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X Chromosome Factor Kdm6a Enhances Cognition Independent of Its Demethylase Function in the Aging XY Male Brain

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Abstract

Males exhibit shorter life span and more cognitive deficits, in the absence of dementia, in aging human populations. In mammals, the X chromosome is enriched for neural genes and is a major source of biologic sex difference, in part, because males show decreased expression of select X factors (XY). While each sex (XX and XY) harbors one active X due to X chromosome inactivation in females, some genes, such as *Kdm6a*, transcriptionally escape silencing in females—resulting in lower transcript levels in males. Kdm6a is a known histone demethylase (H3K27me2/3) with multiple functional domains that is linked with synaptic plasticity and cognition. Whether elevating *Kdm6a* could benefit the aged male brain and whether this requires its demethylase function remains unknown. We used lentiviral-mediated overexpression of the X factor in the hippocampus of aging male mice and tested their cognition and behavior in the Morris water-maze. We found that acutely increasing *Kdm6a*—in a form without demethylase function—selectively improved learning and memory, in the aging XY brain, without altering total activity or anxiety-like measures. Further understanding the demethylase-independent downstream mechanisms of Kdm6a may lead to novel therapies for treating age-induced cognitive deficits in both sexes.

Keywords: Brain aging, Cognitive decline, Epigenetics

Males exhibit shorter life span (1,2) in human populations; they also show more cognitive deficits (3–6) or decreased baseline functions (6,7) in typical aging, particularly when dementia (or its subsequent development) is carefully excluded (3,4,6). Sex chromosomes, and specifically the X chromosome, are a major source of sex difference, such as in life span (8). X-derived, sex difference can result from decreased expression of select X-linked factors in males. The X chromosome is enriched for neural genes and, in females, associates with less cognitive decline in human aging (9). While both males (XY) and females (XX) harbor 1 active X due to X chromosome inactivation (XCI) in females (10), some genes escape transcriptional silencing—resulting in comparatively lower mRNA transcript levels in males (11,12). This results in sex differences in X gene expression. Because cognitive decline is a major biomedical challenge, understanding whether and how specific X factors promote brain health could open avenues for novel treatments for both sexes.

Kdm6a, or lysine demethylase 6a, is an X factor that escapes XCI—and influences synaptic plasticity (13) and cognition (13–15). Due to its escape from XCI, *Kdm6a* levels are lower in XY human and mouse brains (15–17). In XY mice, knockdown of Kdm6a in the hippocampus impairs synaptic plasticity and spatial memory (13); its elevation in the dentate gyrus of the hippocampus—a region central to cognitive networks targeted by aging—rescues cognitive deficits in a model of Alzheimer's disease (15). Kdm6a contains several functional domains and is primarily known for its nuclear histone demethylase (H3K27me2/3) activity (18–20). However, surprisingly,

its location in hippocampal neurons is largely cytoplasmic (15), suggesting that Kdm6a has additional demethylase-independent targets and functions in learning and memory that could lie outside the nucleus. *Kdm6a* is highly enriched in the brains of humans and mice (15,21). In humans, genetic variation leading to higher *Kdm6a* levels in the brain associates with less cognitive decline in both sexes (15).

Whether elevating *Kdm6a* could benefit the aging male brain, and whether this requires its demethylase function remains unknown. Here, we show that acutely increasing *Kdm6a* in the hippocampus—in a form without demethylase function (22,23)—selectively improved memory, without altering total activity or anxiety-like measures, in the aging XY brain.

Materials and Methods

Experimental Animals

Mice for in vivo studies were on a congenic C57BL/6J background and kept on a 12-hour light/dark cycle with ad libitum access to food and water. The standard housing group was 5 mice per cage except for single housing during water-maze studies. Cognitive and behavioral studies were carried out during the light cycle. Studies were approved by the Institutional Animal Care and Use Committee of the University of California, San Francisco, and conducted in compliance with National Institutes of Health guidelines. All cognitive, behavioral, and molecular experiments were conducted in a blinded manner on age-matched littermate offspring.

