, it is significant, reproducible, and consistent with the literature (65–68). We further found that enrichment of the activating hPTM, H3K9/14ac, at the *Cdk5* promoter did not change in female mice following fear conditioning, whereas in male mice, both H3K9/14ac and CDK5 expression were increased. To elucidate the causal relevance of male-specific *Cdk5* promoter acetylation, we targeted histone acetylation to the *Cdk5* promoter, which increased CDK5 expression in mice of both sexes. Surprisingly, *Cdk5* promoter acetylation attenuated STM and LTM retrieval in female mice only. This sexually dimorphic effect was accompanied by a female-specific increase in phosphorylation of tau protein, a CDK5 target implicated in learning and memory. Based on these findings, we propose a model in which *Cdk5* promoter acetylation, expression, and subsequent downstream target phosphorylation is sex-specifically regulated (Figure 7). We posit that in female mice, fear memory activation of *Cdk5* expression is blocked to control its downstream effects (e.g., tau protein phosphorylation) (Figure 7A). When this "break" on *Cdk5* expression is lifted by exogenous, locus-targeted acetylation, female mice display a fear memory deficit (Figure 7B). This finding is in contrast to what was found for male mice, in which both *Cdk5* promoter acetylation and activation are naturally increased following fear memory retrieval, and further, exogenous activation has no effect. Our model is supported by the fact that hyperphosphorylated tau protein affects microtubule dynamics, axonal transport, and neurite outgrowth, resulting in neurodegenerative pathologies (48). Female-specific neurological effects of hyperphosphorylated tau protein are well documented (44–48). For example, transgenic expression of hyperphosphorylated tau protein leads to a greater impairment in spatial learning and memory in female mice compared with that in male mice (45), and overexpression of corticotrophin-releasing factor increases tau protein phosphorylation in female mice, leading to an impairment in working memory (47). Given these sex differences, the 'break' on fear-induced *Cdk5* activation in female CA1 may not be necessary in male CA1. While we investigated tau protein, *N*-methyl-D-aspartate receptor subunit 2B (NR2B) is another target of CDK5 associated with sex-specific synaptic transmission and plasticity (53,69). CDK5 phosphorylates NR2B and reduces its cell membrane expression (25). The literature reveals disagreement, however, on the precise mechanism of CDK5-mediated attenuation of fear memory. Recent studies point to the role of CDK5 in synaptic plasticity through both internalization (25) and reduced degradation of NR2B (70). Future studies to determine the extent of sex-specific downstream targeting by CDK5 will be useful to clarify the precise role of tau protein, NR2B, and other CDK5 targets in fear memory processing.

Sex differences in fear learning in rodents have been documented, with a role for calcium/calmodulin-dependent protein kinase alpha signaling in precipitating greater contextual fear conditioning in male rodents than in female rodents

