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Determinants of Cognitive Resilience in Aging and Alzheimer's Disease: Neuronal Ketone Metabolism and APOE-Dependent Responses to Brain Irradiation

by
Joyce Yang

DISSERTATION

Submitted in partial satisfaction of the requirements for degree of
DOCTOR OF PHILOSOPHY

in

Neuroscience

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

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by

Joyce Yang

Dedicated to my parents, Wei-Lien and Meng-Yu, my sisters, Joanna and Josephine, and my two
cats, Beans and Mochi.

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This dissertation would not have been possible without the unwavering support of friends and family from my hometown of Phoenix, Arizona, the incredible mentors and friends I have made in the Bay Area since arriving at UCSF, and the greater scientific communities that I have had the fortune to be a part of throughout my life.

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Contributions

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Chapter 2 is reproduced in an adapted form from: (*co-first authorship)

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Chapter 3 is reproduced in an adapted form from: (*co-first authorship)

***Yang, J.,** *Oyewole, A., Lee, M., Simms, J., Sei, Y.J., Bui, L., Huang, Y., Nakamura, K., & Nakamura, J.L. Sex and APOE genotype jointly modify memory after brain irradiation. *In submission*.

Chapter 4 is written by Joyce Yang.

**Determinants of Cognitive Resilience in Aging and Alzheimer's Disease:
Neuronal Ketone Metabolism and APOE-Dependent Responses to Brain Irradiation**

Joyce Yang

Abstract

Alzheimer's disease is the most common cause of progressive, age-associated memory loss and neuronal death. Despite recent advances in slowing disease progression, the underlying factors that confer cognitive resilience or accelerated decline remain poorly understood. Here, two potential determinants of resilience are investigated: the intrinsic energetic and metabolic profile of neurons, and the genetic context that governs the brain's response to injury.

To characterize the intrinsic metabolic landscape, we began with metabolomic profiling of iPSC-derived human neurons, which revealed a substantial contribution of the physiologic ketone β -hydroxybutyrate to energetic pathways. Subsequent studies revealed that neurons are capable of rapidly rewiring their metabolism under ketogenic conditions and preferentially oxidize the ketone acetoacetate in place of glucose. Physiologic ketone metabolism was, in fact, essential for maximum mitochondrial respiration and neuronal function. In adult-inducible, neuron-specific *Bdh1* knockout mice, loss of neuronal ketone oxidation in early adulthood accelerated mortality and produced memory deficits under normal dietary conditions—phenotypes that became markedly worse on an Alzheimer's disease background. Together, these findings establish an obligate requirement for neuronal ketone oxidation in meeting the energetic demands of the brain, with direct implications for protecting against neurodegeneration.

To probe the genetic foundations of cognitive resilience, we then asked whether inherited Alzheimer's risk factors modify cognitive change following brain irradiation, a common cancer therapy. Using mice homozygous for humanized APOE3 or APOE4, with or without knock-in mutant amyloid precursor protein (mutAPP), we demonstrated that APOE genotype shapes the cognitive outcome of irradiation in opposing directions: APOE3 mice develop worsened memory, whereas APOE4 mice maintained or improved memory, most notably in females. Introducing a mutAPP background abolished this protective effect in APOE4 mice, leaving memory deficits comparable with or without radiation. Together, these results suggest that radiation converges on a pathway shared with APOE4 and mutAPP to influence memory, and that genotype and sex jointly determine its cognitive consequences.

Taken together, these studies identify metabolic and genetic factors that shape the resilience of memory during aging and Alzheimer's disease, and in response to brain injury. They also chart a hopeful path forward: enhancing neuronal ketone metabolism offers a promising, nontraditional strategy to help preserve cognition, and APOE genotype and sex may serve as predictors for tailoring cancer therapy to protect the brain. More broadly, this work reframes cognitive decline as a process shaped by both intrinsic and extrinsic factors, revealing new avenues toward keeping neurons—and memory—intact.

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List of Abbreviations

82E1: anti-amyloid- β antibody (labels immature A β)

AcAc: acetoacetate

Acetyl-CoA: acetyl coenzyme A

AD: Alzheimer's disease

AMP: adenosine monophosphate

APA: active place avoidance (behavioral paradigm)

APOE / ApoE: apolipoprotein E

APOE ϵ 4 (E4): apolipoprotein E ϵ 4 allele

APP: amyloid precursor protein

App^{NLGF}: App knock-in with Swedish, Iberian, and Arctic mutations

AT8: anti-phospho-tau (Ser202/Thr205) antibody

ATP: adenosine triphosphate

AUC: area under the curve

A β / a β : amyloid-beta

BCS: body condition score

Bdh1 / BDH1: β -hydroxybutyrate dehydrogenase 1

β HB (BHB): β -hydroxybutyrate

BSA: bovine serum albumin

C57BL/6J: mouse strain "Black 6": Cold Spring Harbor strain 57, black coat color, substrain 6,
maintained at the Jackson Laboratory

CD: control diet

CRISPRi: CRISPR interference

DAB: 3,3'-diaminobenzidine

DAPI: 4',6-diamidino-2-phenylindole (DNA-binding dye)

dCas9-KRAB: catalytically dead Cas9 fused to KRAB repressor domain

DPBS: Dulbecco's phosphate buffered saline

EPM: elevated plus maze

F1,6BP / F-1,6-BP: fructose-1,6-bisphosphate

F6P: fructose-6-phosphate

FA: fatty acid

FCCP: carbonyl cyanide-p-trifluoromethoxyphenylhydrazone

FOV: field of view

G6P: glucose-6-phosphate

GFAP: glial fibrillary acidic protein

GMP: guanosine monophosphate

gRNA: guide RNA

hAPPJ20 (J20): human APP J20 transgenic line

Hmgcl: 3-hydroxy-3-methylglutaryl-CoA lyase

Hmgcs2: 3-hydroxy-3-methylglutaryl-CoA synthase 2

HPLC-MS: high-performance liquid chromatography–mass spectrometry

HT7: anti-tau antibody

Iba1: ionized calcium-binding adaptor molecule 1

IMP: inosine monophosphate

iPSC: induced pluripotent stem cell

KD: knockdown (or ketogenic diet; see corresponding notations)

KI: knock-in

KO: knockout

LSD: (Fisher's) least significant difference

MAP2: microtubule-associated protein 2

MCI: mild cognitive impairment

mutAPP: mutant amyloid precursor protein

NAD⁺ / NADH: nicotinamide adenine dinucleotide (oxidized / reduced)

NADPH: nicotinamide adenine dinucleotide phosphate

NeuN: neuronal nuclei antigen (neuron nuclear marker)

NGN2 (Ngn2): neurogenin-2

NOR: novel object recognition

NTG: non-targeting guide RNA (control)

OA: oleate / oleic acid

OCR: oxygen consumption rate

OF: open field

Oxct1: 3-oxoacid CoA-transferase 1 (SCOT)