Lentivirus Production and Stereotaxic Injection

The *Kdm6a* Enzyme-Dead (Kdm6a DeM-dead) plasmid was purified and validated by Addgene (#40619) in which alanine (A) substitutions were made at histidine (H) 1146 (22) and glutamic acid (E) 1148 (22) of a sequence encoding protein, Kdm6a (NCBI Reference Sequence: NM_009483.2; 4,275 bp). This sequence was inserted between AscI and BmtI restriction sites of the pSicoR lentiviral backbone (pSicoR-EF1a-Blast-T2A-EGFP) obtained from the UCSF ViraCore (catalog number: MP394). Kdm6a cDNA is ~4 kB in size. Active lentiviral particles were produced as previously described (15).

Male mice, 17- to 18-month-old, were anesthetized using isofluorane at 2%–3% and placed in a stereotaxic frame. Lentiviral vectors of Kdm6a DeM-dead or control virus (5 µL per hemisphere, multiplicity of infection [MOI] = 2) were stereotactically injected bilaterally into the dentate gyrus of the hippocampus using the coordinates anterior/posterior = −2.1, medial/lateral = ±1.7, and dorsal/ventral = 1.9. All behavioral assays were conducted 3–5 weeks after lentiviral injections.

General Cell Culture

Primary cortical cell cultures were isolated from postnatal days 0–2 congenic C57BL/6J XY mouse pups as described (15). Cells were plated at 1 million cells/mL in 24-well plates for subsequent maturation and treatment in neurobasal media with B-27 supplement (NBA/B27). At day in vitro (DIV) 4, cultures were transduced with Kdm6a DeM-dead lentivirus at MOI = 2. Cells were harvested and RNA isolated at times indicated.

Quantitative Polymerase Chain Reaction [PCR]

Quantitative PCR was performed on RNA isolated from in vitro neurons and in vivo dentate gyrus. To verify Kdm6a DeM-dead lentiviral transfection, the dentate gyrus was dissected from the whole hippocampus under a stereomicroscope (Zeiss, Stemi 2000-C, Dublin, CA) in RNase-free conditions. The RNA was then isolated using Sigma-Aldrich (St. Louis, MO) RNAzol RT (Cat. R4533). Real-time PCR of *18S* and *Kdm6a* was performed. Primers for *Kdm6a* exons 16–17 *5′*-ATAACCGCACAAACCTGACC and *5′*-ACCTGCCAAATGTGAACTCG were used to measure *Kdm6a* mRNA expression.

Elevated Plus-Maze

Testing was carried out as described (15,24,25). Briefly, mice were habituated to the testing room for 1 hour prior to testing. Dim light was maintained in the testing room for both habituation and testing. Mice were placed in the center of an elevated plus-maze facing an open arm and allowed to explore for 10 minutes. Total time spent in open and closed arms was recorded using Kinder Scientific (Chula Vista, CA) Elevated Plus-Maze and MotorMonitor system. Increased time spent in the closed compared to open arms indicates increased anxiety-like behavior.

Open Field

Testing was carried out as described (15,24,25). Briefly, mice were acclimated to the room for 30 minutes and allowed to explore the open field for 5 minutes. Total activity in the open field (clear plastic chamber, 41 × 30 cm) was detected by beam breaks and measured with an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments, San Diego, CA).

Morris Water-Maze

Testing was carried out as described (15,24–26). Briefly, the water-maze pool (diameter, 122 cm) contained white, opaque water with a square, 14-cm^2 platform submerged 2 cm below the surface. During hidden platform training, the platform location remained constant, and the drop location varied between trials. Mice received 2 training sessions, consisting of 2 trials each, daily for 7 days. The maximum time allowed per trial was 60 seconds. Better learning is represented by shorter time to find the hidden platform. For the probe trial, which measures memory, the platform was removed, and the mice were allowed 60 seconds to swim. Shorter time to the platform represents better memory. Following probe testing, mice were tested for their ability to find the platform when marked with a visible cue (15 cm pole placed on the platform).