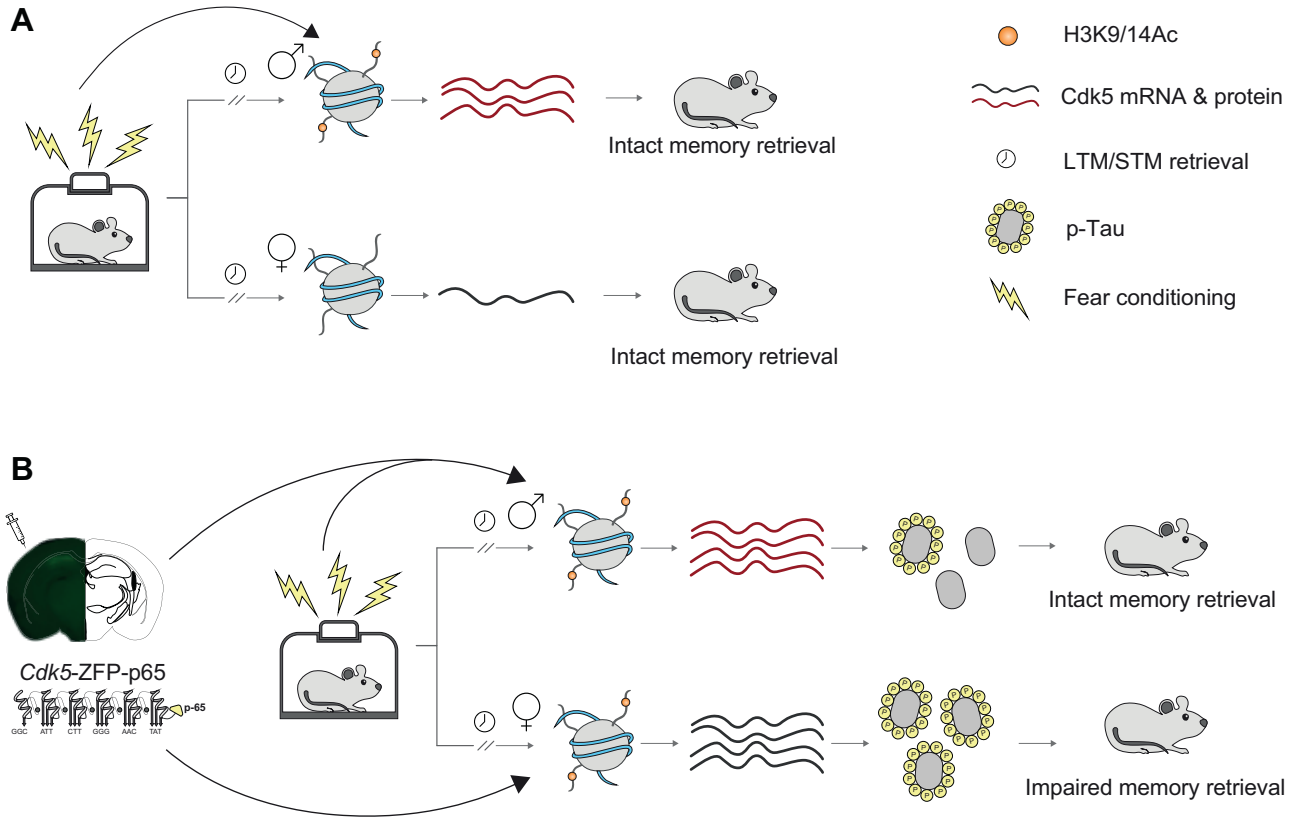

Figure 7. Our model proposes that cyclin-dependent kinase 5 (*Cdk5*) expression is sex-specifically regulated to control its downstream effects. **(A)** Fear conditioning of female mice does not change expression of CDK5 or enrichment of the activating histone posttranslational modification, histone H3 lysine 9/14 acetylation (H3K9/14ac), at the *Cdk5* promoter, whereas in male mice, fear conditioning activates CDK5 expression and enriches H3K9/14ac. **(B)** Locus-specific histone acetylation at the *Cdk5* promoter increases CDK5 expression in CA1 of both female and male mice. Yet this increase in CDK5 causes fear memory loss specifically in female mice, where it is correlated with a female-specific increase in phosphorylation of tau protein. We propose a model in which CDK5 activation in female CA1 is restrained following fear conditioning, to regulate the downstream effects of CDK5 activity. LTM, long-term memory; mRNA, messenger RNA; p-Tau, phosphorylated tau protein; STM, short-term memory; ZFP, zinc finger protein.

(71,72), which supports our similar observation. Additionally, heat exposure of female rats enhances fear extinction and retrieval associated with changes in hippocampal synaptic morphology (73). Sex differences have been observed in fear generalization, in which aversive experiences (e.g., shock) in one context cause unrelated neutral contexts to be processed as threatening. Male mice display greater c-Fos activity in the dorsal HPC during memory retrieval but less fear generalization, whereas female mice show greater fear generalization (74). One caveat to observed sex differences in fear-conditioning paradigms is the recent description of a "darting" phenotype—a rapid, forward movement across the chamber—associated with auditory fear conditioning in female, but not male, rats (75). Failure to quantify darting may contribute to the reduced retrieval measured in female mice. Beyond this, studies of sex-specific effects of fear conditioning have focused on the role of sex hormones (65), finding a role for estradiol in fear generalization (76) and for testosterone in auditory memory and long-term potentiation (77). We found no difference in *Cdk5* expression based on estrous cycle, but additional studies are needed to elucidate the interaction between basal sex hormone levels, estrous cycle stages, and fear memory retrieval in the epigenetic activation of CDK5 expression.