PA: palmitate / palmitic acid

p-tau: phosphorylated tau

PS19: P301S tau transgenic mouse line

qPCR: quantitative polymerase chain reaction

ROCKi: ROCK (Rho kinase) inhibitor

S7P: sedoheptulose-7-phosphate

SA: stearate / stearic acid

SD: standard deviation

SEM: standard error of the mean

TCA: tricarboxylic acid cycle

ThioS: thioflavin S

Thy1: thymocyte differentiation antigen 1 (Thy1-CreERT2 / SLICK-H line)

VC: visual cliff

WT: wild-type

WTC11: wild-type control 11 human iPSC cell line

List of Symbols

β: beta; used in β-hydroxybutyrate, amyloid-β, β-actin

Δ: delta; change or difference

ε: epsilon; APOE allele designation (e.g., ε4)

Gy: gray; SI unit of absorbed radiation dose

¹³C: carbon-13 stable isotope label (U-¹³C = uniformly labeled)

CHAPTER 1: Modifiers of Memory in Aging and Neurodegeneration

Alzheimer's disease (AD) is the most common cause of age-associated cognitive decline, but the progressive memory loss that defines it remains without a durable treatment. As the global population ages, understanding why certain individuals harbor heightened cognitive resilience while others exhibit accelerated decline has become a central scientific question. Here, we investigate two broad classes of factors that shape this trajectory: the intrinsic properties of neurons themselves—including how this particularly vulnerable cell type generates the energy required to sustain function—and the genetic context under which those neurons operate, such as inherited risk alleles, biological sex, and exposure to external stressors. Using a combination of *in vitro* and *in vivo* genetic models, we ask how metabolism, genotype, and sex impact the resilience or vulnerability of memory in aging and neurodegeneration.

The brain is an extraordinarily energy-demanding organ. Under physiologic conditions, glucose is its primary oxidative fuel (1). Yet, the brain retains the capacity to oxidize alternative substrates: during fasting or starvation, the ketone bodies β -hydroxybutyrate (β HB) and acetoacetate (AcAc) can support a large proportion of the body's energetic needs (2). Glucose and energy utilization in the brain decline with age and, more steeply, in AD, as well as in cognitively normal carriers of the APOE ϵ 4 allele who are at elevated risk for the disease (11–17). This has motivated interest in ketones as an alternative fuel that might bypass glucose-related energetic deficits in aging. Our understanding of brain ketone metabolism, however, derives largely from exogenous supplementation or dietary manipulation, rather than from the low, constitutive levels of ketones present under ordinary conditions. Whether neurons actually require ketone oxidation for normal function throughout adult life, and whether this requirement becomes more critical in

neurodegeneration, has remained unresolved—in part because brain tissue is a heterogeneous mixture of neurons and glia, and because prior genetic models of ketone metabolism were confounded by developmental or systemic effects. Here, we address this question directly through a combination of induced pluripotent stem cell (iPSC)-derived human neurons and adult-inducible, neuron-specific mouse knockouts of β -hydroxybutyrate dehydrogenase 1 (*Bdh1*), the enzyme that interconverts β HB and AcAc and gates their oxidation for energy metabolism.

A second determinant of cognitive fate is inherited genetic risk, and no common variant carries more weight than APOE. The APOE ϵ 4 allele is the strongest genetic risk factor for late-onset AD, present in 20–25% of the population and the majority of patients, with dose-dependent effects that are stronger in women than in men (67–69, 71). Such genetic susceptibility may shape not only the course of neurodegeneration, but also how the brain responds to external insults. One increasingly relevant insult is cancer therapy: brain-focused radiation is a common and often life-saving treatment, but it can produce lasting deficits in memory and other cognitive domains (74–77). There are currently no reliable biomarkers to predict these outcomes. Whether inherited risk for neurodegeneration—through APOE or amyloid-driven factors—modifies cognitive response to brain irradiation, and whether sex interacts with that risk, is essentially unknown. To investigate these risk factors and interactions, we use AD mouse models homozygous for human APOE3 or APOE4—with or without knock-in mutant amyloid precursor protein (mutAPP)—exposed to whole-brain irradiation and evaluated through comprehensive neurobehavioral and neuropathological analyses.

Although the two presented studies employ different models and interventions, they are unified by a common question and conceptual frame. Both query factors that modify memory function in aging and Alzheimer's disease, and both converge on a set of modifiers: cellular

metabolism, genetic background, amyloid pathology, and biological sex. In Chapter 2, adult-onset impairments to neuronal ketone oxidation shorten lifespan and worsen memory, with effects that are amplified on an amyloid (hAPPJ20) background and that differ between males and females. In Chapter 3, APOE genotype dictates whether brain irradiation harms or spares memory, but the addition of a mutant APP background overrides these genotype-specific effects. Sex again emerges as a significant variable in determining outcomes. Thus, sex, genotype, and their interactions with endogenous metabolism and exogenous stressors recur as organizing threads throughout this dissertation.

Together, these chapters advance a view of cognitive resilience as something actively maintained by the underlying metabolic machinery of neurons, and by the genetic context that governs response to injury. Chapter 2 establishes an obligate requirement for neuronal ketone body oxidation in normal brain function and aging, identifying a potentially targetable metabolic vulnerability. Chapter 3 demonstrates that inherited AD risk and sex strongly influence cognitive outcomes following a common cancer therapy, with direct implications for how such therapies may be tailored to individual patients. Chapter 4 synthesizes these findings, considers their translational implications, and outlines directions for future work at the intersection of brain metabolism, genetic risk, and cognitive aging.

CHAPTER 2: Requirement for Oxidation of Neuronal Ketone Bodies in Aging and Neurodegeneration

ABSTRACT

Glucose is the brain's primary fuel, but the brain can also use alternative energy substrates, especially during development or starvation. Emerging evidence suggests ketone metabolism may help the brain adapt to energy stress in neurodegenerative diseases such as Alzheimer's disease, although its role in constitutive brain function in normal aging is poorly understood. Using iPSC-derived human neurons and adult-inducible, neuron-specific *Bdh1* knockout mice, we show that ketone body metabolism is essential for maximum energy production, neuronal function, and mouse survival—even under normal nutritional conditions. Mechanistically, phenotypes of *Bdh1* knockout neurons are mitigated by provision of acetoacetate, a downstream energy metabolite. Moreover, loss of neuronal ketone oxidation markedly increases mortality and memory deficits in Alzheimer's disease model mice. These findings identify ketones as critical neuronal fuels, with particular importance during neurodegeneration. While non-energetic activities of ketone bodies are increasingly appreciated, oxidation for energy provision is an essential mechanism for normal function in neurons and mice. Targeting the energetic function of ketones may thus offer new therapeutic strategies for both aging and neurodegenerative diseases such as Alzheimer's.

INTRODUCTION

Glucose is the primary oxidative fuel for the mammalian brain under physiologic conditions (1). However, in energetically compromised states, the brain can rewire to utilize alternative substrates including ketone bodies, which can support the majority of energetic needs during starvation (2). Little is known, however, about neuronal requirements for ketone metabolism under non-fasting physiological or pathological conditions in the adult brain (3).

