The Inactive X Chromosome in Resilience Against Brain Aging and Cognitive Decline

by
Cayce Katherine Shaw

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Approved:

DocuSigned by:
Barbara Panning
B18F20197C95417...

Barbara Panning
Chair

DocuSigned by:
Saul Villeda
DocuSigned by:

Saul Villeda

DocuSigned by:
Vijay Ramani
12BA217F83A048B...

Vijay Ramani

Committee Members

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Contributions

The work in this dissertation was developed, led, executed and written by me. The hypotheses, interpretations, and conclusions were consulted with published literature and valuable discussions with colleagues and mentors.

Chapter 1 includes previously published work:

Arnold, A.P., Abdulai-Saiku, S., Chesselet, M.F., Dubal, D.B., **Shaw, C.K.**, Gold, S., McCullough, L., Werling, D. (2022). Sex Differences and Neurological Disease chapter. In Zigmond, M.J., Wiley, C.A., Chesselet, M.F. Neurobiology of Diseases, 2nd Edition. Elsevier. doi.org/10.1016/B978-0-323-85654-6.00034-4.

AUTHOR CONTRIBUTIONS

All authors discussed results and commented on the manuscript.

Chapter 2 is a reprint of the previously published work:

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AUTHOR CONTRIBUTIONS

All authors discussed results and commented on the manuscript.

The Inactive X Chromosome in Resilience Against Brain Aging and Cognitive Decline

Cayce K. Shaw

Abstract

Sex biology influences vulnerability to brain aging and cognitive decline. Females show advantage in lifespan and cognitive deficits in aging human populations – while males are more vulnerable. Historically, female biology has been largely understudied – specifically it is unknown how sex chromosomes influence resilience to age-related cognitive decline. In mammals, the X chromosome is enriched for neural genes and is a major source of biologic sex difference, in part, because females (XX) show increased expression of select X factors. Both sexes (XX and XY) harbor one active X due to random X chromosome inactivation (XCI) in female cells. However, some genes, such as *Kdm6a*, transcriptionally escape silencing from the inactive X (Xi) during development – leading to higher transcript levels in females. Escapee gene *Kdm6a*, encodes a lysine demethylase, best known for interaction with tri-/di-methylated histone 3 lysine 27 (H3K27me3/2). Kdm6a contains additional functional domains and is linked with synaptic plasticity and cognition. Previous studies in our lab found that the second X in females promotes neuronal resilience against Alzheimer's disease (AD)-related toxicity partially through increased expression of *Kdm6a*.

Here (Chapter 2), we used lentiviral-mediated overexpression of escapee *Kdm6a* – in a form without its demethylase function – to improve spatial learning and memory, in aging male mice, as measured using the Morris water-maze. Then (Chapter 3), we go onto further highlight the importance of the inactive X chromosome (Xi) by identifying and characterizing the

transcriptional signatures of Xi during female brain aging using a mouse assay based on strain-specific detection of differing SNPs crossed with a well-established mouse line that contains an *Xist* deletion, leading to forced activation of the X chromosome. We found that aging preferentially changes female hippocampal gene expression on the X chromosome – which causes an increase in the transcription of several Xi genes: *Plp1*, *Tspan7*, *Gpm6b*, and *Pck1n*. Top Xi factor *Plp1*, encodes the myelin proteolipid protein and has increased expression in aging female hippocampal oligodendrocytes, possibly contributing to enhanced cognition. We used oligodendrocyte-specific adeno-associated virus (AAV)-mediated overexpression to increase *Plp1* expression in aged XY and XX brains and tested male and female cognition using behavioral tasks. We found that increasing *Plp1* – specifically in oligodendrocytes – improved hippocampal-dependent spatial working memory in both aging males and females, as measured using two-trial Y-maze. *Plp1* additionally enhanced spatial learning in aging males, but not females, as tested in the Morris water-maze.

These findings highlight a role of the inactive X, via baseline and age-induced transcriptional escape, in countering age-related cognitive decline. Understanding how the inactive X may confer female cognitive advantage, and specifically how it is regulated throughout the lifespan, may lead to novel therapeutic targets for age-related cognitive decline in both sexes.

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List of Abbreviations

Xi = inactive X chromosome

XCI = X-chromosome inactivation

Kdm6a = lysine demethylase 6a

H3K27me2/3 = Histone H3 lysine 27 di-/tri-methylated

AD = Alzheimer's disease

PD = Parkinson's disease

Xist = X-inactive specific transcript

Aβ = Amyloid beta

SCR = scrambled

Sh = short hairpin

CTL = control

OE = overexpression

Kdm6a DeM-dead = Kdm6a demethylase-dead

TPR = tetratricopeptide repeat

ANOVA = analyses of variance

A = alanine

H = histidine

E = glutamic acid

Plp1 = proteolipid protein 1

AAV = Adeno-associated virus

SNP = single nucleotide polymorphism

Mbp = myelin binding protein

DIV = day *in vitro*

NLS = nuclear localization signal

NES = nuclear export signal

Chapter 1 - Introduction

SUMMARY

This chapter provides an overview of the biology of sex differences, focusing on the role of the inactive X chromosome and its significance in relation to neuroscience, aging and cognitive decline. An introduction to X-chromosome inactivation, baseline escapee genes, such as *Kdm6a*, and age-induced reactivation is discussed. A summary of the specific research aims of this dissertation is given.

Importance of studying sex differences.

Sex matters in science (*1*). Females have been largely understudied in preclinical and clinical biomedical research (*2, 3*), resulting in an incomplete understanding of disease mechanisms, diagnoses, and treatment. In the case of aging, females live longer (*4, 5*) and are more resilient to cognitive decline (*6, 7*), while males show vulnerability to cognitive deficits in typical aging – especially when dementia is carefully excluded (*6, 8, 9*). Aging is the predominant risk factor for cognitive decline (*10*) and several neurodegenerative disorders, including Alzheimer's disease (AD) and Parkinson's disease (PD) (*11, 12*). Major neurological and neurodegenerative diseases affect females and males differently, suggesting that inherent sex-based biological factors contribute to disease susceptibility, incidence, progression, and response to treatment (*13*). Understanding what makes one sex more vulnerable, or what makes the other sex more resilient, can extend our understanding of fundamental sex biology and unravel new therapeutic targets that benefit cognitive aging in both sexes.

Sex chromosomes are a fundamental difference between males and females.

In mammals, the inequality of sex chromosomes encompasses a fundamental biological process that makes males (XY) and females (XX) inherently different (*14*). The X chromosome is a major source of biological sex difference, such as in lifespan (*15*) and disease susceptibility against age-related cognitive decline (*16*). The X- and Y- sex chromosomes evolved from a pair of autosomes but, due to sequence divergence and lack of recombination, the Y-chromosome contains very few genes compared to the X (*17*). Thus, the loss of Y-linked genes means X-linked genes are present in two copies in female (XX) cells, and only one copy in male (XY) cells.

To compensate for this inequality in gene dosage of X-chromosome genes, mammalian XX cells evolved the conserved process of random X-chromosome inactivation (XCI) (*18*). During embryonic development in females, the long non-coding RNA X-inactive specific transcript (*Xist*) gets randomly upregulated from the X-inactivation center of one of the X chromosomes and coats this X-chromosomes *in cis* (*19, 20*). *Xist* then recruits various proteins, including the Polycomb group protein complexes, responsible for repressive histone modifications of the inactive X chromosome (Xi), resulting in a heterochromatic region (*21*).

At baseline, a small subset of X-linked genes escape XCI and show biallelic expression – leading to higher transcript levels in XX females (25% of X-linked genes in humans) (*22-27*). An even smaller subset (3%) escapes XCI in both mice and humans (*28*). A role for XCI gene escape is emerging for sex-biased diseases and disorders (*29, 30*). Our lab found that a second X chromosome promotes cognitive resilience against Alzheimer's disease-related toxicity – partly through increased expression of baseline escapee gene, *Kdm6a*, in females.

Baseline *Kdm6a* robustly escapes XCI and plays a role in resilience against AD-related toxicities.

Baseline XCI escapee gene *Kdm6a* is an X chromosome factor that influences synaptic plasticity (*31*) and cognition in mice (*16, 31, 32*), and causes cognitive deficits in humans with loss-of-function mutations (*33-38*). *Kdm6a* contains several functional domains and is primarily known for its nuclear enzymatic function which catalyzes removal of di- and tri-methylated histone 3 lysine 27 (H3K27me2/3), a repressive chromatin modification (*39-41*), though its location in hippocampal neurons is largely cytoplasmic (*16*). While its catalytic, epigenetic function is exerted in the nucleus, its cytoplasmic localization points to additional, undiscovered functions (*42*).

The second X chromosome confers resilience in female mouse and neuronal models of AD, in part, through increasing expression of *Kdm6a* (*16*). *Kdm6a* knockdown in XX neurons worsened, while *Kdm6a* overexpression in XY neurons attenuated amyloid beta (Aβ)-induced toxicity *in vitro* (**Figure 1.1A-D**) (*43*). Additionally, *Kdm6a* overexpression in male XY-hAPP, a model of AD, mice partially reversed cognitive impairment *in vivo (16)*. Human relevance for *KDM6A* in AD is supported by the finding that this gene is increased in the brains of women compared to men – and that a genetic variant of *KDM6A* linked to its increased brain expression associates with slower cognitive decline in individuals at risk for AD (*16*).

In summary, female XX-specific levels of *KDM6A* protected against Aβ-induced neuronal toxicity *in vitro* and *in vivo,* suggesting that higher levels of *KDM6A* expression may contribute to slower progression of AD (*16*). Understanding whether Kdm6a-mediated neuronal resilience requires its demethylase activity (*44*), its transcription factor binding function (*45*), or another action altogether, is an important next step that is explored in this thesis.

Potential for Xi reactivation during aging.

Another possible contribution to sex differences in cognitive decline and aging is the potential for Xi reactivation and epigenetic modification during aging. It is widely known that genomic instability, including loss of repressive epigenetic marks, occurs with aging (*46, 47*). Dynamic and intricate epigenetic alterations at heterochromatic regions, such as on Xi, are proposed to lead to changes in X-linked gene expression levels with time (*22, 48*). These changes include histone modifications, removal of repressive protein complexes, and increased accessibility of chromatin for transcription factor binding (*11, 49, 50*). Furthermore, age-induced alterations of the transcriptome causes a loss of epigenetic silencing maintenance and re-expression of silenced Xi gene (*51*). These mechanisms propose a potential for partial reactivation of the Xi across the female lifespan allowing for transcription of resilient factors.

Yet, whether this reactivation phenomenon occurs in the brain, and how cell type-specific transcriptional changes of Xi contribute to improved cognition in female brain aging remain unknown.

Aims of this thesis.

The purpose of this thesis is to highlight the importance of sex chromosome biology, particularly the role of the inactive X chromosome, in understanding sex differences in brain aging and cognitive decline. Chapter 2 aims to understand if the X chromosome factor, *Kdm6a*, enhances cognition independent of its catalytic demethylase function in the aging XY male brain. Chapter 3 tests a novel idea that the inactive X chromosome reactivates with aging, and that certain escapee genes increase expression during aging providing protection against cognitive decline in females. The final chapter, Chapter 4, outlines future directions in the exploration of how the inactive X-

chromosome influences brain aging and cognitive decline. Together, these findings highlight the influence of sex differences, specifically focusing on sex chromosome biology and the inactive X, on brain aging and age-related cognitive decline.