Statistical Analyses

GraphPad Prism (version 7.0) was used for *t* tests and visualization of data. R (nmle package) was used for analyses of variance (ANOVAs) and post hoc tests. Differences between 2 means were assessed by 2-tailed *t* tests unless otherwise indicated. Differences among multiple means were assessed by 2-way ANOVA. A mixed-model ANOVA was used for analyses of Morris water-maze data and included effects of repeated measures. Only significant *p* values were stated for 2-way ANOVA results. Multiple comparisons of post hoc *t* tests were corrected for with the Bonferroni–Holm test to control for a family-wise error rate of $\alpha = 0.05$. Exclusion criteria (greater than 2 *SD*s above or below the mean) were defined a priori to ensure unbiased exclusion of outliers. Error bars represent ±SEM. Null hypotheses were rejected at or below a *p* value of .05.

Results

Kdm6a Is Decreased in the Hippocampus of Old XY Mice, and Its Demethylase-Dead Form (Kdm6a DeM-Dead) Was Overexpressed in Aged XY Brains

We first assessed whether sex difference in *Kdm6a* mRNA levels extends to the aging hippocampus. Indeed, similar to the young

hippocampus, *Kdm6a* levels were significantly lower in the aging male hippocampus (Figure 1A); *Kdm6a* did not decrease with age in either sex (Figure 1A). To test if elevating *Kdm6a* could benefit the aging XY brain in a histone demethylase (H3K27me2/3)-independent manner, we utilized a *Kdm6a* lentivirus construct containing point mutations at the catalytic jumonji-C domain (H1146A/E1148A), rendering dead its demethylase function as previously demonstrated (22) (Figure 1B). The Kdm6a demethylase-dead (Kdm6a DeM-dead) construct increased *Kdm6a* mRNA expression levels via lentiviral-mediated transfection in XY primary neurons compared to the control (Figure 1C and D).

We next increased Kdm6a DeM-dead expression in the hippocampus of aging XY mice. We injected lentivirus with (Kdm6a DeM-dead) or without (control) the *Kdm6a* construct bilaterally into the dentate gyrus, a region that affects spatial learning and memory, and analyzed mice behaviorally 1 month later (Figure 1E). Lentiviral-mediated overexpression of Kdm6a DeM-dead increased *Kdm6a* mRNA expression in the dentate gyrus (Figure 1F).