The ketone bodies β -hydroxybutyrate (β HB) and acetoacetate (AcAc) are small molecules synthesized primarily in the liver from lipid substrates. These ketones circulate and provide a readily oxidizable source of Acetyl-CoA and ATP to meet the energetic needs of diverse tissues (4). β HB and AcAc are interconverted by the bidirectional enzyme β -hydroxybutyrate dehydrogenase 1 (Bdh1) as the final step in ketone body production and the first step in oxidation (Fig. 2.1A). While blood levels of ketone bodies are induced 10- to 100-fold under glucose-deprived states such as fasting, starvation, or the low-carbohydrate ketogenic diet, a small amount of ketone bodies are produced constitutively, resulting in blood levels in the 50-100 micromolar range (5). Astrocytes may also generate β HB and AcAc from fatty acids *in situ* (6, 7), a process required in *Drosophila* for normal memory function (8). While the role of ketones in supporting the mammalian brain during starvation is well known, their function under normal conditions is less understood.

Ketogenic metabolism is required in the neonatal state for normal mammalian brain and sensory neuron development (9, 10), but less is known about its requirement beyond this period. Glucose and energy utilization in the brain decreases with age and in Alzheimer's disease (AD) (11-15), as well as in cognitively normal ApoE4 carriers (16, 17) who are at increased risk for AD. Baseline brain usage ratios of 29:1 for glucose to other fuels can shift as low as 2:1 in incipient

AD (18, 19). The capacity to oxidize ketone bodies remains intact in AD (20), and in heart failure, cardiomyocytes preferentially utilize ketone bodies as an adaptive means to rescue energy deficits (21, 22). Ketogenic therapies are therefore under clinical investigation in both heart failure and AD (12, 21). Data on ketone body utilization in AD is largely derived from exogenous administration or ketogenic diets (23, 24), but not the study of endogenous constitutive ketogenesis. It is unknown whether neuronal metabolism of endogenous ketone bodies is compensatory for bioenergetic deficits in AD, or more broadly, whether this process is required for normal neuronal and brain function throughout adult life.

Here we use a combination of induced pluripotent stem cell (iPSC)-derived human neurons and adult-inducible, neuron-specific mouse knockouts of *Bdh1* to interrogate the role of ketone bodies in neuronal metabolism and organismal aging, in both wild-type and AD genetic backgrounds. Using our models, we find a neuronal requirement for ketone bodies to generate maximal acetyl-CoA, with distinct roles for AcAc and β HB. Further, mice with adult-induced neuron-specific *Bdh1* knockout show accelerated early mortality and memory impairments in both wild-type and AD genetic backgrounds, supporting a constitutive requirement for neuronal ketone body oxidation throughout mammalian adulthood.

RESULTS

Human neurons rapidly rewire metabolic pathways in response to ketogenic environments

Although β -hydroxybutyrate (β HB) is constitutively present in blood, crosses the blood-brain barrier, and is oxidized in the brain (2, 12), there is little direct evidence of neuronal uptake and metabolism of β HB, in part because brain tissue and primary neuronal cultures contain a

significant proportion of glial cells. To overcome this hurdle, we studied β HB metabolism in near-homogenous human excitatory neurons derived from iPSCs (25, 26) co-expressing dCas9-KRAB and neurogenin-2 (Ngn2) (27).

iPSC lines were differentiated into neurons by doxycycline induction of Ngn2 and cultured for 20 days (**Fig. 2.1B**). Neurons were then incubated for 4, 24, or 48 h in media with different ratios of ^{13}C - β HB to glucose—physiologic (1.5 mM glc + 0.2 mM β HB; based on brain microdialysis (28-32)), moderately ketogenic (0.5 mM glc + 3 mM β HB; simulating bloodstream levels during ketogenic diet (33)), or extreme ketogenic (10 mM β HB)—before metabolite isolation. Isotope tracing of ^{13}C - β HB revealed rapid uptake and incorporation of β HB into the TCA cycle and lipid synthesis, but not into other major pathways (**Fig. 2.1C,F**). Surprisingly, a small amount of glucose, glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), and fructose-1,6-bisphosphate (F1,6BP) also contained ^{13}C (**Fig. 2.1F**), suggesting gluconeogenesis may be occurring in neurons.

The metabolomic profiles of cells exposed to moderate and extreme β HB/glucose ratios differed from cells exposed to a physiologic ratio (**Fig. 2.1D**), as illustrated by a decreased flux through glycolytic metabolites detectable within 4 h of incubation and an increased level of some TCA metabolites by 24 h. There was also a substantial elevation in nucleotide (NT) biosynthesis, with sedoheptulose-7P and hypoxanthine appearing as two of the strongest hits across the panel (**Fig. 2.1E**). Changes were similar across moderate and extreme ketogenic conditions, suggesting that large changes in fuel availability are not necessary to significantly alter the neuronal metabolome.

Human neurons utilize β HB for oxidative metabolism under physiologic states

Labeling of TCA metabolites from ^{13}C - β HB after 24 h under physiologic β HB/glucose ratio was substantial, supporting that human neurons actively utilize the constitutive ketones present in the brain microenvironment. For instance, approximately 30% of acetyl-CoA—the TCA cycle entry-point metabolite—was at least partially derived from β HB under physiologic conditions, a proportion that increased only modestly as β HB availability increased from 0.2 mM to 3 mM and 10 mM (37% and 41%, respectively) (**Fig. 2.1G, blue**).

Given this substantial usage of ketones for oxidative metabolism, we wondered whether β HB is a dominant neuronal fuel source, similar to glucose. We repeated HPLC-MS targeted metabolomics with isotope tracing of ^{13}C -glucose for 24 or 48 h under physiologic and moderately ketogenic conditions. Neurons in ketogenic conditions exhibited a significant shift away from glucose-based TCA derivation, and towards β HB by 24 h (**Fig. 2.1G, green vs. blue**). The shift was even more pronounced by 48 h (**Fig. 2.1H**). Again, large changes in fuel availability were not necessary to generate fuel shifts (physiologic vs. moderately ketogenic), suggesting that human neurons readily utilize β HB. Total Acetyl-CoA levels were also elevated in ketogenic relative to physiologic conditions by 48 h (**Fig. 2.1I**). Taken together with the isotope tracing, these observations establish that human neurons directly import and metabolize β HB, and that β HB is a major fuel source contributing to neuronal oxidative metabolism across a range of conditions, including under normal glucose availability.