FIGURE AND LEGEND

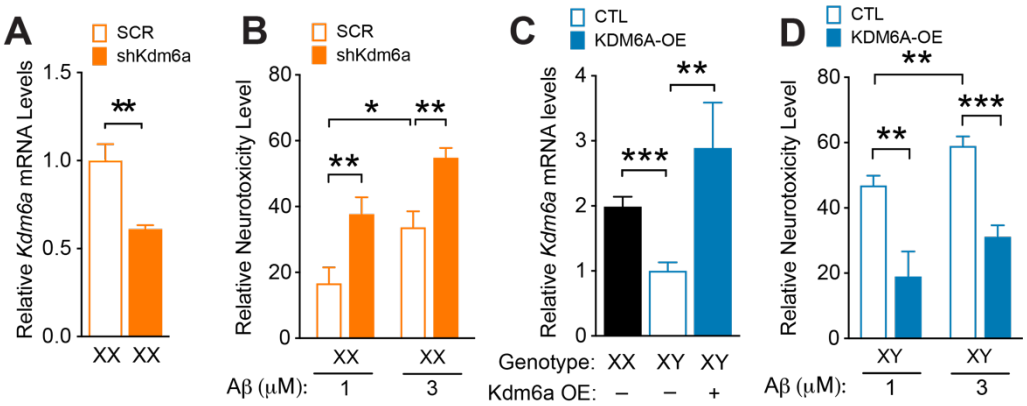

Figure 1.1. *Kdm6a* knockdown in XX mouse neurons worsens, whereas *Kdm6a* overexpression (*Kdm6a*-OE) in XY neurons attenuates Aβ toxicity *in vitro*.

(**A**) *Kdm6a* mRNA expression in neurons transfected with lentivirus expressing scrambled (SCR) or short hairpin (sh) *Kdm6a* for knockdown (n = 5-6 wells/experimental group from eight XX pups, from two litters). (**B**) Knockdown of *Kdm6a* in XX neurons worsened Aβ toxicity (n = 24-25 wells/experimental group from 14 XX pups, from three independent litters). (**C**) *Kdm6a* mRNA expression in neurons transfected with lentivirus expressing control (CTL) or overexpressing *Kdm6a* (*Kdm6a*-OE) (n = 3-8 wells/experimental group from 12 XY pups, from two independent litters). (**D**) Overexpression of *Kdm6a* in XY neurons attenuated Aβ toxicity (n = 12-13 wells/ experimental group from 26 XY pups, from three independent litters). *P < 0.05; **P < 0.01; ***P < 0.001 (Bonferroni-Holm corrected for more than one comparison). Data are presented as means ± SEM. *Figure and legend adapted from Science Translational Medicine Davis et al 2020.*

Chapter 2 - X chromosome factor Kdm6a enhances cognition independent of its demethylase function in the aging XY male brain

SUMMARY

Males exhibit shorter lifespan 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.

KEY WORDS: Brain aging, cognitive decline, epigenetics

Authors:

Cayce K. Shaw, B.S.[1,2], Samira Abdulai-Saiku, Ph.D. [1], Francesca Marino, B.A. [1,3], Dan Wang, M.S.[1], Emily J. Davis. Ph.D.[1], Barbara Panning, Ph.D.[4], Dena B. Dubal, M.D., Ph.D. [1, 2, 3]

Affiliations:

[1]Department of Neurology and Weill Institute for Neurosciences, University of California, San Francisco, CA 94158

[2]Rehabilitation Sciences Graduate Program, University of California, San Francisco, CA 94158

[3]Neuroscience Graduate Program, University of California, San Francisco, CA 94158

[4]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94158

INTRODUCTION

Males exhibit shorter lifespan (*4, 5*) in human populations; they also show more cognitive deficits (*6, 7, 9, 52*) or decreased baseline functions (*9, 53*) in typical aging, particularly when dementia (or its subsequent development) is carefully excluded (*6, 7, 9*). Sex chromosomes, and specifically the X chromosome, are a major source of sex difference, such as in lifespan (*15*). 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 (*54*). While both males (XY) and females (XX) harbor one active X due to X chromosome inactivation (XCI) in females (*18*), some genes escape transcriptional silencing – resulting in comparatively lower mRNA transcript levels in males (*21, 23*). This results in sex differences in X gene expression. Since 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 males and females.

Kdm6a, or lysine demethylase 6a, is an X factor that escapes XCI – and influences synaptic plasticity (*31*) and cognition (*16, 31, 32*). Due to its escape from XCI, *Kdm6a* levels are lower in XY human and mouse brains (*16, 55, 56*). In XY mice, knockdown of Kdm6a in the hippocampus impairs synaptic plasticity and spatial memory (*31*); its elevation it 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 (*16*). Kdm6a contains several functional domains and is primarily known for its nuclear histone demethylase (H3K27me2/3) activity (*39-41*). However, surprisingly, its location in hippocampal neurons is largely cytoplasmic (*16*), 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 (*16, 57*). In humans, genetic variation leading to higher *KDM6A* levels in the brain associates with less cognitive decline in both sexes (*16*).

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 (*58, 59*) – selectively improved memory, without altering total activity or anxiety-like measures, in the aging XY brain.

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 2.1A**); *Kdm6a* did not decrease with age in either sex (**Figure 2.1A**). 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 (*58*) (**Figure 2.1B**). 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 2.1C-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 2.1E**). Lentiviral-mediated overexpression of Kdm6a DeM-dead increased *Kdm6a* mRNA expression in the dentate gyrus (**Figure 2.1F**).

Kdm6a **enhances learning and memory, independent of its demethylase function, in aging XY male mice.**

We tested XY aged mice in cognitive and behavioral tasks (**Figure 2.2A**) 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 XY aged mice did not alter time spent in open arms (**Figure 2.2B**) in the elevated plus-maze or movements in the open field task (**Figure 2.2C**), indicating no changes in anxiety-like behavior or total activity. In contrast, increasing Kdm6a DeM-dead expression in XY aged mice improved learning, measured by shorter distance traveled to the hidden platform in the Morris water-maze (**Figure 2.2D**).

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 2.2E**). 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 wildtype increases of *Kdm6a* is not known since 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 (*60*), 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 (TPR) domain (*61, 62*). Kdm6a can also indirectly regulate levels of H3K27 methylation by controlling enhancer activity of other demethylase enzymes (*62*). 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.

MATERIALS AND METHODS

Experimental Animals.

Mice for *in vivo* studies were on a congenic C57BL/6J background and kept on a 12-hr light/dark cycle with ad libitum access to food and water. The standard housing group was five 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 (*58*) and glutamic acid (E) 1148 (*58*) of a sequence encoding protein, Kdm6a (NCBI Reference Sequence: NM_009483.2; 4275 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 ~4kB in size. Active lentiviral particles were produced as previously described (*16*).

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 uL per hemisphere, MOI = 2) were stereotactically injected bilaterally into the dentate gyrus of the hippocampus using the coordinates AP=-2.1, ML=±1.7 and DV=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 (*16*). 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 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) in RNase-free conditions. The RNA was then isolated using Sigma Aldrich 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 (*16, 63, 64*). 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 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 (*16, 63, 64*). 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 x 30 cm) was detected by beam breaks and measured with an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments).

Morris water-maze.

Testing was carried out as described (*16, 63-65*). 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 two training sessions, consisting of two trials each, daily for seven 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 two means were assessed by two-tailed *t* tests unless otherwise indicated. Differences among multiple means were assessed by two-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 two-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 SDs 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 0.05.

CONFLICTS OF INTEREST: None declared.

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FIGURES AND LEGENDS

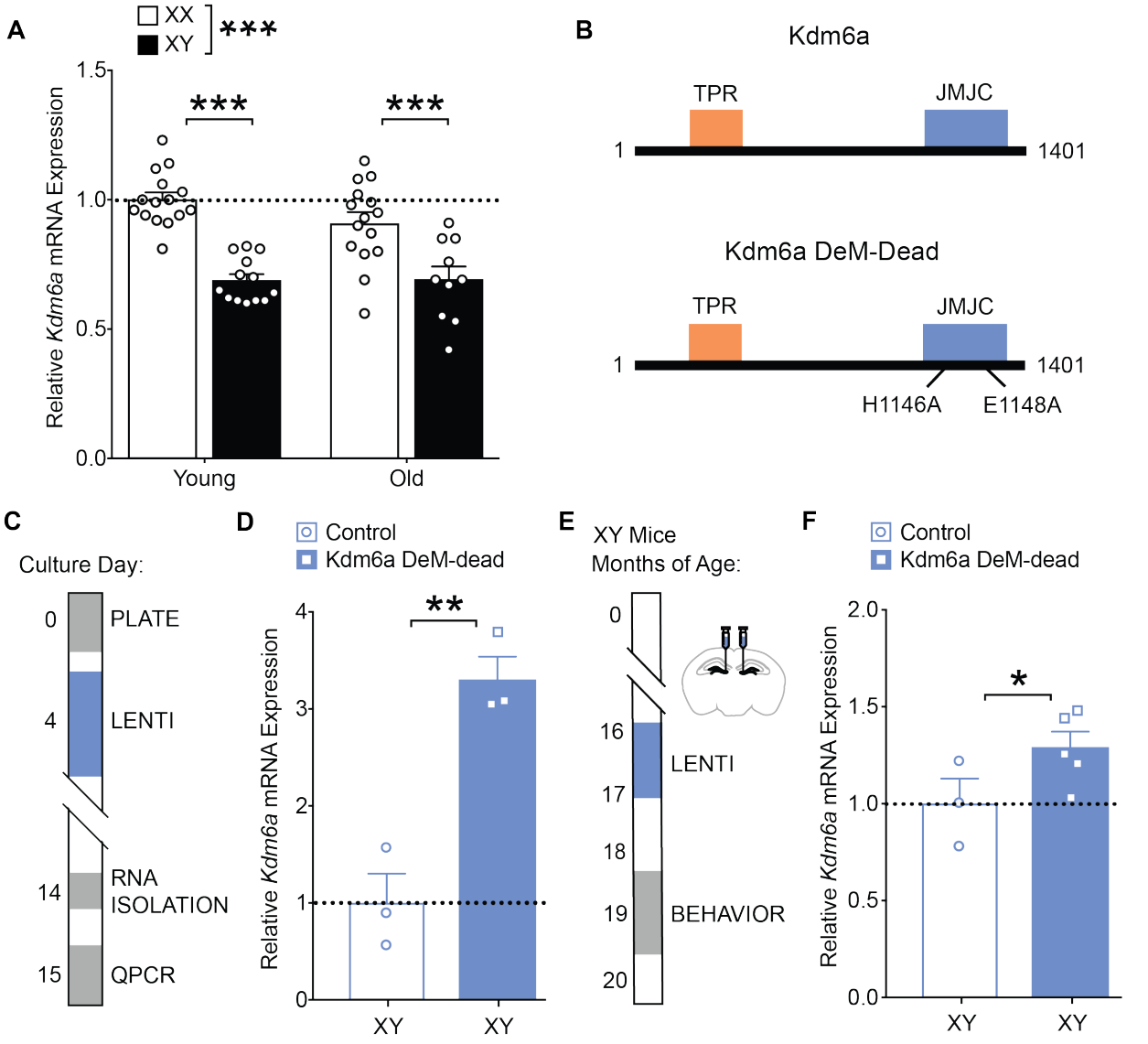

Figure 2.1. *Kdm6a* is decreased in the hippocampus of aged XY mice, and its demethylase-dead form (Kdm6a DeM-Dead) was overexpressed in aged XY brains.