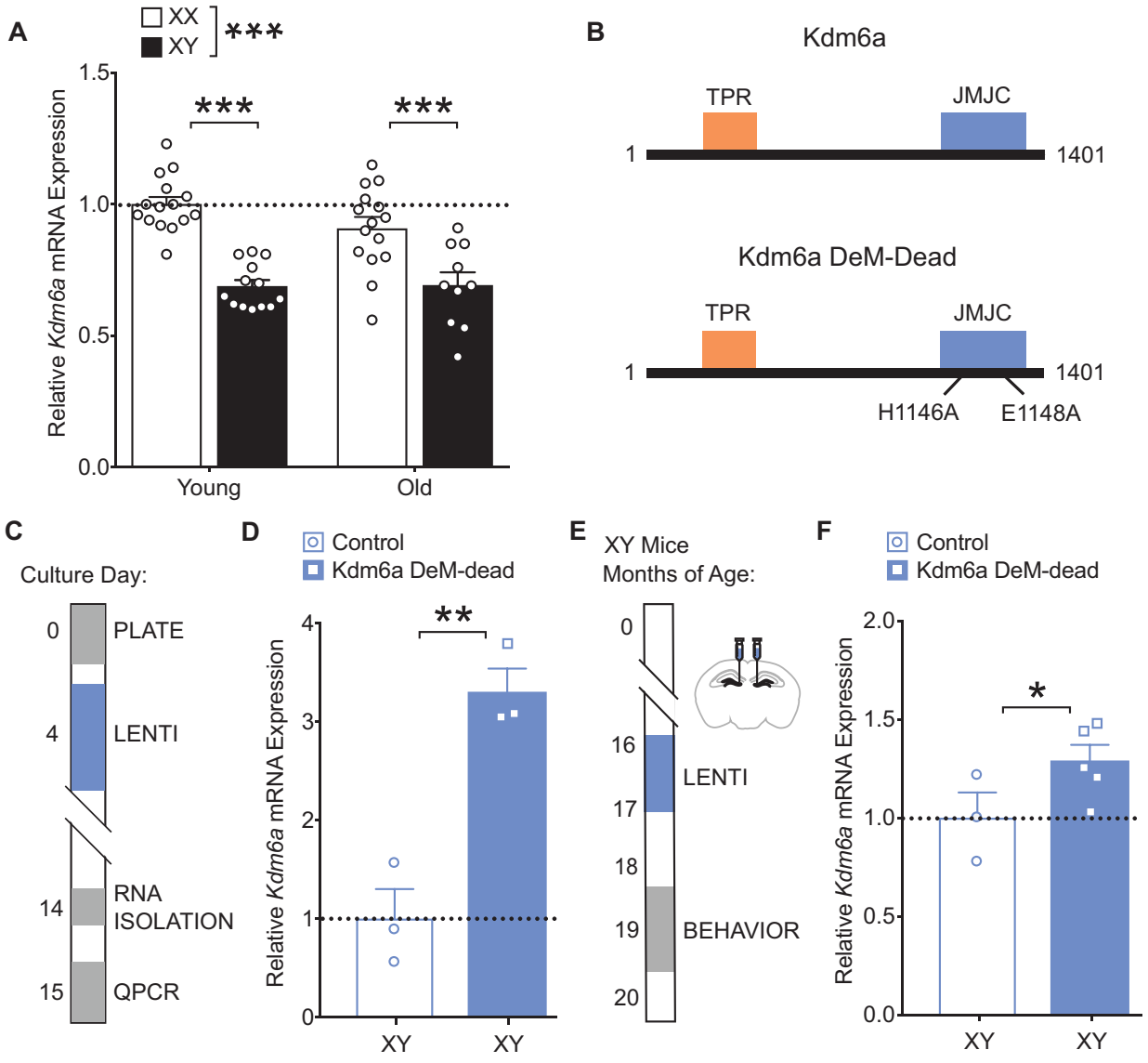

Figure 1. *Kdm6a* is decreased in the hippocampus of aged XY mice, and its demethylase-dead form (Kdm6a DeM-dead) was overexpressed in aged XY brains. (A) Hippocampal *Kdm6a* mRNA expression in young (age 3 months; n = 13–15 mice per experimental group) and old (age 20–35 months; n = 10–15 mice per experimental group) XX and XY mice. Data shown relative to XX young mice. Two-way analysis of variance: genotype ***$p < .0001$ (Bonferroni–Holm). (B) Construct maps showing the mutations rendering dead the demethylase activity of Kdm6a (Kdm6a DeM-dead) along with the tetrapeptide repeat (TPR) domain. (C) Experimental strategy of lentivirus-mediated overexpression of Kdm6a DeM-dead in XY mouse primary cortical neurons. (D) *Kdm6a* mRNA levels in primary XY neurons transfected with lentivirus expressing control or Kdm6a DeM-dead, shown relative to control (n = 3 wells per experimental group from 10 XY pups). **$p < .01$ (2-tailed t test). (E) Experimental strategy of lentiviral injection followed by testing in behavioral tasks. (F) *Kdm6a* mRNA expression following lentiviral transfection of Kdm6a DeM-dead measured in the dentate gyrus, microdissected from the hippocampus, relative to XY controls (n = 3 mice per experimental group). *$p < .05$ (1-tailed t test). Data are presented as means + SEM.