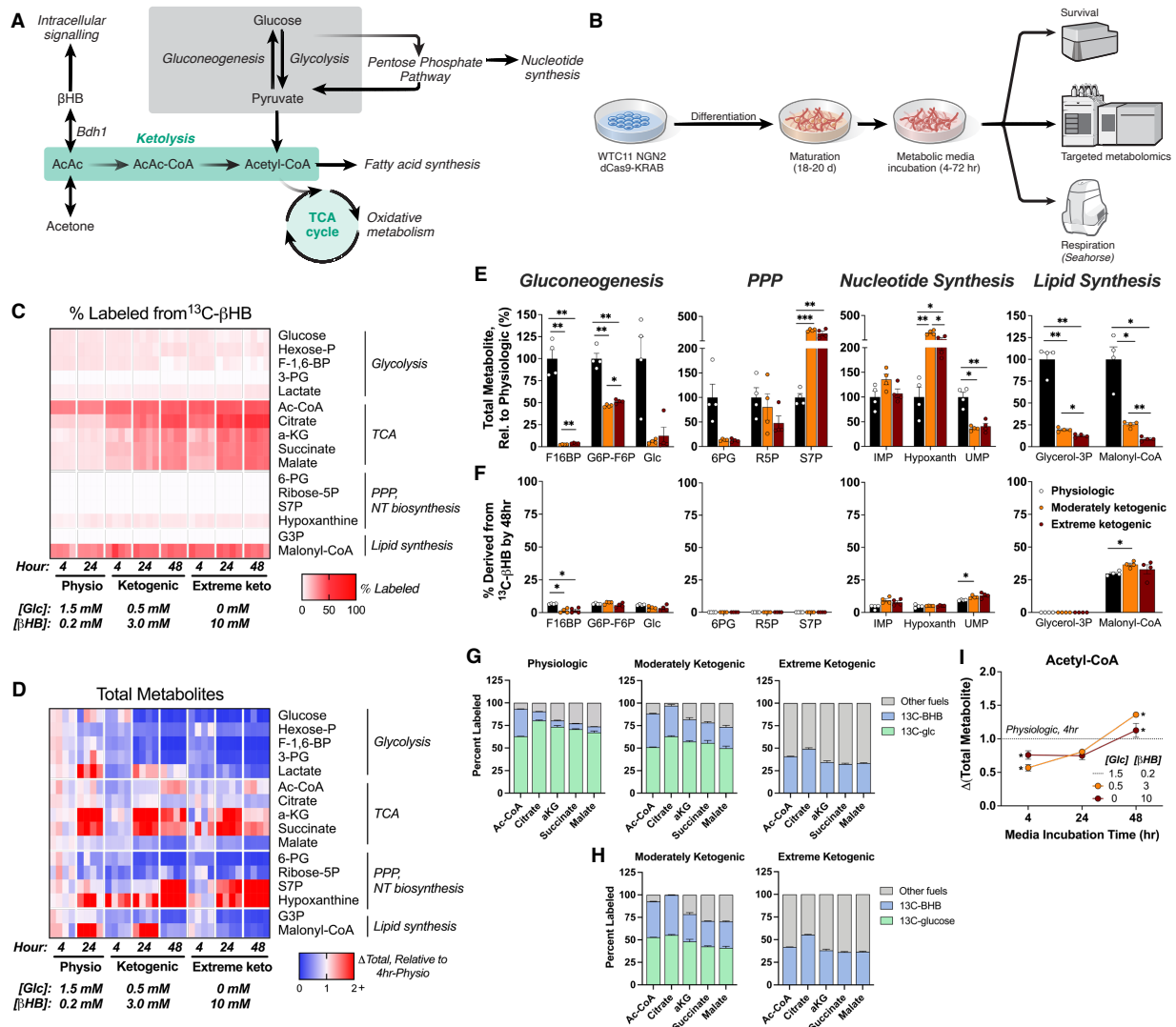


Fig. 2.1. Neurons use β -hydroxybutyrate in oxidative metabolism under physiologic and ketogenic conditions. (A) Pathways of interest relative to ketones β -hydroxybutyrate (β HB), acetoacetate (AcAc), and acetone. (B) Experimental timeline for WTC11 human iPSCs expressing doxycycline-inducible Neurogenin-2. (C) HPLC-MS targeted metabolomics conducted on 20-22d neurons incubated with ^{13}C - β HB under physiologic, moderately ketogenic, or extreme conditions for 4, 24, or 48h. Rows: metabolites; columns: percent labeled from ^{13}C - β HB; and (D) total metabolite change per replicate, normalized to 4hr physiologic average. N=4 per condition. (E) Physiologic-normalized total metabolite and (F) percent derivation from ^{13}C - β HB after 48hr incubation in metabolic media. N=4 per condition. (G) Fractional labeling of TCA metabolites from isotope tracing of ^{13}C -glc or ^{13}C - β HB after 24hr or (H) 48hr incubation in ketogenic conditions. N=4, except 24 h “moderately ketogenic” with ^{13}C -glc (3). (I) Changes in total Acetyl-CoA over time, relative to 4hr physiologic. N=4 per condition. Data are means $\pm$ SEM. *p < 0.05, **p < 0.01, and ***p < 0.001 by Brown-Forsythe and Welch one-way ANOVA with Dunnett’s T3 multiple comparisons (E-F), or 2-way ANOVA with Dunnett’s multiple comparisons vs. physiologic 4hr average (I).

AcAc is a better alternative to glucose than β HB for neuronal energy production

We next investigated whether other lipid-based fuels—ketones AcAc and acetone, and common fatty acids palmitate (PA), oleate (OA), and stearate (SA)—induce similar metabolic phenotypes as β HB does. We first generated dose-survival curves in 21-day human neurons incubated for 48 to 72 h in lipid-based media, and selected 8 mM (ketones) and 50 μ M (fatty acids) as concentrations that maximized fuel availability with little cell death (**Extended Data Fig. 2.1A**).

Using HPLC-MS targeted metabolomics, we observed decreased total and labeled glycolytic metabolites across all conditions relative to physiologic (**Fig. 2.2A**), consistent with a shift to lipid-based metabolism. We performed ranked analyses on the metabolite panel for fold-change comparisons and found large elevations in inosine monophosphate (IMP) and guanosine/adenosine monophosphate (GMP, AMP) without substantial incorporation of initial ^{13}C -fuels (**Extended Data Fig. 2.1B-C**). Given that these metabolites are precursors of ATP, NADH, and NADPH, these peripheral elevations suggest a possible mechanism through which neurons accommodate metabolic changes by increasing coenzymes for energy production. Surprisingly, sole provision of ^{13}C -AcAc resulted in TCA labeling similar to that of glucose under physiologic conditions (**Fig. 2.2B**, “AcAc” vs. “ $^{13}\text{Glc}+\beta\text{HB}$ ”), while sole provision of ^{13}C - β HB resulted in more intermediate TCA labeling similar to before (**Fig. 2.1C** “extreme keto” vs. **fig 2B** “ βHB ”). This suggests that AcAc is a more effective substitute for glucose than β HB in maintaining neuronal oxidative metabolism.

Ketones are vital to maximize mitochondrial respiration in restricted & physiologic states

To further explore contributions of lipid-based fuels to mitochondrial oxidative phosphorylation, we recorded oxygen consumption rates (OCR) via Seahorse assay in mature

neurons incubated for 72 h in the previously-described conditions. Of the sole-provided fuels, only AcAc (but not β HB) matched the reserve capacity observed under physiologic conditions containing both glucose and β HB (**Fig. 2.2C-F**), supporting the hypothesis that AcAc is a more effective respiratory substrate than β HB, that can substitute for both glucose and β HB to maintain neuronal mitochondrial respiration.