(**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 ANOVA: genotype ***P < 0.0001 (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. (Figure caption continued on the next page.)

(Figure caption continued from the previous page.)
(**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 < 0.01 (two-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 < 0.05 (one-tailed t-test). Data are presented as means ± SEM.

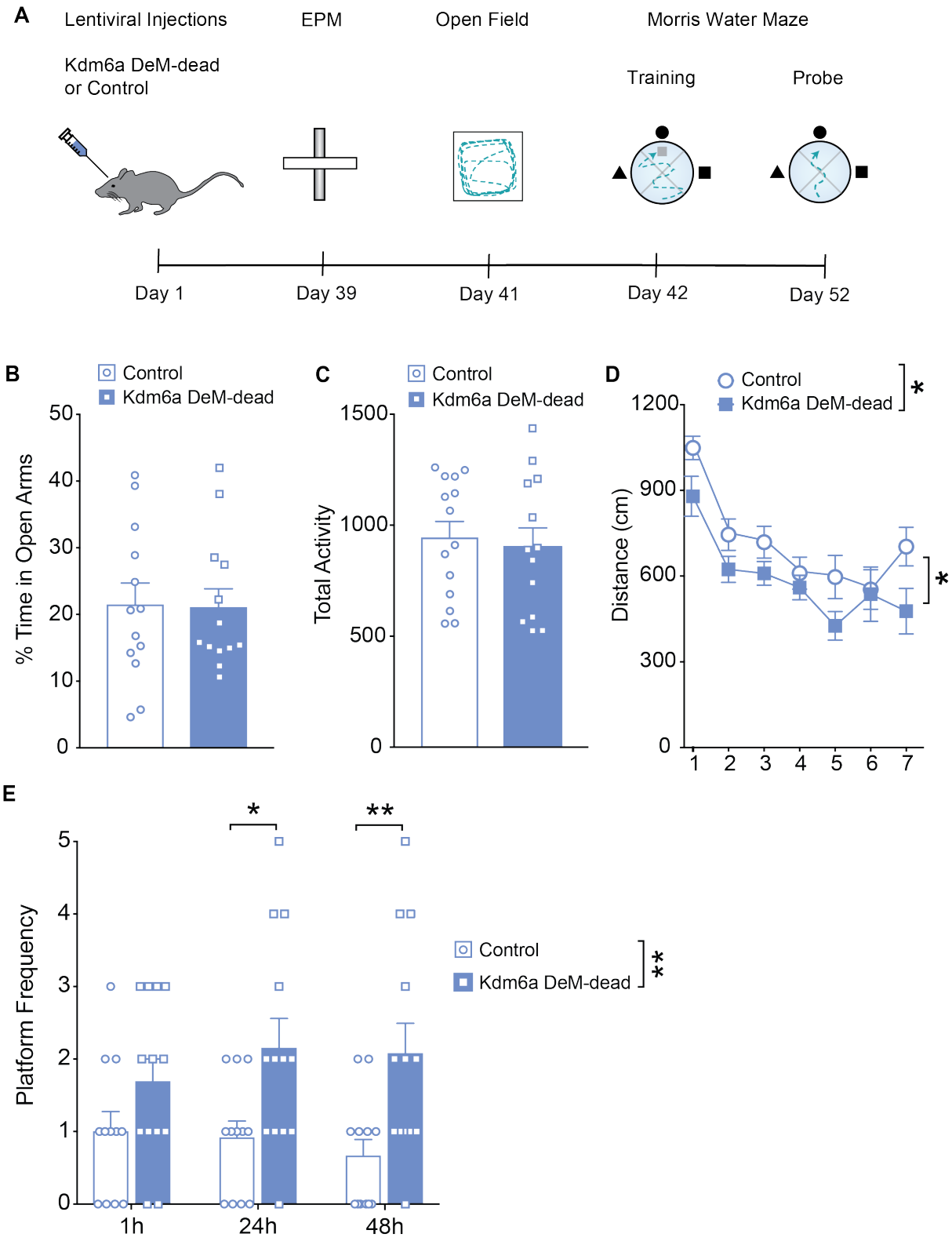

Figure 2.2. Kdm6a demethylase-dead (DeM-dead) overexpression in hippocampus improves cognition in XY aged mice. (Figure caption continued on the next page.)

(Figure caption continued from the previous page.)
(**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 min 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 min 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 ANOVA: treatment $^{*}P < 0.05$. (**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 $^{*}P < 0.05$; $^{**}P < 0.01$ (Bonferroni-Holm). Data are presented as means + SEM.

Chapter 3 - Age-induced XCI escape of *Plp1* in oligodendrocytes enhances cognition in aging XX and XY brains

SUMMARY

Females show advantage in lifespan and cognitive deficits in aging human populations – while males are more vulnerable. The X chromosome is enriched for neural genes and is a major source of biologic sex difference, in part, because females show increased expression of select X factors (XX). One of the two X chromosomes in female cells randomly inactivates during development resulting in an active (Xa) and inactive (Xi) X chromosome. At baseline, some genes from Xi escape silencing, leading to higher expression levels in XX compared to XY cells. Since aging induces loss of epigenetic repression of the genome, we wondered whether aging further increases X gene dosage in females by reactivating and increasing Xi expression in the XX brain. To identify and characterize the transcriptional signatures of Xi and Xa during female brain aging, we used a mouse assay based on strain-specific detection of differing SNPs. Next, we performed single-nuclei RNA sequencing to profile the young (3 months) and aged (22 months) XX mouse hippocampus, a brain region vulnerable to cognitive decline during aging. We assessed patterns governing baseline gene escape and age-induced reactivation of Xi. Aging preferentially changes female hippocampal gene expression on the X chromosome – which causes an increase in the transcription of several Xi genes: *Plp1*, *Tspan7*, *Gpm6b*, and *Pck1n*. Top Xi factor *Plp1*, encodes the myelin proteolipid protein and has increased expression in aging female hippocampal oligodendrocytes, possibly contributing to enhanced cognition. We used oligodendrocyte-specific adeno-associated virus (AAV)-mediated overexpression to increase *Plp1* expression in aged XY and XX brains and tested male and female cognition using behavioral tasks. We found that increasing *Plp1* – specifically in oligodendrocytes – improved hippocampal-dependent spatial

working memory in both aging males and females. *Plp1* additionally enhanced learning in aging males, but not in females. Understanding how the inactive X may confer female cognitive advantage, and specifically how it is regulated throughout the lifespan, may lead to novel therapeutic targets for age-related cognitive decline in both sexes.

KEY WORDS: Brain aging, sex differences, glia biology

Authors:

Cayce K. Shaw, B.S.[1,2], Samira Abdulai-Saiku, Ph.D.[1], Scott Nanda, Ph.D.[4,5], Dan Wang, M.S.[1], Vijay Ramani, Ph.D.[4,5], Barbara Panning, Ph.D.[3], Dena B. Dubal, M.D., Ph.D. [1,2,3]

Affiliations:

[1]Department of Neurology and Weill Institute for Neurosciences, University of California, San Francisco, CA 94158

[2]Rehabilitation Sciences Graduate Program, University of California, San Francisco, CA 94158

[3]Neuroscience Graduate Program, University of California, San Francisco, CA 94158

[4]Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94158

[5]Gladstone Institute, San Francisco, CA 94158

INTRODUCTION

Females (XX) show advantage in lifespan (*4, 5, 66*) and cognitive deficits (*6, 7, 43, 52*) in aging human populations, while males (XY) are vulnerable to cognitive deficits in typical aging – especially when dementia is carefully excluded (*6, 8, 9*). The X chromosome is enriched for neural genes and is an inherent source of biologic sex difference, in part, because females show increased expression of select X factors (XX). To compensate for the imbalance in X gene dosage between males (XY) and females (XX), XX cells evolved the process of random X chromosome inactivation (XCI) in which one X chromosome is transcriptionally silenced in each somatic XX cell (*18, 21, 67*). A small subset of X-linked genes escape XCI and show biallelic expression – leading to higher transcript levels in females (*23*). Yet, it is unknown how aging impacts XCI gene escape, and how this contributes to improved cognition during female aging.

Plp1, or proteolipid protein 1, escapes from XCI in the brain (*27*) and plays an indispensable structural role in oligodendrocyte-driven myelination of the central nervous system (*68-70*) – directly impacting cognition (*71, 72*). The intricate myelination process is crucial for establishing connections within the brain and for facilitating rapid and synchronized transfer of information, which is essential for learning and memory formation. Regions highly composed of myelinated axons, referred to as white matter, are a foci for accelerated aging and may contribute to cognitive decline (*73*). Interestingly, during aging there are sex-specific transcriptional modifications in oligodendrocytes (*74*) contributing to overall sex differences in age-related alterations to white matter regions of the brain and by association, leads to cognitive consequences.

In this study, we investigated how the X chromosome contributes to female cognitive resilience – and encountered XCI escapee gene *Plp1*, a myelinating factor expressed in

hippocampal oligodendrocytes, which increases expression during female aging and improved measures of cognition when overexpressed in aging males and females.

RESULTS

Aging preferentially changes female hippocampal gene expression on the X chromosome.

To identify and characterize the transcriptional signatures of the active and inactive X chromosome during female brain aging, we used a mouse assay based on strain-specific detection of differing single nucleotide polymorphisms (SNPs) and skewed X inactivation (**Figure 3.1A**). Next, we performed single-nuclei RNA sequencing to profile the young (3 months) and aged (22 months) female mouse hippocampus, a brain region highly vulnerable to cognitive decline (**Figure 3.1B**). Aging preferentially changes female hippocampal gene expression on the X chromosome; of the top aging-related differentially expressed genes, 46% were X-linked compared to 34% autosomal (**Figure 3.1C-D**). Unexpectedly, the inactive X, a chromosome previously thought to be inactive and lacking transcriptional activity, significantly contributed to this effect. Several alleles on the inactive X (Xi) changed expression during female hippocampal aging, providing evidence of Xi epigenetic remodeling (**Figure 3.1E-F**). Xi alleles *Gpm6b*, *Plp1*, *Pcsk1n*, *Tspan7*, and *Igbp1* were significantly upregulated in aged female hippocampus, whereas Xi alleles *Pola1*, *Fam3a*, and *Ubqln2* were significantly downregulated (**Figure 3.1E-F**).

Age-related XCI escape of *Plp1* in oligodendrocytes.