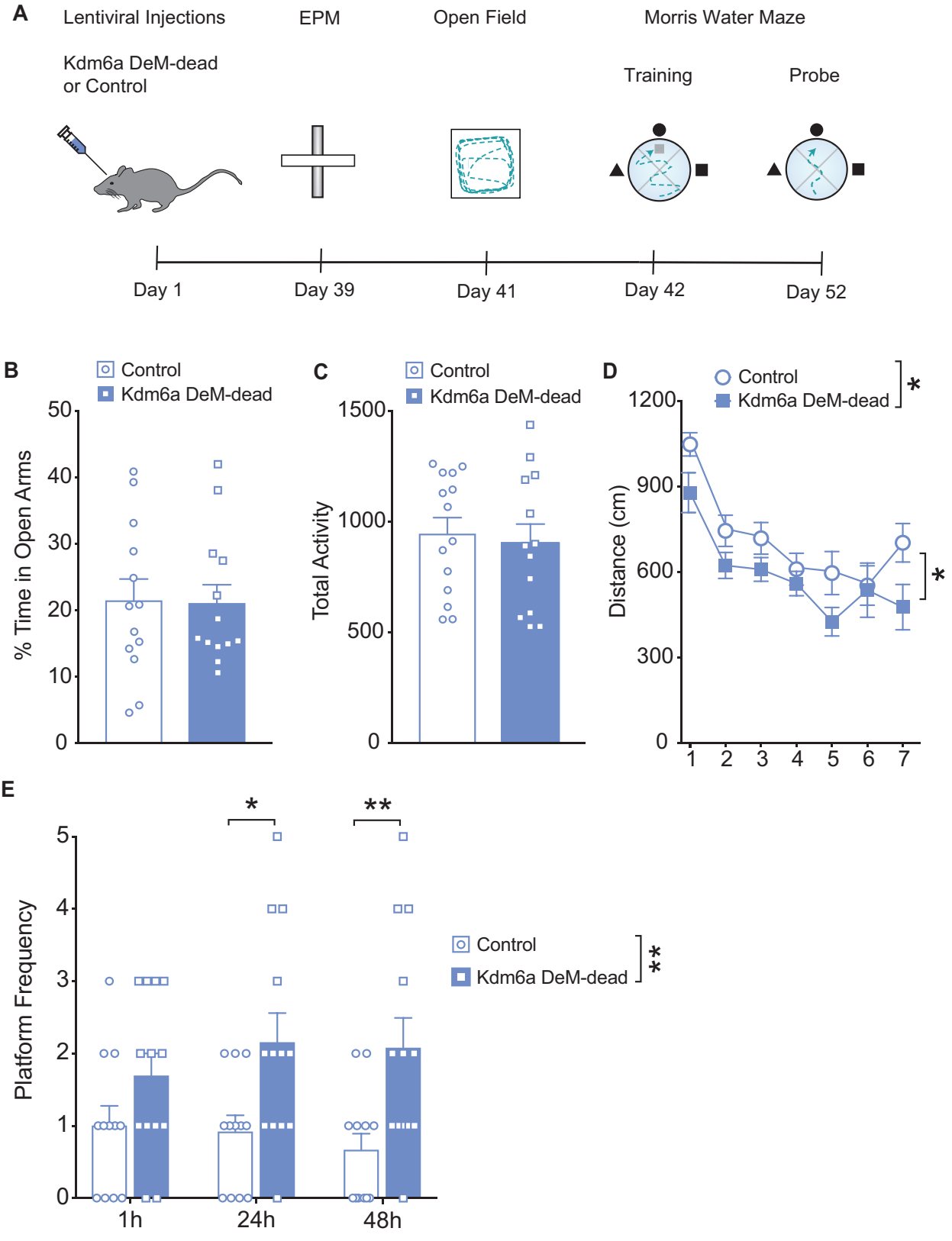

Figure 2. Kdm6a demethylase-dead (DeM-dead) overexpression in hippocampus improves cognition in aged XY mice. (A) Diagram of the experimental strategy for cognitive testing in the elevated plus-maze, open field testing, and the Morris water-maze in aged XY mice (age 17–20 months). (B) Anxiety-like behavior, measured by percentage of time spent in the open arms of the elevated plus-maze during 10-minute exploration period, did not differ between groups ($n = 13$ mice per experimental group). (C) Total number of movements during exploration of the open field for 5 minutes did not differ between groups ($n = 13$–14 mice per experimental group). (D) Spatial learning curves (hidden platform), measured by distance to find platform, of aged XY mice, control or Kdm6a DeM-dead, in the Morris water-maze ($n = 13$–14 mice per experimental group). Overexpressing Kdm6a DeM-dead mRNA enhanced learning. Two-way analysis of variance (ANOVA): treatment $^*p < .05$. (E) Probe trial results 1 hour, 24 hours, and 48 hours after completion of hidden platform learning, indicating spatial memory, showed that Kdm6a DeM-dead overexpressing mice had attenuated spatial deficits measured by increased frequency of entries into the target zone, compared to control mice ($n = 12$–13 mice per experimental group). Two-way ANOVA: treatment $^*p < .05$; $^{**}p < .01$ (Bonferroni–Holm). Data are presented as means + SEM.

Kdm6a Enhances Learning and Memory, Independent of Its Demethylase Function, in Aging XY Male Mice

We tested aged XY mice in cognitive and behavioral tasks (Figure 2A) to determine if elevating *Kdm6a* mRNA levels, in its demethylase-dead form, could improve learning and memory, cognitive measures decreased by aging. Increasing Kdm6a DeM-dead expression in aged XY mice did not alter time spent in open arms (Figure 2B) in the elevated plus-maze or movements in the open field task (Figure 2C), indicating no changes in anxiety-like behavior or total activity. In contrast, increasing Kdm6a DeM-dead expression in aged XY mice improved learning, measured by shorter distance traveled to the hidden platform in the Morris water-maze (Figure 2D). Additionally, in probe trials, which measures the ability to remember hidden platform location, increasing XY Kdm6a DeM-dead robustly improved spatial memory, compared to XY control mice (Figure 2E). Swim speeds and distance to find a visible cue, experimental controls, did not differ between the groups (data not shown). Thus, overexpression of *Kdm6a* in its demethylase-dead form selectively improved learning and memory, without altering total activity or anxiety-like measures, in the aging XY brain.