The lack of a fear memory deficit in male mice following *Cdk5* promoter acetylation was unexpected given that fear conditioning activated CDK5 expression and that conditional deletion of *Cdk5* in forebrain excitatory neurons results in poor spatial learning and memory in male mice (20). Such deletion is associated with hyperactivity, impaired cognitive function, and deficits in neurotransmitter release (20). Prior studies have used either pharmacological manipulation or conditional deletion of *Cdk5*, suggesting that the mechanism of manipulation may affect outcome measure. Targeted epigenetic editing recapitulated the endogenous mechanism and magnitude of CDK5 expression to reveal a previously unobserved, sex-specific epigenetic role for this kinase in fear memory retrieval. Importantly, we confirmed that CPP is not affected by *Cdk5* promoter acetylation in either CA1 or the NAc (15), indicating a specific role for CDK5 expression in contextual fear memory but not contextual reward memory. One conflicting outcome of these studies was the lack of CDK5 activation following fear conditioning of male mice injected with HSV-control. We reasoned that this deficit in *Cdk5* activation may be due to the effects of surgery and anesthesia in the context of viral manipulations but not in naïve mice. In support of this hypothesis, general anesthesia

is reported to cause histone modifications. For example, brains of 7-day-old rat pups show decreased histone acetylation following nitrous oxide and isoflurane anesthesia, while the suprachiasmatic nucleus of mice anesthetized with sevoflurane/oxygen shows decreased histone H4 acetylation of period circadian regulator 2 (37,78). We found that anesthesia reduced *Cdk5* expression, which could be overcome by HSV-*Cdk5*-ZFP-p65–activated expression. Fear conditioning alone (with HSV-control) was not sufficient to overcome repression of *Cdk5* expression by anesthesia and/or surgery, accounting for the lack of activation in this context.

The relevance of sex-specific gene expression to affective disorders is underscored in several studies of global and specific gene expression (23,79) and DNA methylation (79) in both male and female subjects. As reviewed recently (51), sex plays a key role in the extent of PTSD in the human population such that women develop this disorder at twice the rate of men (80). PTSD severity is associated with DNA methylation of the SLC6A4 locus in female but not in male persons (6). PTSD may be associated with hormonally regulated DNA methylation of histone deacetylase 4, the expression of which is greater in the amygdala following auditory fear conditioning in wild-type female subjects compared with ovariectomized female subjects (8). In addition, increased promoter DNA methylation and reduced expression of the nuclear receptor subfamily 3 group C member 1 is associated with reduced PTSD risk in male, but not female, survivors of the Rwandan genocide (81). These compelling examples of sex-specific epigenetic regulation of DNA methylation at PTSD risk loci point to the potential for additional modes of gene regulation including hPTMs, like that identified in our study, as well as noncoding RNA (82–84) and chromatin conformation (85,86), mechanisms that are also implicated in susceptibility to stress disorders.

To contribute to understanding of sex-specific epigenetic regulation in fear memory, we specifically investigated the transcriptional regulation of CDK5 and found that the *Cdk5* promoter in male but not female CA1 is hyperacetylated after fear conditioning. Histone acetylation plays a vital role in gene expression related to fear memory (87–89) and anxiety (90–92). Sex-specific regulation of hPTMs has been documented, with male neonatal brain showing increased H3K9/14ac and H3K9me3 relative to female mice (93–95). Global chromatin profiling of female rat astrocytes finds a greater number of H3K4me3 peaks and greater H3K4–specific methyltransferase activity in young adult than in middle-aged female mice (96). There are also sex differences in the expression of histone acetyltransferases and deacetylases, which correlate with sex-specific gene expression (97). Quantification of histone acetyltransferase and/or histone deacetylase enrichment at *Cdk5*, as well as targeted deacetylation of *Cdk5*, can be applied to further our understanding of sex-specific epigenetic regulation of this locus.

Conclusions

Cdk5-targeted histone acetylation in CA1 attenuates fear memory retrieval in female, but not male, mice. This difference may be due to the increase in *Cdk5* expression and acetylation in male, but not female, mice after fear memory retrieval, as well as female-specific tau protein phosphorylation following *Cdk5* activation. These results point to the relevance of *Cdk5* promoter acetylation in sexually dimorphic fear memory formation and related disorders.

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ARTICLE INFORMATION

From the Department of Systems Pharmacology and Translational Therapeutics (ASS, SIL, BAS, RRW, CJL, EAH) and the Penn Epigenetics Institute (EAH), University of Pennsylvania, Philadelphia, Pennsylvania; and Gene Delivery Technology Core (RLN), Massachusetts General Hospital, Boston, Massachusetts.

Address correspondence to Elizabeth A. Heller, Ph.D., 10-115 Smilow Center for Translational Research, 3400 Civic Center Boulevard, Building 421, Philadelphia, PA 19104–5158; E-mail: eheller@pennmedicine.upenn.edu.

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