Top Xi factor, *Plp1*, escapes XCI in the brain (*27*) and plays a known structural role in oligodendrocyte-mediated myelination of the central nervous system (*68, 69*). X-linked *PLP1* mutations cause intellectual disabilities in Pelizaeus-Merzbacher disease, a recessive human

disease which predominant affects males, due to compromised myelin formation (*75*). Since *Plp1* escapes from XCI in the brain, it has higher transcript levels in females – but it is unknown whether these higher transcript levels in female aging brains contributes to enhanced cognition. We tested this question using AAV-mediated overexpression, specific to oligodendrocytes, in aging XY male and XX female brains.

Endogenous *Plp1* is increased in the aging XX female hippocampus compared to males.

We first assessed whether a sex difference in *Plp1* mRNA levels extends to the entire aging hippocampus. At baseline, there was no discernable difference between males and females (**Figure 3.2A**). However, in the aged female hippocampus *Plp1* levels were significantly increased (**Figure 3.2A**), but *Plp1* mRNA levels did not change in aging males (**Figure 3.2A**).

Oligodendrocyte-specific overexpression of *Plp1* enhances spatial working memory in aging males and females.

We then tested if elevating *Plp1* could benefit the aging XY and XX brain. To do this, we utilized an adeno-associated virus serotype 2/5 (AAV2/5) *Plp1* mRNA construct driven by the Myelin basic protein (*Mbp*), an oligodendrocyte-specific promoter (*76, 77*) (**Figure 3.2B**). The oligodendrocyte-specific *Plp1* overexpression (Plp1-OE) construct increased *Plp1* mRNA expression levels via AAV-mediated transfection in XY and XX primary mixed glia cultures compared to control (**Figure 3.2C-D**).

Next, we increased *Plp1* expression in the hippocampus of aging XY and XX mice (**Figure 3.2E**). We stereotaxically injected AAV2/5 with (Plp1-OE) or without (Control) the *Plp1* construct

bilaterally into the XY and XX hippocampi, a region that affects spatial learning and memory, and analyzed the mice behaviorally 1 month later (**Figure 3.2E**). Co-localization of Gfp and Olig2, a known marker of mature oligodendrocytes (*78*), confirmed successful Plp1-OE transfection in aged male and female hippocampi (**Figure 3.2F**). We then tested aged XY and XX mice in cognitive and behavioral tasks (**Figure 3.3A**, **Figure 3.4A**) to determine if elevating *Plp1* mRNA levels could improve hippocampal-dependent learning and memory, cognitive measures that decrease drastically during aging.

Increasing *Plp1* expression in aged XY and XX mice did not alter time spent in open arms (**Figure 3.3B**, **Figure 3.4B**) of elevated plus-maze or movements in the open field task (**Figure 3.3C**, **Figure 3.4C**), indicating no change to anxiety-like behavior or total activity for either sex. In contrast, during the two-trial Y maze, aging male and female mice overexpressing *Plp1* showed improved hippocampal-dependent spatial working memory, measured by more time spent exploring the novel compared to familiar arm (**Figure 3.3D-E**, **Figure 3.4G-H**). Additionally, *Plp1* overexpression enhanced learning in aging males measured by shorter distance traveled to the hidden platform in the Morris water-maze (**Figure 3.3F**) – yet this improvement was not seen in females (**Figure 3.6A**).

These data indicate that selectively increasing *Plp1* in hippocampal oligodendrocytes is sufficient to improve age-related deficits of cognitive aging in both males and females.

DISCUSSION

In this work, our study shows a role for the inactive X chromosome, specifically through action of escapee gene *Plp1*, in enhancing cognition in aging males and females. This work implies that the balance of dosage and timing of *Plp1* expression is critical for learning and memory function in the hippocampus, a region targeted during aging. Upregulating oligodendrocyte-specific expression of *Plp1* within the hippocampus enhanced spatial memory in both aging males and females. Additionally, *Plp1* overexpression improved learning in aging males, but not females.

The second X chromosome is important for conferring female advantage in lifespan (*15*), but it is unknown if and how the X chromosome impacts brain health and cognition during aging. Our study reveals that aging preferentially changes X chromosome gene expression in female hippocampal cells – and this effect is specifically driven by inactive X alleles. These findings are consistent with evidence that the inactive X contributes to cognition via escapee genes (*16, 79*), and that expression of XCI master regulators, such as *Xist* and *Jpx* are dynamic during female brain aging (*80*). Future studies can decipher the extent of inactive X remodeling during female aging and whether this effect is global or specific to the brain, including understanding its mechanism of regulation.

Our study uncovered Xi factor *Plp1* as an effective mediator of learning and memory in aging male and females. This suggests that *Plp1*-mediated myelin formation, driven in oligodendrocyte cells, is important for hippocampal memory formation throughout life. It remains unknown whether this effect is driven specifically by myelinating oligodendrocytes, or if other subtypes of the oligodendrocyte lineage may be responding heterogeneously (*81*) to *Plp1* overexpression.

However, recent studies suggest that enhancing myelination in aging could be beneficial for combatting age-related impairment in memory retention (*82*). It will be important to investigate the specific mechanism of *Plp1*-mediated cognitive improvement in aging males and females.

Unexpectedly, a sex difference in *Plp1*-mediated resilience to cognitive decline emerged in select behavioral measures. Specifically, during water-based tasks like the Morris water-maze and radial arm water-maze, sex differences in *Plp1*-mediated cognitive enhancement were observed, potentially linked to stress-induced or learning-based alterations in myelination. This suggests possibilities of *Plp1*-derived sex differences in myelin formation, a crucial structure governing cognition across a lifetime. Notably, females produce more *Plp1* (*28*) due to the presence of two X chromosomes transcribing this gene. This dosage difference potentially influences the delicate balance of *Plp1* levels and subsequently the modulation of myelination (*83*) in our overexpression paradigm.

In conclusion, our study reveals a role of the inactive X chromosome, through age-related escapee gene *Plp1*, in improving cognitive function in the aging XY and XX brains. Understanding sex-specific and age-related cognitive decline may unravel novel pathways to target with treatments that could benefit one or both sexes.

MATERIALS AND METHODS

Experimental Animals for RNA-sequencing. Young (3-4 month) and aged (23–24 month) *Xist* loxP +/-, Zp3-cre female mice were used for hippocampal single-nuclei isolation. Studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of

California, San Francisco, and conducted in compliance with National Institutes of Health guidelines. Animals were kept on a 12-hr light/dark cycle with ad libitum access to food and water. The standard housing group was five mice per cage. Mice were sacrificed and fresh hippocampal tissue was dissected and frozen.

Single Nuclei RNA-sequencing. Nuclei extraction was performing using the Nuclei EZ Prep kit (Millipore Sigma) according to the manufacturer's instructions. Frozen hippocampal tissue was transferred using a transfer pipette into a refrigerated Dounce homogenizer with 5 ml lysis solution following kit instructions. Tissue was homogenized with the Dounce B and the lysate was transferred into a 15-ml Falcon tube through a 30-μm filter to remove excess debris. Samples were spun for 20 min at 500g at 4 °C. Nuclei were counted using a hemocytometer and 5,000 nuclei per sample were loaded onto the Chromium Single Cell 3′ Chip (10X Genomics) and processed with the Chromium Controller (10X Genomics). Samples: Young_1, Young_2, Young_3, Young_4, Aged_1, Aged_2, Aged_3, and Aged_4 were prepared using the Chromium Single Cell 3′ Library and Gel Bead kit v3.1 according to manufacturer's instructions. Samples were sequenced at Novogene USA on an Illumina NovaSeq PE150, with a target of 100,000 reads per sample.

Quality control, data processing and analysis. We performed sequence alignment to the Mus musculus (house mouse) and Mus musculus castaneus genome assembly GRCm38 (mm10) (2020). Cell Ranger was used to perform sample de-multiplexing, barcode processing, and single-cell gene unique molecular identifier (UMI) counting, whereas a digital expression matrix was obtained mapped to the 10x reference for mm10.

Identification of cell clusters. The Scanpy Python package (version 1.9) (*84*) was used to project all sequenced cells onto two dimensions using UMAP and the Leiden graph-clustering method. We defined cell cluster-specific marker genes from our snRNAseq dataset using a bimodal likelihood ratio test (Wilcoxon rank-sum test). We named each cluster using the top five genes to come out of the bimodal likelihood ratio test that define a cluster identity.

Identification of differentially expressed genes between young and old female samples.

Differential expression analysis between young and aged samples was conducted to identify genes associated with aging. This was achieved using statistical tests such as the Wilcoxon rank-sum test. The Scanpy Python package (version 1.9) was used to create volcano plot.

AAV production and stereotaxic injection surgery.

To upregulate Proteolipid protein 1 (*Plp1*) expression in oligodendrocytes, an AAV vector (AAV 2/5; serotype 5(*85*)) encoding *Plp1* (NCBI Reference Sequence: NM_011123; 894 bp) was obtained from Applied Biological Materials (catalog number: #370271040110). The CMV promoter was changed to the Mouse myelin basic protein (*Mbp*) (NCBI Reference Sequence: NC_000084; 1661 bp); and green fluorescent protein (Gfp) was expressed under a separate SV40 promoter.

Male and female mice, 18-month-old, on a congenic C57BL/6J background were anesthetized using isofluorane at 2-3% and placed in a stereotaxic frame using earbars and tooth bar. AAV vectors of *Plp1*-overexpression or control virus (5 uL per hemisphere) were stereotaxically injected bilaterally into the dentate gyrus of the hippocampus using the coordinates AP=-2.1, ML=±1.7 and

DV=1.9 at a rate of 3uL/min and allowed to diffuse for 10 min. All behavioral assays were conducted 3-5 weeks after injections.

Behavior and Cognitive Tasks.

All studies were carried out in a blinded manner on age-matched littermate offspring. 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. Arenas, objects, or chambers were cleaned with 70% alcohol between testing sessions in all tasks except the water maze.

Elevated Plus-Maze. Testing was carried out as described (*16, 63, 64*). Briefly, mice were habituated to the testing room for 1 hour before 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 5 minutes. Total time spent and distance traveled in open and closed arms was recorded using Kinder Scientific Elevated Plus-Maze (Chula Vista, CA) 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 (*16, 63, 64*). Briefly, mice were acclimated to the room for 30 minutes and allowed to explore the open field for 10 minutes. Total activity in the

open field (clear plastic chamber, 41 x 30 cm) was detected by beam breaks and measured with an automated Flex-Field/Open Field Photobeam Activity System (San Diego Instruments).

Radial Arm Water-Maze. Spatial learning and memory was assessed using the radial arm water-maze (RAWM) paradigm according to established protocol (*86*). In this task, the mouse is trained to the location of a constant goal arm throughout the training and testing phase. The start arm changes each trial. Entry into an incorrect arm is scored as an error, and errors are averaged over training blocks (three consecutive trials). During training (day 1), mice are trained for 12 trails (blocks 1-4), with trials alternating between a visible and hidden platform. Following an hour break, learning is tested for 3 trials (block 5) using only a hidden platform. During testing (day 2), mice are tested for 15 trials (blocks 6-10) with a hidden platform. Investigators were blinded to treatment when scoring.

Two-Trial Y-Maze. Mice were tested in the two-trial Y-maze (Noldus Ethovision), which assesses spatial and working memory, as described (*87*). Briefly, mice underwent training by exploring the maze with a visual cue in one arm and another arm blocked off. 16 hours after training mice underwent testing with all three arms open (start arm, familiar arm, novel arm) and the distance traveled exploring the novel arm compared to the familiar arm, an index of memory, was measured.