Given the previously demonstrated contribution of β HB to the acetyl-CoA pool under physiological conditions (**Fig. 2.1G**), we next assessed the effect of physiologic fuel availability on OCR. Removal of this low concentration of β HB led to a significant decrease in reserve capacity (**Fig. 2.2G-H**), underscoring the requirement for β HB during physiologic states. Furthermore, incremental changes in fuel availability produced corresponding changes in reserve capacity, suggesting that neurons are highly sensitive to small fluctuations in environmental ketone (**Extended Data Fig. 2.1D**) and glucose (**Extended Data Fig. 2.1E**) levels. Therefore, although β HB alone cannot compensate for loss of glucose (**Fig. 2.2C**), β HB supplementation can boost energy production beyond glucose alone.

Human neurons preferentially convert excess lipid-based fuels into oleic acid

The fuel concentrations utilized for metabolomics and Seahorse were selected to maximize availability while minimizing toxicity (**Extended Data Fig. 2.1A**). To gain insight into whether neurons perform ketogenesis or store excess ketones as fatty acids, we screened interconversion patterns between each of the tested fuels from the metabolomics panel. Analyses of total metabolite production (**Fig. 2.2I**) and percent derived from each starting fuel (**Fig. 2.2J**) revealed the expected conversion of AcAc to β HB, but no indication of *de novo* ketogenesis, and instead preferential conversion of all substrates towards storage as oleic acid via fatty acid synthesis (**Fig. 2.1A**).

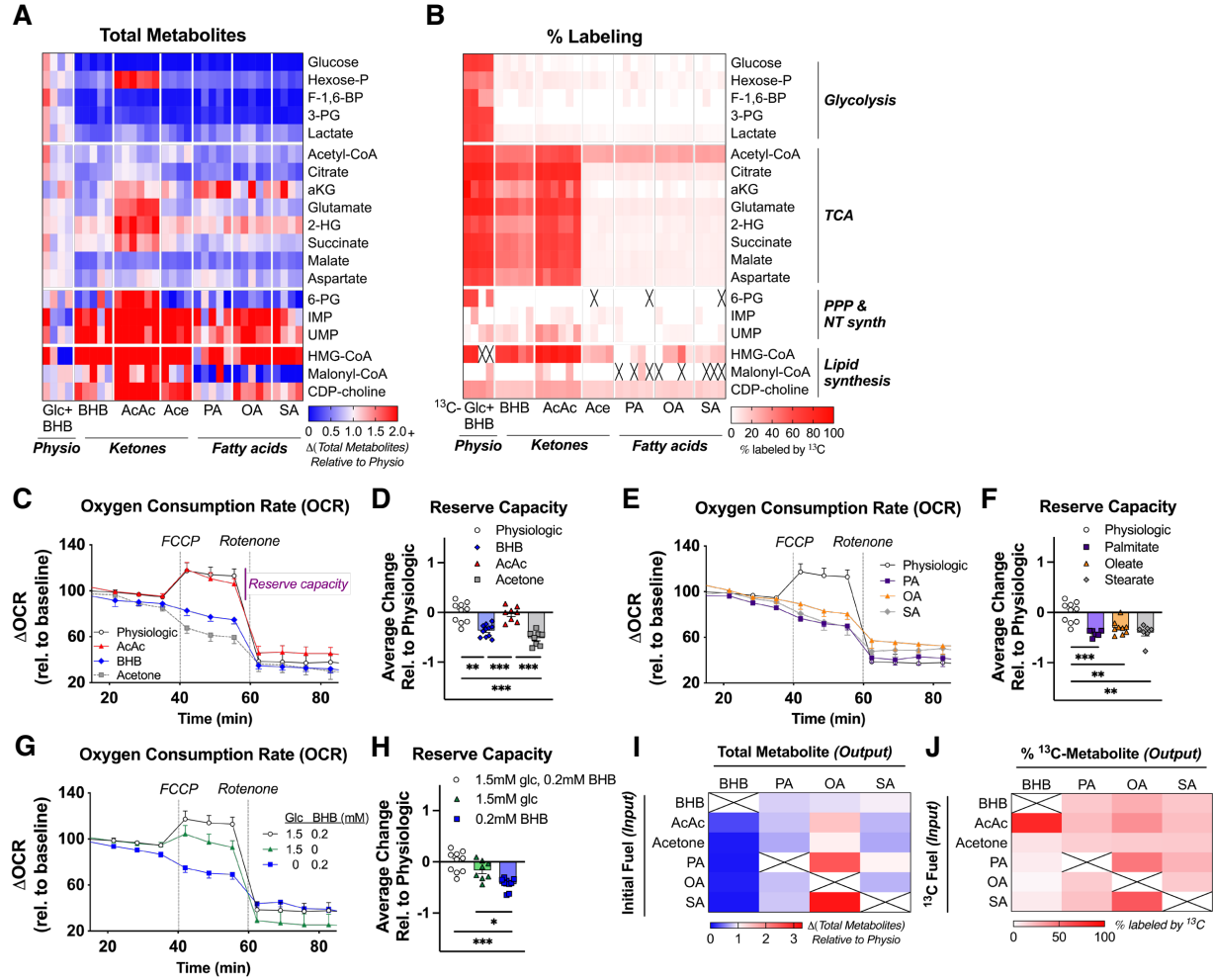


Fig. 2.2. Neurons preferentially utilize acetoacetate in oxidative metabolism to support cellular respiration. (A) Targeted metabolomics conducted on 21d neurons after 3d incubation in physiologic, ketone-, or fatty acid (FA)-based medias with initial ^{13}C -fuel of interest. Rows: metabolites; columns: total metabolite change, normalized to physiologic average; and (B) corresponding % labeled from ^{13}C -fuel. N=4 (Glc+ β Hb, Ace, SA), 5 (β Hb, PA, OA), or 6 (AcAc) samples/condition. (C) Oxygen consumption rate (OCR) after 3d incubation in ketone-based or (E) FA-based conditions. Cells were treated sequentially with carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) and rotenone. Data normalized to average of baseline readings per condition, highlighting differences in maximum OCR (“reserve capacity”). (D, F) Quantification of relative reserve capacity. N=6 (PA), 7 (SA), 8 (AcAc, Ace), 9 (OA), 10 (Physio), or 11 (β Hb) samples/condition across 2 experimental replicates. (G) OCR changes after 3d incubation in physiologic media variants (+-glucose, +- β Hb), and (H) quantification of reserve capacity. N=8 (Glc), 9 (β Hb), 10 (Glc+ β Hb) samples/condition across 2 experimental replicates. (I) Total metabolite levels of β Hb, PA, OA, and SA (columns) based on initial fuel (rows), relative to physiologic levels; and (J) percent of each “output” containing ^{13}C from “input”. N=4 (Ace, SA), 5 (β Hb, PA, OA), or 6 (AcAc) samples/condition. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by Brown-Forsythe and Welch one-way ANOVA with Dunnett’s T3 multiple comparisons.

Knocking down Bdh1 causes oxidative and survival deficits in human neurons that are mitigated by AcAc supplementation

Bdh1 is the bidirectional enzyme that converts β HB into AcAc, which proceeds via Acetyl-CoA into the TCA cycle. To test the importance of this conversion for neuronal health and metabolism, we knocked down Bdh1 in iPSCs (*BDHI-kd* line) via inducible dCas9-KRAB (27) and differentiated the iPSCs into neurons. As controls, we generated an iPSC line expressing a non-targeting gRNA (*NTG* line) and cultured for 21d to validate stable knockdown (**Fig. 2.3A**).