Discussion

Our studies in mice collectively reveal that *Kdm6a* is decreased in the aging XY hippocampus and overexpressing it in the dentate gyrus—in a form without demethylase function—selectively improved learning and memory in aging XY mice. These data support the hypothesis that elevating Kdm6a enhances the brain in aging.

An acute and modest increase of *Kdm6a*, without its demethylase activity, in the dentate gyrus of aged XY mice improved learning and memory. Whether the extent of improvement could match wild-type increases of *Kdm6a* is not known because this was not tested in parallel. It is noteworthy that a small increase, similar to or less than levels found in females, was sufficient to improve learning and memory in males. This suggests that lower levels of *Kdm6a* in males, compared to females, may confer vulnerability to cognitive aging. It remains unknown whether even higher levels could achieve further protection—and whether increasing *Kdm6a* in females, beyond its endogenously higher levels, could also improve cognition in aging.

The small *Kdm6a* increase in males occurred during the old life stage and acutely increased cognition, suggesting that Kdm6a manipulations, even late in life, could be beneficial. Consistent with findings that manipulating cells within functional hubs can affect larger networks (27), increasing *Kdm6a* levels in a small subregion of the XY hippocampus improved cognition. It will be important to investigate pharmacologic or lifestyle pathways to increase *Kdm6a* in the brains of both sexes.

In our studies, the lack of demethylase function in Kdm6a-mediated cognitive improvement in males indicates that other yet-identified functions of Kdm6a improve learning and memory. For example, Kdm6a can act as a scaffold for a larger transcription factor complex based on its tetratricopeptide repeat domain (28,29). Kdm6a can also indirectly regulate levels of H3K27 methylation by controlling enhancer activity of other demethylase enzymes (29). Future studies will identify specific mechanisms of Kdm6a demethylase-independent functions in cognition.

Strategies for targeting demethylase-independent mechanisms of Kdm6a in the brain could potentially lead to novel therapies for treating cognitive deficits in aging males, females, or both.

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Conflict of Interest

None declared.

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