Morris Water-Maze. Testing was carried out as described (*16, 63-65*). Briefly, the water-maze pool (diameter, 122 cm) contained white, opaque water (21°± 1°C) 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 two training sessions,

consisting of two trials each, daily for seven days. The maximum time allowed per trial was 60 seconds. For the probe trial, the platform was removed, and the mice were allowed 60 seconds to swim. 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).

Immunohistochemistry.

Mice were perfused with cold PBS (10 ml/min) for 4 min using peristaltic pump. Whole brains were isolated and post-fixed in 4% (w/v) PFA for 48 hours before preservation in 30% (w/v) sucrose in PBS. Whole brains were sectioned coronally at a thickness of 40 μm on a freezing sliding microtome (Leica). Sections were stored in the cryoprotective medium at -20°C. Free-floating sections were blocked with 10% normal donkey serum and incubated with primary antibodies at 4°C overnight at the following concentration: mouse anti-Gfp (1:1000, ThermoFisher A-11120) and rabbit anti-Olig2 (1:1000, Abcam ab109186). After washing, sections were incubated with Alexa Fluor-conjugated secondary antibodies (1:1000) and 300nM of 4′,6-diamidino-2-phenylindole (DAPI) at room temperature for 2 hrs. Sections were washed and mounted with Vectashield before imaging on fluorescence microscope with spinning disk confocal microscope (Nikon CSU-W1).

Mixed glia cell culture.

Primary mixed glial cortical cell cultures were isolated and cultured from postnatal days 0-2 congenic C57BL/6J male XY and female XX mouse pups as described (*88*). Cells were plated at 1 million cells/mL in 24-well plates for subsequent maturation and treatment in DMEM media. At day *in vitro* (DIV) 5, cultures were transfected with *Plp1* AAV at MOI=2. Cells were treated with

anti-mitotic agent cytosine arabinoside (AraC), to reduce glial proliferation (*89*). Cells were harvested and RNA isolated at times indicated.

Quantitative PCR.

Quantitative PCR was performed on RNA isolated from *in vitro* XY and XX mixed glia. To verify *Plp1* AAV transfection, RNA was isolated from mixed glial cultures using Sigma-Aldrich (St. Louis, MO) RNAzol RT (Cat. R4533). Real-time PCR of *Actin*, *Gapdh*, *Mbp*, and *Plp1* was performed. Primers for mouse *Plp1* transcript variant 2 5'CTTCCTTTATGGGGCCCTCC and 5' GGTGGTCTTGTAGTCGCCAA were used to measure relative *Plp1* mRNA expression. Primers for mouse *Mbp* transcript variant 8 5' CAGCCAGCACCACTCTTGA and 5' TGCCCACGCTTCTCTTCTTT were used to measure relative *Mbp* mRNA expression.

Statistics and reproducibility. Statistical analyses were executed with GraphPad Prism (version 7.0) or R for *t*-tests and analysis of variance (ANOVAs). Differences between two means were assessed by two-tailed *t*-tests unless otherwise indicated. One-tailed t-tests were applied in an unbiased manner to experiments that were independent replications of previous findings because of prior knowledge of expected direction of change. For validation and replication of AAV overexpression in XX mixed glia, one-tailed *t*-test was conducted. Differences among multiple means were assessed by two-way ANOVA. In mouse studies, exclusion criteria (greater than two standard deviations above or below the mean) were defined a priori to ensure unbiased exclusion of outliers. No statistical methods were used to pre-determine sample sizes, but our sample sizes are similar to those reported for behavior in our previous publications (*63-65, 87*). Error bars

represent ± standard error of the mean (S.E.M.). Null hypotheses were rejected at or below a *P* value of 0.05.

CONFLICTS OF INTEREST: None declared.

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FIGURES AND LEGENDS

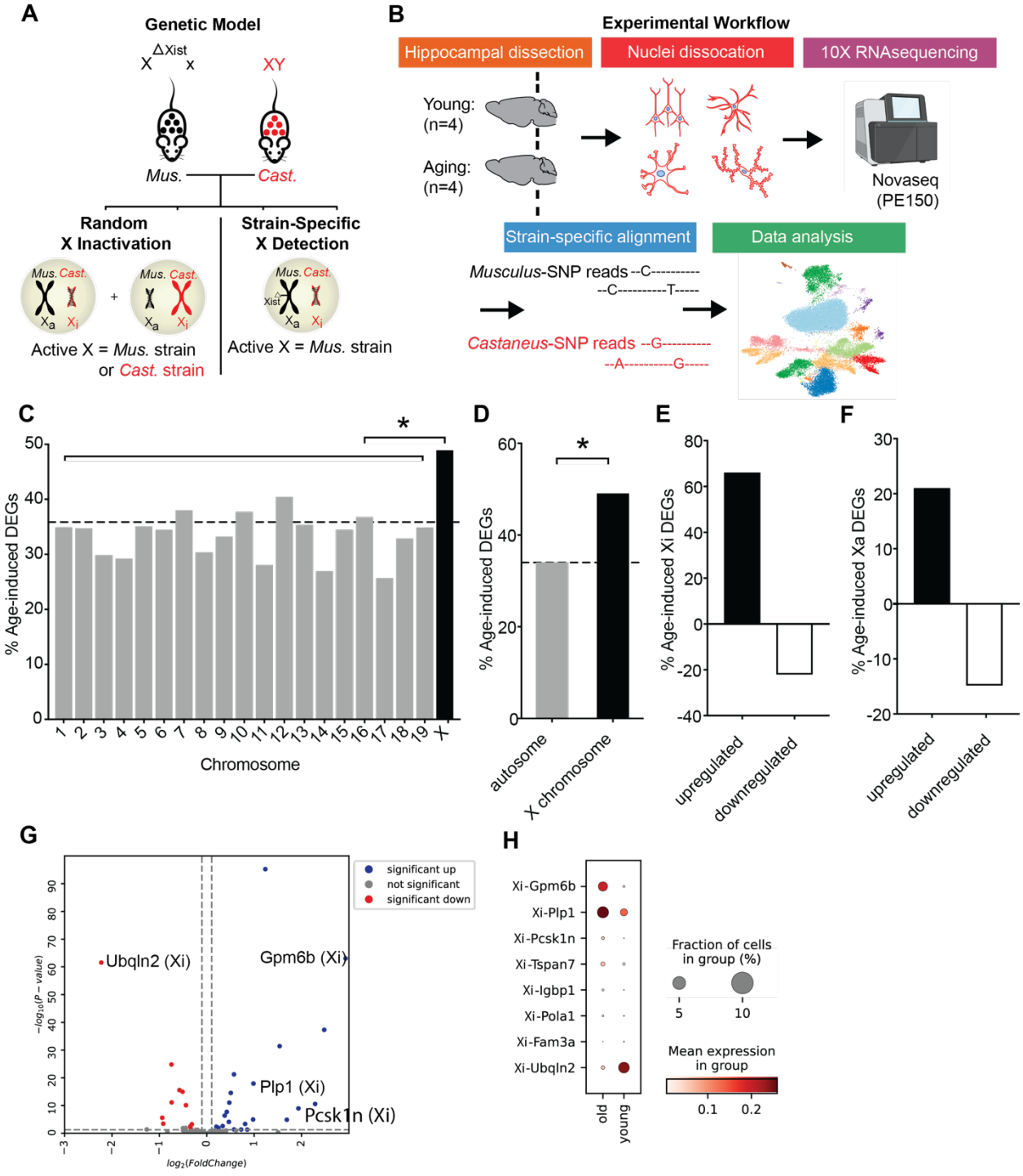

Figure 3.1. Aging preferentially changes X chromosome allelic expression in the female hippocampus. (Figure caption continued on the next page.)

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(**A** and **B**) Active X and inactive X allele detection using a genetic mouse model of strain-specific X detection combined with single nuclei RNA sequencing of young (age 3 months, n=4) and aging (age 22 months, n=4) female hippocampi. (**C** and **D**) Age-induced hippocampal gene changes across genome. **P* = 0.0442 (Fisher's exact test). (**E** and **F**) Xa and Xi allele-specific age-induced differential gene expression changes. (**G**) Volcano plot showing allele-specific X changes *P < 0.05; log-fold change > 1.0 (Wilcoxon rank-sum test). (**H**) Top Xi alleles that significantly change expression with age in female hippocampus: Xi alleles *Gpm6b*, *Plp1*, *Pcsk1n*, *Tspan7*, and *Igbp1* were significantly upregulated; Xi alleles *Pola1*, *Fam3a*, and *Ubqln2* were significantly downregulated.

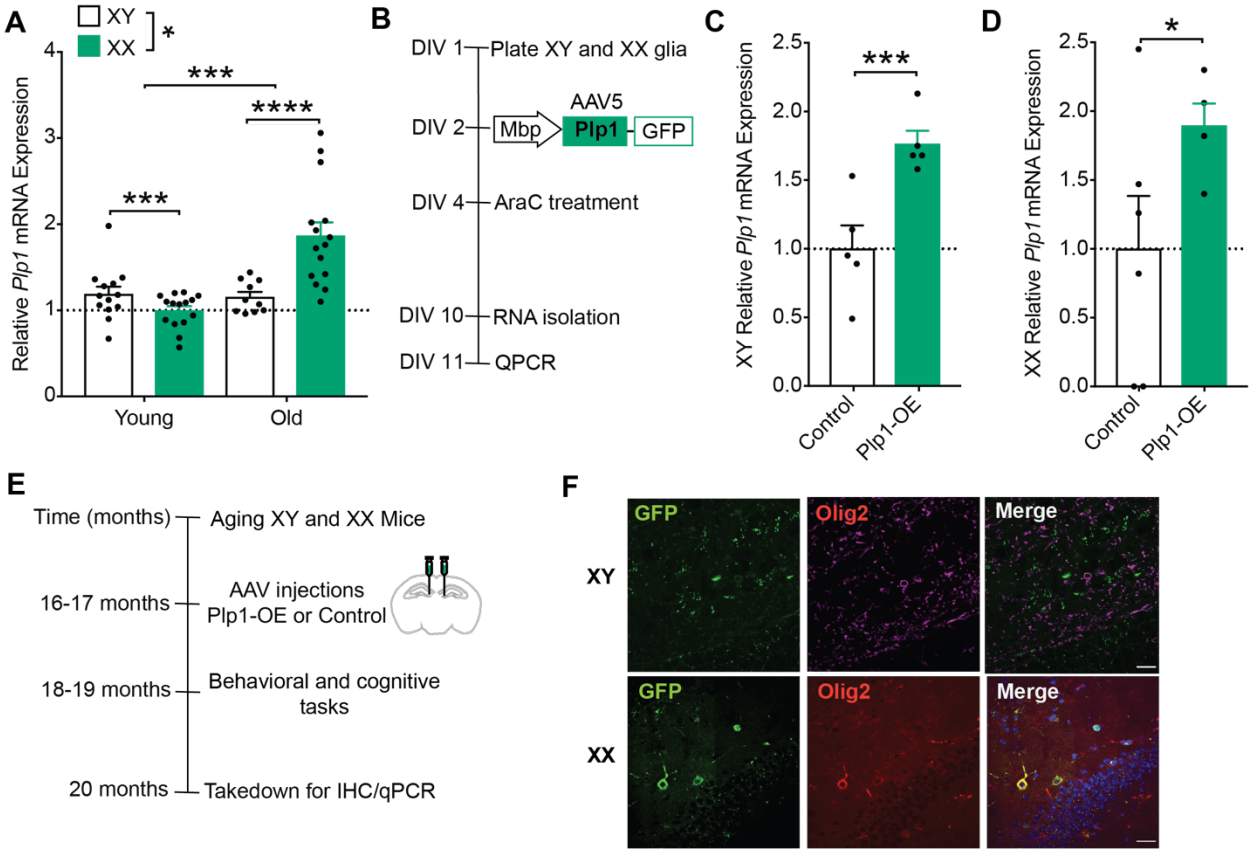

Figure 3.2. *Plp1* is increased in the hippocampus of aged XX mice, and it was overexpressed in aged XY and XX brains.