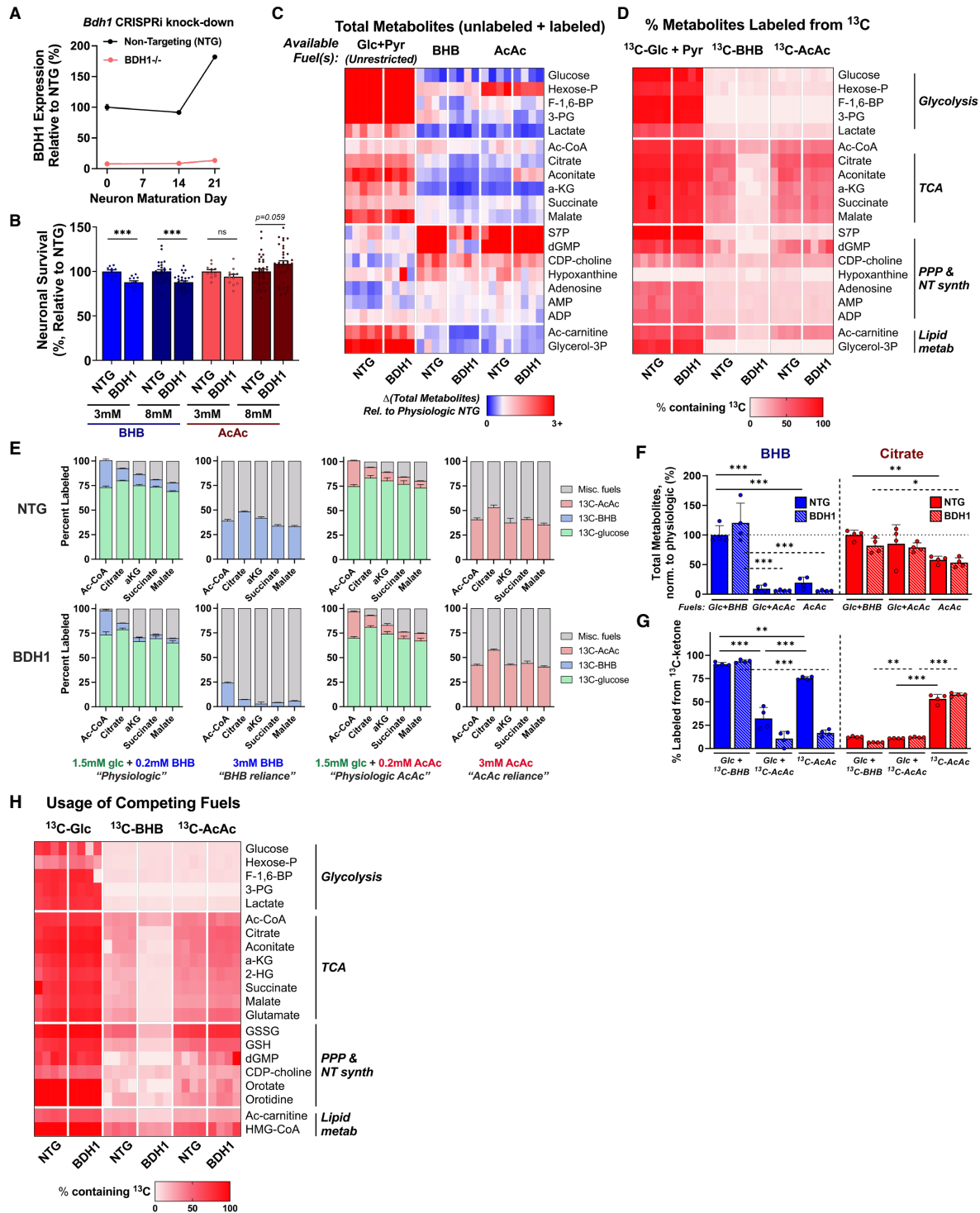
Survival curves showed that incubation with either 3 mM or 8 mM β HB, but not AcAc, decreased the survival of *BDHI-kd* neurons relative to *NTG* control (**Extended Data Fig. 2.2A-B, Fig. 2.3B**). To determine if these differences may be attributed to discrepancies in energy production, we conducted metabolomics in basal (unrestricted glc + pyruvate), physiologic (1.5 mM glc + 0.2 mM β HB), physiologic AcAc (1.5 mM glc + 0.2 mM AcAc), β HB-only (3 mM β HB), or AcAc-only (3 mM AcAc) medias. As expected, *BDHI-kd* neurons largely stopped metabolizing ^{13}C - β HB (**Fig. 2.3D**), resulting in reduced TCA metabolites relative to physiologic (**Fig. 2.3C**). General usage of ^{13}C -AcAc was unaffected (**Fig. 2.3D**). *BDHI-kd* neurons incubated in β HB exhibited reductions in S7P and dGMP compared to control, and to AcAc-incubated *BDHI-kd* & control neurons. This suggests the elevations are linked to ketone oxidation via Bdh1.

To explore the possibility that AcAc mitigates Bdh1-related oxidative deficits, we compared the generation of TCA metabolites in physiologic conditions relative to either 0.2 mM β HB or 0.2 mM AcAc, and from either ^{13}C -glc or ^{13}C - β HB/-AcAc. In physiologic states, the relative contribution of ^{13}C - β HB was reduced in *BDHI-kd* neurons (**Fig. 2.3E**). These differences were mitigated in physiologic AcAc conditions, where β HB was swapped for AcAc. We observed similar mitigation when comparing ^{13}C - β HB versus ^{13}C -AcAc reliance conditions.

Bdh1 strongly favors ketolysis in human neurons

In extrahepatic tissues, the canonical function of Bdh1 is ketolysis, the conversion of imported β HB to AcAc as the first step towards oxidation of β HB. But this reaction is reversible, and its direction is determined by substrate concentration gradients and NAD^+/NADH balance. Since β HB is now known to be a multifaceted signaling metabolite (4), the reverse reaction could enhance these signaling activities (**Fig. 2.1A**). To test the directionality of Bdh1 in neurons, we analyzed β HB and citrate production as readouts for the reverse (β HB-producing) and canonical (AcAc-generating) directions, respectively. In *NTG* neurons, only citrate was substantially produced from ^{13}C -AcAc (**Fig. 2.3F-G**), supporting the primacy of the canonical reaction in neurons. Moreover, the fraction of every metabolite in the panel derived from ^{13}C -AcAc was nearly identical in *NTG* and *BDHI-kd* (**Extended Data Fig. 2.2D**; $y = 1.04x - 0.14$, $R^2 = 0.99$), indicating no impact of Bdh1 activity on ^{13}C -AcAc metabolic fate.