(A) Hippocampal *Plp1* mRNA expression in young (age 3 months; XY, n = 13; XX, n = 15) and old mice (age 20–35 months; XY, n = 10; XX, n = 15). Data shown relative to XX young mice. Two-way ANOVA: genotype **P*=0.0163, age ****P*=0.0002 (Bonferroni–Holm). **(B)** Experimental strategy for AAV-mediated overexpression of *Plp1*, under *Mbp* oligodendrocyte-specific promoter, in XY and XX mouse primary mixed glia. **(C)** *Plp1* mRNA levels in primary XY mixed glia transfected with AAV expressing empty control or Plp1-OE, shown relative to control (n=4 wells for control and n=4 wells for Plp1-OE from 8 XY pups). **p=0.0044 (two-tailed *t*-test). **(D)** *Plp1* mRNA levels in primary XX mixed glia transfected with AAV expressing empty control or Plp1-OE, shown relative to control (n=4 wells for control and n=4 wells for Plp1-OE from 8 XX pups). *p=0.0386 (one-tailed *t*-test). **(E)** Experimental strategy of AAV injections in aged XY and XX mice followed by behavioral tasks. (**F**) Representative confocal images of GFP staining (left), Olig2 (middle), and merged with Neuronal nuclei (NeuN) staining (right) in the hippocampal dentate gyrus region of an aged male XY mouse (top row) and an aged female XX mouse (bottom row). GFP is shown in green, Olig2 in red, and DAPI nuclear stain is shown in blue.
Scale bar, 50µm; 60x magnification. Data are presented as means +/- SEM.

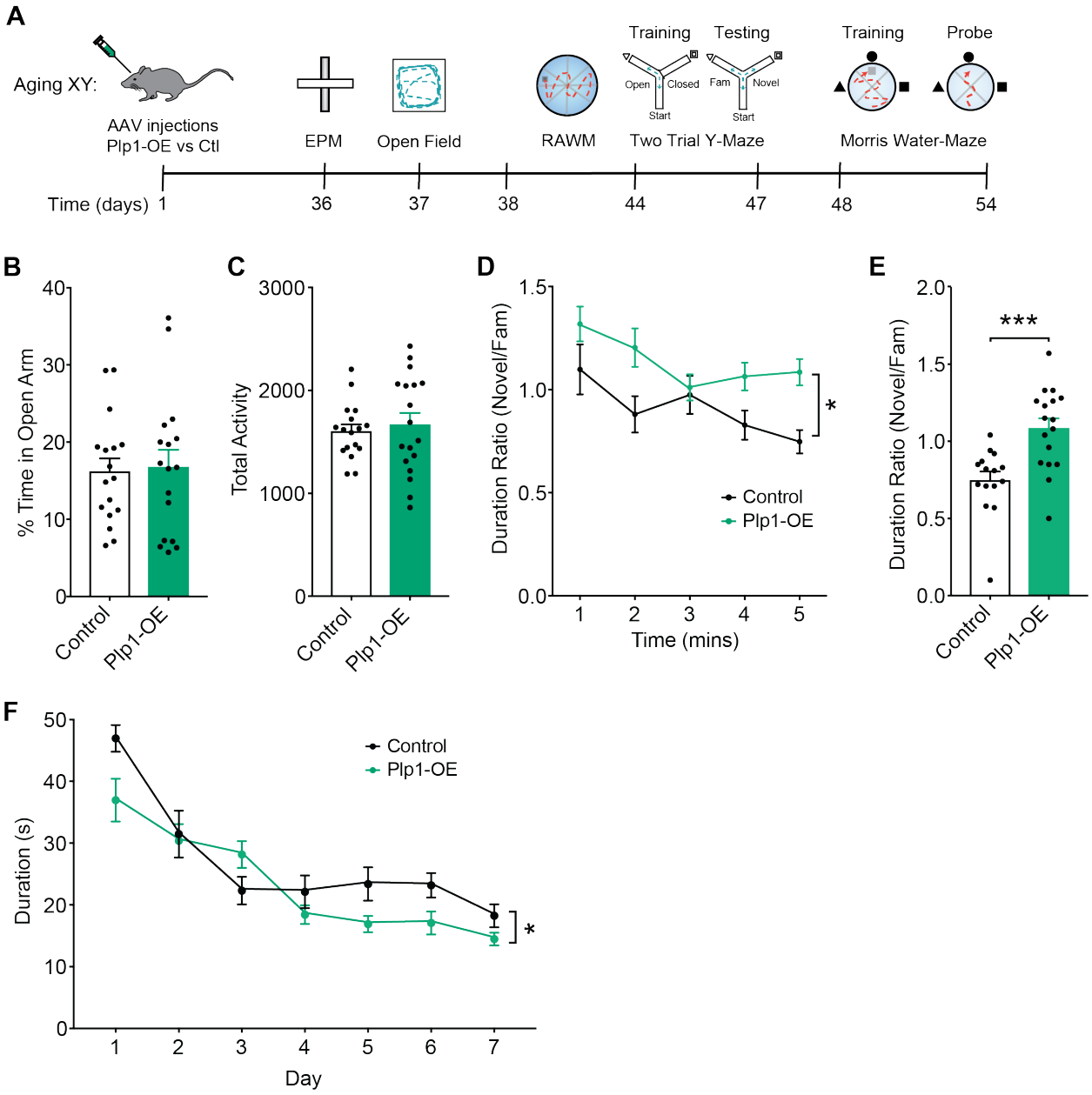

Figure 3.3. Oligodendrocyte-specific *Plp1* overexpression in hippocampus attenuates spatial working memory and learning impairments in male aged mice.

(A) Diagram of the experimental strategy for cognitive testing in the elevated plus-maze, open field testing, two trial Y-maze, radial arm water-maze, and Morris water-maze in aging male mice (age 18-20 months, n=16-18 per experimental group). (**B**) Total number of movements during exploration of the open field for 10 minutes did not differ between groups (age 19 months, n=18 mice for control and n=18 mice for Plp-OE).
(Figure caption continued on the next page.)

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(**C**) 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 (age 19 months, n=18 mice for control and n=18 mice for Plp-OE). **(D)** Spatial and working memory of aged XY mice injected with control or Plp1-OE was assessed by two-trial Y-maze (age 19 months, n=15 mice for control and n=17 for Plp-OE). The ratio of the percentage of duration in the novel to the familiar arm during testing was measured 16 hours following training. Two-way ANOVA: interaction Plp1-OE by time, **P*=0.0193. Data are presented as means ± SEM. **(E)** 5 min data for two-trial Y-maze. ***P=0.0005 (two-tailed *t*-test). **(F)** Spatial learning curves (hidden platform), measured by distance to find platform, of aged XY mice, control or Plp1-OE, in the Morris water-maze (age 19 months, n=18 mice for control and n=18 mice for Plp-OE). Two-way analysis of variance (ANOVA): treatment *p < .05. (n=18 mice for control and n=16 mice for Plp1-OE). Two-way ANOVA: interaction Plp1-OE by time, **P*=0.0198. Data are presented as means +/- SEM.

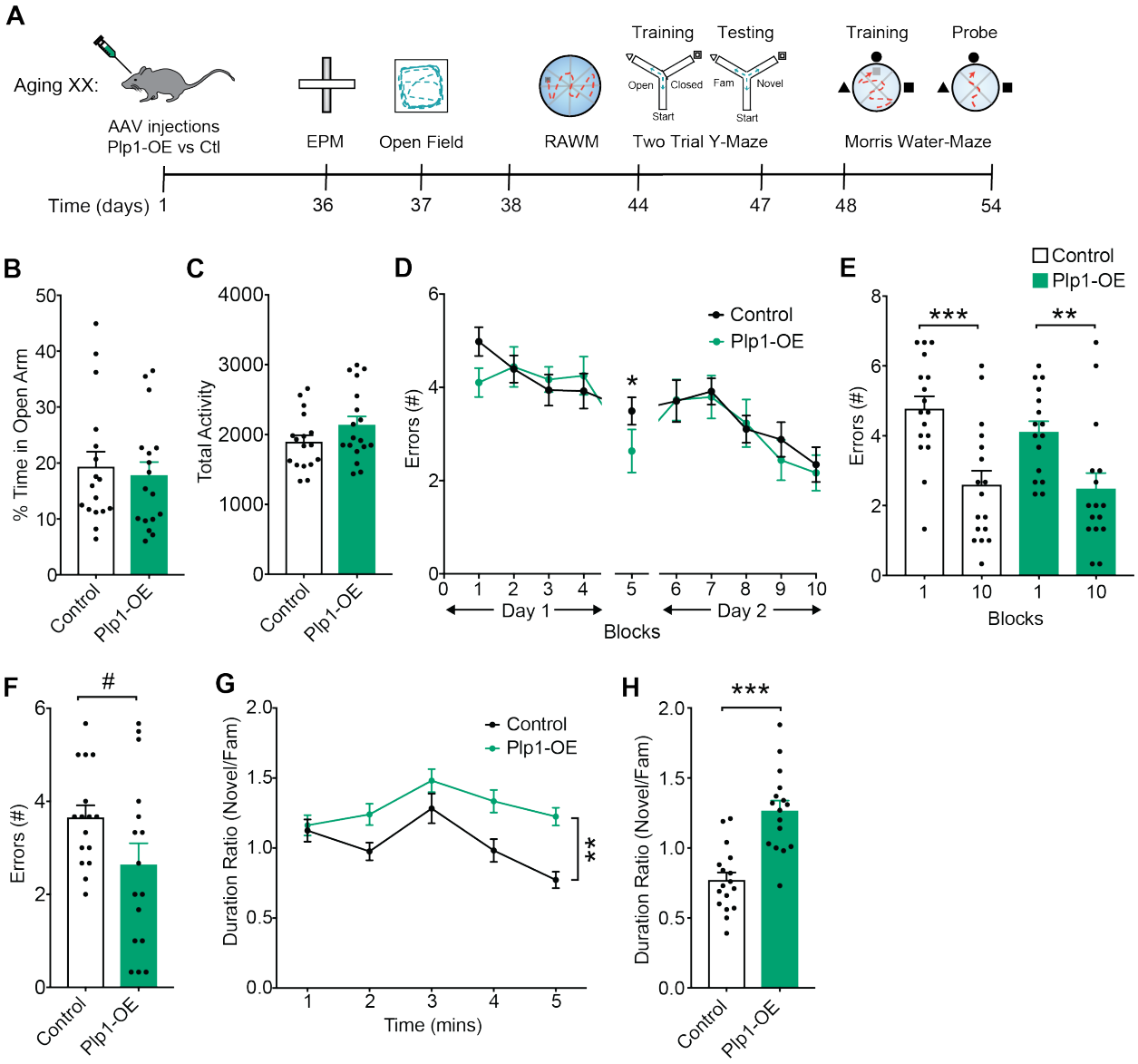

Figure 3.4. Oligodendrocyte-specific *Plp1* overexpression in hippocampus improves spatial working memory in female aged mice.

(**A**) Diagram of the experimental strategy for cognitive testing in the elevated plus-maze, open field testing, two trial Y-maze, radial arm water-maze, and Morris water-maze in aging female mice (age 18-20 months, n=16-18 per experimental group). (**B**) Total number of movements during exploration of the open field for 10 minutes did not differ between groups (age 19 months, n=16 mice for control and n=18 mice for Plp-OE). (**C**) 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 (age 19 months, n=17 mice for control and n=16 mice for Plp-OE).
(Figure caption continued on the next page.)

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(**D** and **E**) Spatial learning and memory were assessed by RAWM as the number of entry errors committed during the training and testing phases. Overall learning and memory were analyzed between block 1 and block 10 (1 block = 3 trials; age 19 months n=16 mice for control and Plp1-OE). #P=0.0681 (two-tailed t test). (**F**) Spatial memory was assessed by number of entry errors committed in RAWM during testing phase. Spatial working memory was analyzed at block 5 (1 block = 3 trials). Overexpressing *Plp1* mRNA in aged XX mice enhanced spatial working memory compared to control (age 19 months, n=16 mice for control and Plp-OE). #P=0.0681 (two-tailed t test). (**G**) Spatial and working memory of aged XX mice injected with control or Plp1-OE was assessed by two-trial Y-maze (age 19 months, n=17 mice for control and Plp-OE). The ratio of the percentage of duration in the novel to the familiar arm during testing was measured 16 hours following training. Two-way ANOVA: interaction Plp1-OE by time, **P=0.0028. Data are presented as means ± SEM. (**H**) 5 min data for large Y-maze. ***P<0.0001 (two-tailed t-test). Data are presented as means +/- SEM.

SUPPLEMENTARY FIGURES AND LEGENDS

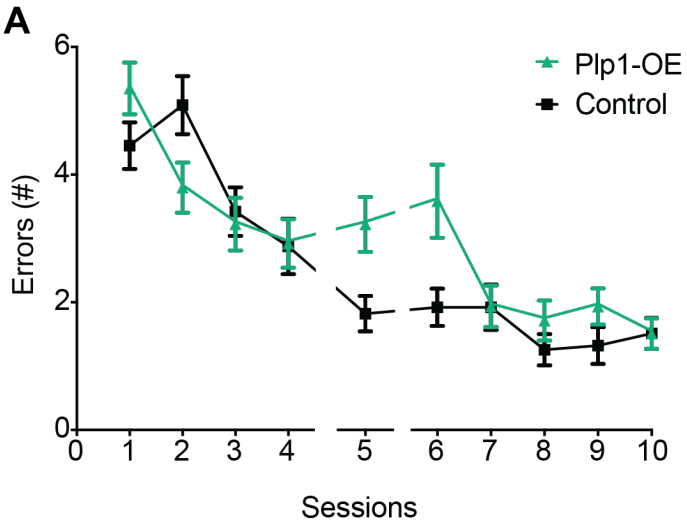

Figure 3.5. *Plp1* overexpression in aging male mice did not affect some measures of spatial memory.

(**A**) Spatial memory was assessed by number of entry errors committed in radial arm water-maze during testing phase. Spatial working memory was analyzed at block 5 (1 block = 3 trials). Overexpressing *Plp1* mRNA in aged XY mice did not improve spatial working memory compared to control (age 19 months, n=18 mice for control and n=18 for Plp-OE).

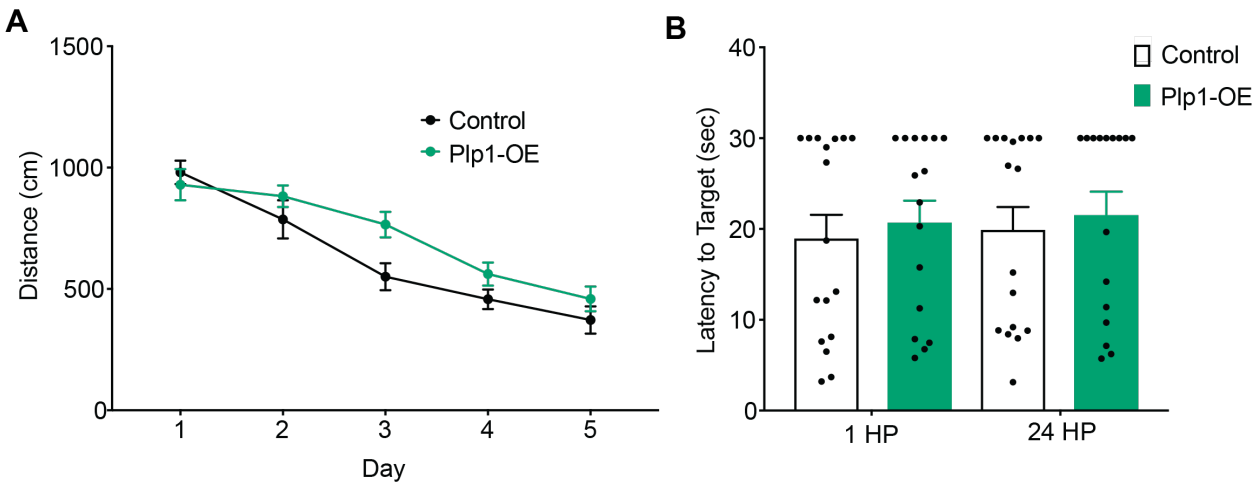

Figure 3.6. *Plp1* overexpression in aging female mice did not affect measures of spatial learning.

(**A**) Spatial learning curves (hidden platform), measured by distance to find platform, of aged XX mice, control or Plp1-OE, in the Morris water-maze (age 19 months, n=16 mice for control and n=16 mice for Plp-OE). (**B**) Probe trial results 1 hour and 24 hours after completion of hidden platform learning, indicating spatial memory, showed that Plp1 overexpressing XX mice did not have attenuated spatial deficits measured by increased frequency of entries into the target zone, compared to control mice (age 19 months, n=16 mice for control and n=16 mice for Plp-OE).

Chapter 4 - Conclusion and Future Directions

SUMMARY

This thesis demonstrates the importance of the inactive X chromosome in resilience against cognitive decline, through baseline escapee *Kdm6a* (Chapter 2) and age-induced reactivation of *Plp1* in oligodendrocytes (Chapter 3). While this work expands our understanding of X chromosome biology and its role in cognitive aging, questions remain unanswered – some of which I explore in this chapter. I propose further inquiries into 1) the downstream transcriptional and post-translational effects of Kdm6a demethylase-independent mechanisms in neuronal resilience, 2) the epigenetics of inactive X chromosome changes during brain aging and 3) understanding how Plp1-mediated myelination can improve cognition in aging male and female brains.

Characterization of Kdm6a Demethylase-independent Mechanisms in Neuronal Resilience

XCI escapee, *Kdm6a*, plays a known epigenetic role in cellular and developmental processes linked to its demethylase domain – a catalytic site that facilitates removal of di- and tri-methyl groups from histone H3 lysine 27 (H3K27me2/3), an important step for the induction of gene expression in the nucleus. Along with its demethylase domain, Kdm6a contains six additional tetrapeptide-repeat regions (TPR), which are likely to mediate transcription factors interactions for the assembly of multiprotein complexes (*40*). Surprisingly, in mouse hippocampal neurons (*16*), and *in vitro*, Kdm6a is predominantly localized in the cytoplasm (**Figure 4.1A**), suggesting that it acts on cytoplasmic and demethylase-independent targets. As described in Chapter 2, we observed that using lentiviral-mediated transfection to acutely overexpress *Kdm6a* – in a form without demethylase function – selectively improved learning and memory in the aging male brain (*79*), adding further evidence to a novel neuronal function of Kdm6a.

These findings converge and introduce a novel role of Kdm6a demethylase-independent and cytoplasmic mechanisms of resilience in the brain. To address this hypothesis, I propose that future studies investigate: 1) Kdm6a demethylase-independent protein binding partners in neurons, 2) Kdm6a downstream effectors and transcriptional targets using RNA sequencing analysis, and 3) whether Kdm6a requires both its cytoplasmic and nuclear localization to improve cognitive dysfunction. Strategies for targeting Kdm6a mechanisms in the brain could potentially lead to novel therapies for treating cognitive deficits in aging males, females, or both.

To identify Kdm6a demethylase-independent neuronal protein binding partners, the same lentiviral construct from the above-described studies (Chapter 2) could be used *in vitro* to perform a co-immunoprecipitation assay and mass spectrometry analysis. The proteome of XX and XY neurons, transfected the demethylase-dead construct, could be compared to understand whether sex differences exist in Kdm6a demethylase-independent targets. One possibility is that Kdm6a may indirectly regulate levels of H3K27 methylation by controlling enhancer activity of other demethylase enzymes through its TPR domain (*62*). It would also be important to investigate Kdm6a demethylase-independent transcriptional effects that are responsible for cognitive resilience in the brain. I recommend that RNA sequencing analysis can be used to compare gene expression in aging male mice with overexpression of wild-type, with unperturbed catalytic function, versus the demethylase-dead construct. This will result in the identification of demethylase-independent downstream effectors and candidate genes that may rely on Kdm6a demethylase-independent regulation of gene expression and enhancer activation.

Interestingly, in contrast to its known nuclear demethylase function, Kdm6a resides primarily in the cytoplasm of hippocampal neurons (*16*), and *in vitro* (**Figure 4.1A**). To elucidate whether the subcellular localization of Kdm6a is necessary for neuronal resilience, I recommend

that lentiviral constructs lacking specific known domains (*90*) for the nuclear localization signal (NLS), along with control, (**Figure 4.1B-C**) are transfected *in vitro* and *in vivo*. Lentiviral-mediated overexpression of the NLS mutated construct increases overall *Kdm6a* mRNA expression (**Figure 4.1D**), and the NLS-null mutant has exclusive cytoplasmic localization in primary XY neurons (**Figure 4.1E**). Since Kdm6a does not require its demethylase function for resilience against cognitive decline, this suggests that it is acting on demethylase-independent targets in subcellular specific compartments.

It will be important to overexpress and compare the Kdm6a DeM-dead, NLS-null, and wild-type constructs using previous measures of resilience, such as neuronal survival against Aβ -induced toxicity and *in vivo* cognitive testing, to ask whether Kdm6a-mediated resilience against brain aging requires the dynamic regulation of Kdm6a's subcellular localization, and/or its demethylase-independent targets.