To probe the impact of Bdh1 on the relative usage of energetic metabolites, we conducted targeted metabolomics on 21-day *NTG* and *BDHI-kd* neurons incubated with all three substrates (β HB, AcAc, and glucose) at 3 mM each, run in triplicate with alternating ^{13}C -labeled fuels. ^{13}C -glc labeled a high fraction of most metabolites in our panel in *NTG* neurons, as expected, whereas ^{13}C -AcAc and ^{13}C - β HB labeled a subset of the panel and less strongly than ^{13}C -glc (**Fig. 2.3H**). *BDHI-kd* substantially reduced labeling by ^{13}C - β HB only, consistent with the metabolic fate of β HB being primarily via conversion to AcAc through Bdh1. There was a corresponding small relative increase in labeling from *both* ^{13}C -glc and -AcAc in response to *BDHI-kd*. Altogether, these data are consistent with Bdh1 functioning primarily in the canonical direction in neurons, and with neurons actively metabolizing ketones even in the presence of abundant glucose.



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(C) Targeted metabolomics after 3d incubation in basal (^{13}C -Glc + Pyr, unrestricted), ^{13}C - βHB -only (3mM), or ^{13}C -AcAc-only (3mM) media. Rows: metabolites; columns: total metabolite change, normalized to physiologic NTG (1.5mM Glc + 0.2mM βHB); and (D) percent labeled from ^{13}C -fuel. N=4 samples/condition, except NTG unrestricted (5). (E) Fractional labeling of TCA metabolites from isotope tracing of ^{13}C -glc, - βHB , or -AcAc after 72hr. N=4 per condition. (F) Total levels of βHB and citrate after incubation in physiologic (1.5mM Glc + 0.2mM βHB), physiologic AcAc (1.5mM Glc + 0.2mM AcAc), or AcAc-only (3mM AcAc) conditions; and (G) percent of βHB or citrate containing ^{13}C label from βHB or AcAc. N=4 samples/condition. Data are means $\pm$ SEM. (H) Metabolomics on neurons after 3d incubation in 3mM glc + 3mM βHB + 3mM AcAc. Columns: percent of metabolite labeled from each initial ^{13}C -fuel in NTG vs. BDH1 neurons. N=4 samples/condition. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ by unpaired two-tailed t-test with Welch's correction (B), or 2-way ANOVA with Tukey's multiple comparisons; select comparisons shown (F-G).

Knockout of *Bdh1* in neurons exacerbates mortality & memory impairments in aging mice

Given the high rate of neuronal ketone oxidation *in vitro*, even in the presence of abundant glucose, we hypothesized that neuronal oxidation of βHB may be required for maintenance of normal cognitive function *in vivo*, even under normal diet and housing conditions (*ad libitum* feeding, typical diet) (34). To test this hypothesis, we generated neuron-specific *Bdh1* knockout (KO) (*Bdh1*^{Thy1-/-}) mice using a *Bdh1*^{fl/fl} line (22) and the Thy1-Cre^{ERT2} (SLICK-H) line (35). First, we generated a preliminary cohort for validation and to test acute effects of the knockout, injecting two-month-old *Bdh1*^{+/+} (*Bdh1*^{fl/fl} without Thy1-Cre^{ERT2}) and *Bdh1*^{Thy1-/-} mice with tamoxifen. We validated knockout of *Bdh1* in NeuN-positive neurons, but not GFAP-positive astrocytes (Extended Data Fig. 2.3, A and B). The deletion of *Bdh1* in neurons did not result in any significant alterations in *ad libitum* or fasted body weight, blood glucose levels, or plasma βHB levels (Extended Data Fig. 2.3C). *Bdh1* KO did not alter expression of ketogenic or ketolytic genes in the brain, heart or liver, except for *Bdh1* expression in the brain (Extended Data Fig. 2.3D). Glucose tolerance and insulin tolerance, assessed at nine months of age, were also unaltered (Extended Data Fig. 2.4, A and B). The KO did not alter the effect of a ketogenic diet (KD) on body weight, blood glucose levels, plasma βHB levels, or the expression of ketogenic and ketolytic

genes in the brain other than *Bdh1* (**Extended Data Fig. 2.4, C and D**). These findings indicate that KO of *Bdh1* in neurons does not generate acute toxicity, alter systemic glucose or ketone metabolism, or alter ketone-related transcription in the short term.

We then tested the role of neuronal ketolysis in lifespan and healthspan in well-powered mouse cohorts which were administered Tamoxifen at four months of age. Surprisingly, *Bdh1*^{Thy1-/-} mice of both sexes had decreased survival and reduced median lifespan, with a stronger negative effect in females (**Fig. 2.4A**). At 24 months of age, *Bdh1*^{Thy1-/-} mice also displayed reduced memory performance compared to controls in the novel object recognition (NOR) test (**Fig. 2.4B**), as well as impaired spatial working memory in the Y-maze (**Extended Data Fig. 2.5C**). There were no differences in body weight (**Extended Data Fig. 2.5A**) or in other aspects of behavior or physical performance, as assessed by the open field (**Extended Data Fig. 2.5B**), elevated plus maze (EPM) (**Extended Data Fig. 2.5D**), and rotarod (**Extended Data Fig. 2.5E**).

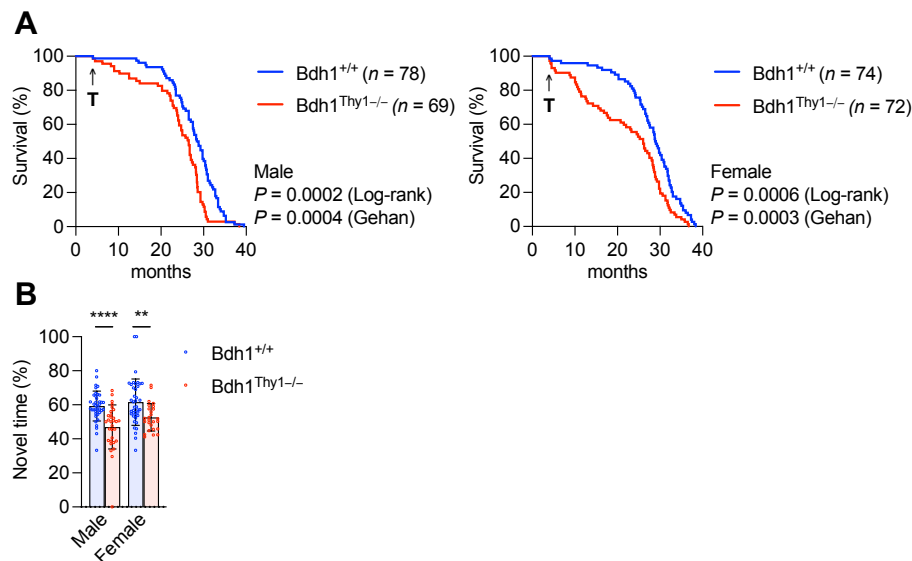


Fig. 2.4. Knockout of *Bdh1* in neurons exacerbates mortality and memory impairments in normal aging mice. (A) Survival curves. Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to calculate p-values. **(B)** Novel object recognition test at 24 months old. N=39 (*Bdh1*^{+/+} male), 31 (*Bdh1*^{Thy1-/-} male), 39 (*Bdh1*^{+/+} female), and 26 (*Bdh1*^{Thy1-/-} female) mice per group. P < 0.0001 (male, t = 4.72, df = 68) and p = 0.0036 (female, t = 3.02, df = 64) by unpaired two-tailed t-test assuming Gaussian distribution. Mice were given tamoxifen at four months old to knockout *Bdh1* in neurons.