In summary, the integration of Kdm6a demethylase-independent protein binding partners, transcriptional effects, and localization-based mechanisms of resilience can enable a more comprehensive understanding of Kdm6a's contributions to neuronal resilience. The studies outlined above will support therapeutic strategies for selectively targeting XCI escapee, Kdm6a, and its mechanisms in resilience against cognitive decline in aging males and females.

Epigenetics of Xi Reactivation in Brain Aging

In addition to individual XCI escapees, like *Kdm6a*, conferring resilience against cognitive decline, it is possible that previously silenced genes on the inactive X chromosome reactivate during brain aging to aid in this. When using a mouse model to distinguish the active and inactive X chromosome transcripts, we found compelling evidence that the X chromosome is preferentially changing gene expression in the female hippocampus during aging. Several genes on the inactive

X chromosome increased expression - an example being *Plp1* in oligodendrocytes. As detailed in Chapter 3, we observed that using AAV-mediated transfection to overexpress *Plp1* – specifically in hippocampal oligodendrocytes – selectively improved learning and memory in aging females and males. However, more work needs to be done to unravel the Xi reactivation mechanism and understand the interplay of Xi genes during female brain aging.

The above-described model, in which mRNA transcripts coming from Xi and Xa can be distinguished in specific cell types of the female hippocampus, provides an abundance of information that one can explore to gain a more comprehensive understanding of X-driven mechanisms in brain aging. Studying Xi reactivation in brain aging would involve a multidisciplinary approach that integrates 1) cell type-specific RNA sequencing analysis, and 2) ChIP-sequencing analysis to study epigenetic modifications, focusing on DNA methylation and known histone modifications. This deeper knowledge could pave a way for targeted interventions and therapeutic strategies aimed at reactivating Xi genes important for resilience to cognitive decline during aging.

Although it represents only 5% of the genome, the X chromosome is highly enriched with neural genes important for cognition (*91*). Notably, our data shown in Chapter 3 illustrates a preferred modulation of X chromosome gene expression in the aging female hippocampus. Therefore, utilizing high-throughput single nuclei RNA sequencing of the above-described mouse model can provide more insights into the heterogeneity of Xi reactivation across different cell types of the hippocampus. A deep dive into this already constructed allele-specific snRNA sequencing database can answer questions such as: whether Xi genes are reactivated in specific hippocampal cell types, or if it is a global phenomenon, and if Xa alleles undergo similar or divergent gene modulation. These studies can also look into the expression of long non-coding

RNAs, such as *Xist*, *Jpx*, *Ftx*, and *Tsix*, which act as master regulators of inactive X-chromosome silencing, maintenance, and regulation (*92*).

Subsequently, ChIP-Seq can be employed to assess gene expression profiles and epigenetic modifications associated with Xa and Xi in hippocampal brain aging. Focus can be placed on key epigenetic marks, such as DNA methylation and histone modifications, known to regulate gene silencing on the inactive X chromosome (*21, 93*). We could use this data in unison with the above-described RNA-sequencing studies, to infer the crosstalk between histone marks and DNA methylation with gene expression levels on the inactive X chromosome during aging. Understanding whether Xi silenced genes reactivate and how the Xi heterochromatin gets remodeled during brain aging will inform our general knowledge of sex differences in brain aging and the underlying transcriptional pathways. The proposed studies can inform potential strategies for selectively targeting Xi reactivation (*94*), in a cell type- and gene-specific manner.

Plp1-mediated Mechanisms of Protection in the Aging Brain

As described in Chapter 3, we found that aging increases expression of XCI escapee gene, *Plp1*, in females and that selectively overexpressing it in aging males and females improved cognition. This is an exciting result since Plp1 plays a structural role in oligodendrocyte-driven myelination of the central nervous system (*68-70*) – directly impacting cognition (*71, 72*). Recent studies show that during aging, sex-specific transcriptional modifications in oligodendrocytes (*74*) contribute to overall sex differences in age-related alterations to white matter regions of the brain and by association, cognitive consequences. Therefore, investigating Plp1-mediated myelination mechanisms in the aging female and male brains can shed light on its contribution to sex differences in cognitive decline.

Myelination is crucial for establishing connections within the brain and for facilitating rapid and synchronized transfer of information, which is essential for learning and memory formation. During aging, disruption and loss of myelin occur, leading to impaired cognition (*83*). It will be important for future studies to decipher whether X factor Plp1 rescues age-induced cognitive decline by 1) increasing overall myelin production of oligodendrocytes to counteract aging deficits, or 2) maintaining the integrity and cellular health of already present myelin. Expanding our knowledge at the intersection of sex differences, brain aging, and Plp1-mediated myelination could reveal new pathways to counter cognitive decline associated with aging and myelin deficits, in males and females.

Oligodendrocytes have a long lifetime, but there is deterioration of myelin integrity that occurs with age leading to impaired cognition (*83*). Due to this, there may be increased vulnerability to myelin breakdown during aging. To explore if XCI escapee, Plp1, mediates effects on myelin structural integrity, future studies could involve *in vitro* and *in vivo* approaches to visualize and analyze myelin sheaths. *In vitro* methods would use AAV-mediated transfection, as described in Chapter 3, to overexpress Plp1 in myelin-forming oligodendrocytes of the hippocampus. Immunocytochemistry and high-resolution microscopy methods could be used to visualize myelin structure and organization by focusing on myelin binding protein (Mbp) and lipophilic dyes, highlighting the lipid-rich membrane layer. *In vivo* methods could also utilize histological approaches, enabling post-mortem examination of myelin architecture and distribution in aging male and female brains with *Plp1* overexpression. These findings would inform our understanding of Plp1-mediated myelination mechanisms in oligodendrocytes of the hippocampus and Plp1's overall role in sex differences of cognitive decline during aging.

CONCLUSION

To conclude, this thesis covered the importance of studying sex biology, particularly the role of the inactive X chromosome, in countering age-related cognitive deficits. In Chapter 2, I demonstrated that X-chromosome escapee gene, *Kdm6a*, functions independent of its canonical demethylase domain in resilience against cognitive decline in aging males. Chapter 3 highlighted a fundamental idea that brain aging preferentially changes gene expression on the X chromosome. In this study, we tested the functional relevance of X escapee gene, *Plp1*, increasing expression in oligodendrocytes during aging. My data showed that selectively overexpressing *Plp1* in the aging male and female brain improved cognition in measures of learning and memory. I hope that this work can inspire and inform future discoveries aimed at treating cognitive decline, a major biomedical problem and immense societal burden.

FIGURE AND LEGEND

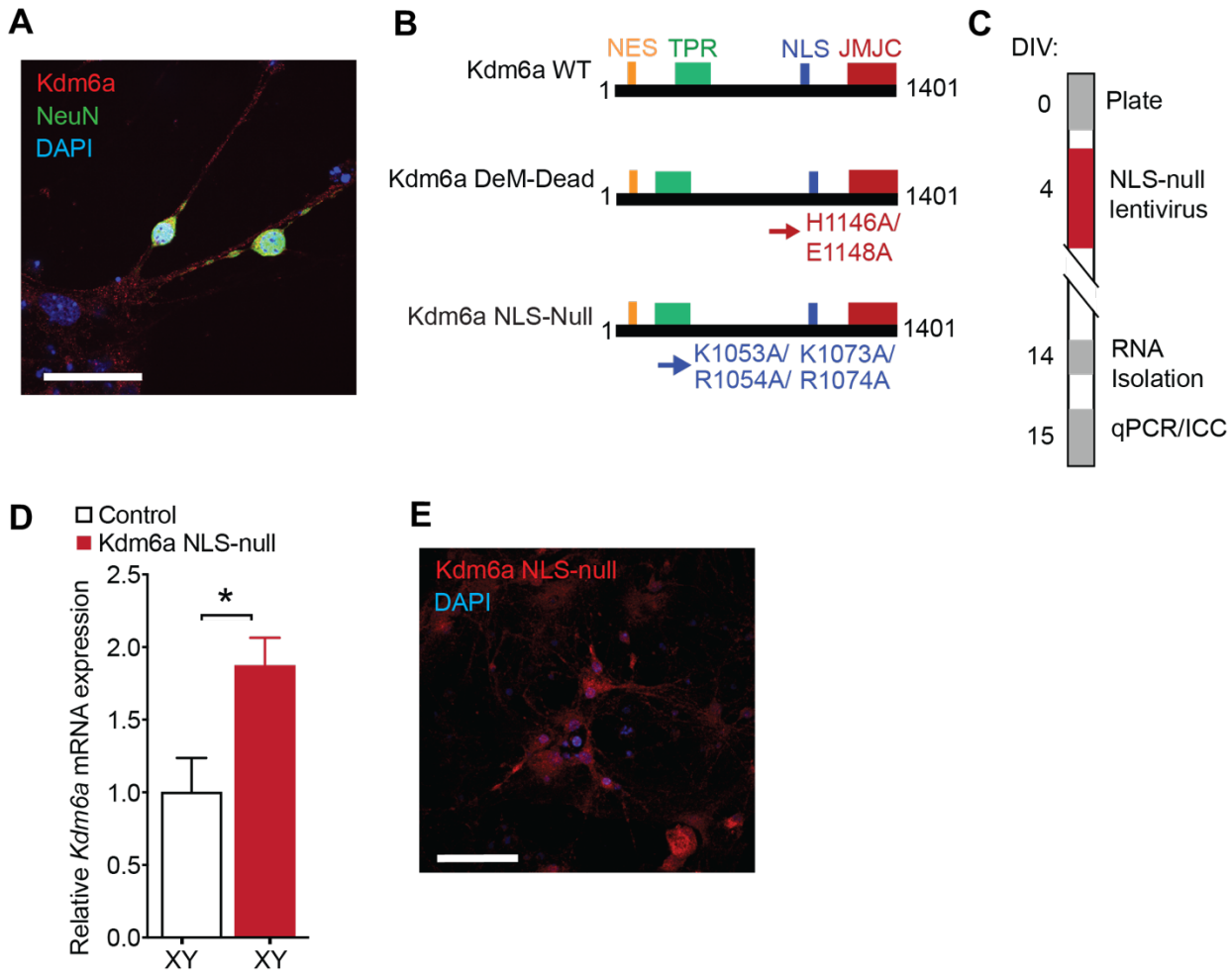

Figure 4.1. Endogenous Kdm6a is cytoplasmic, and lentiviral overexpression of nuclear localization signal mutated (NLS-null) construct trapped Kdm6a is the cytoplasm of primary XY mouse neurons.

(**A**) Primary XY neurons with Kdm6a immunofluorescence (Red, Kdm6a) (Blue, DAPI) (Green, NeuN). Scale bar=50µm; 60x magnification. **(B)** Kdm6a construct domain map. **(C)** Experimental strategy of lentivirus-mediated overexpression of Kdm6a NLS-null construct in XY mouse primary neurons. **(D)** *Kdm6a* mRNA levels in primary XY neurons transfected with lentivirus expressing control or Kdm6a NLS-null, shown relative to control (n = 6 wells per experimental group from 10 XY pups). $^{**}P < .01$ (2-tailed *t*-test). **(E)** Primary XY neurons with Kdm6a NLS-null immunofluorescence (Red, anti-flag tag) (Blue, DAPI). Scale bar=50µm; 60x magnification.

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