Knockout of Bdh1 in neurons exacerbates mortality and memory defects in hAPPJ20 mice

Finally, we hypothesized that the deleterious effects of neuronal Bdh1 deletion would be exacerbated and become apparent at younger ages in AD models which are characterized by energetic stress (36-38). We crossed Bdh1^{Thy1-/-} mice with hAPPJ20 AD model mice, which overexpress a human APP gene containing several disease-associated mutations (39), and administered tamoxifen at 4 months of age. The knockout (hAPPJ20/Bdh1^{Thy1-/-}) demonstrated dramatically reduced survival, with males more strongly affected (**Fig. 2.5A**). Body weights were similar (**Extended Data Fig. 2.6A**). At 12-14 months of age we observed more significant memory impairment (NOR) (**Fig. 2.5B**), margin distance (open field) (**Extended Data Fig. 2.6B**), and anti-anxiety behavior (EPM) (**Extended Data Fig. 2.6D**) in the female KO mice compared to hAPPJ20/Bdh1^{+/+}. At 19-22 months of age, long-term memory in the Barnes maze was impaired in female hAPPJ20/Bdh1^{Thy1-/-} mice, but not in males (**Fig. 2.5C**). Conversely, in a simultaneously tested cohort on wild-type background, impaired memory in Barnes maze was observed only in male KO mice (**Fig. 2.5C**). There were no differences in tests of spatial working memory (Y-maze) (**Extended Data Fig. 2.6C**) and physical function (Rotarod) (**Extended Data Fig. 2.6E**). Overall, in both WT background and, more dramatically, in the hAPPJ20 AD model, impaired neuronal metabolism of BHB was associated with substantial mortality and memory function deficits, consistent with a requirement for neuronal ketone body oxidation under normal dietary conditions in both aging and AD.

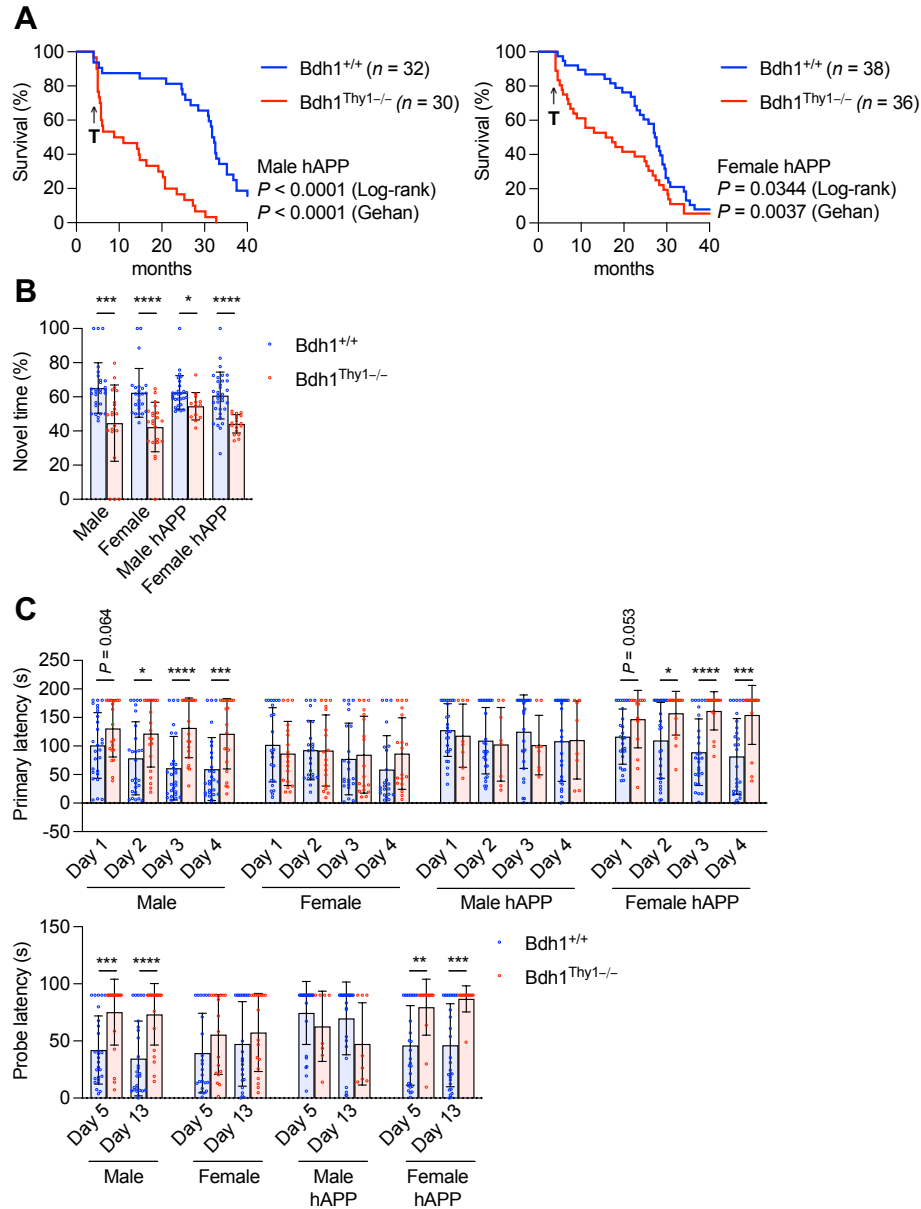


Fig. 2.5. Knockout of *Bdh1* in neurons exacerbate mortality and memory impairments in hAPPJ20 mice. (A) Survival curves. Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to calculate p-values. (B) Novel object recognition test at 12 months old. N=30 (*Bdh1*^{+/+} male), 23 (*Bdh1*^{Thy1-/-} male), 27 (*Bdh1*^{+/+} female), 22 (*Bdh1*^{Thy1-/-} female), 28 (hAPPJ20/*Bdh1*^{+/+} male), 14 (hAPPJ20/*Bdh1*^{Thy1-/-} male), 33 (hAPPJ20/*Bdh1*^{+/+} female), and 20 (hAPPJ20/*Bdh1*^{Thy1-/-} female) mice per group. $P = 0.00033$ (male, $t = 3.88$, $df = 47$), $p < 0.0001$ (female, $t = 4.77$, $df = 45$), $p = 0.014$ (hAPPJ20 male, $t = 2.57$, $df = 39$), and $p < 0.0001$ (hAPPJ20 female, $t = 5.02$, $df = 50$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (C) Barnes maze at 19-22 months old. N=27 (*Bdh1*^{+/+} male), 21 (*Bdh1*^{Thy1-/-} male), 23 (*Bdh1*^{+/+} female), 18 (*Bdh1*^{Thy1-/-} female), 27 (hAPPJ20/*Bdh1*^{+/+} male), 8 (hAPPJ20/*Bdh1*^{Thy1-/-} male), 28 (hAPPJ20/*Bdh1*^{+/+} female), and 16 (hAPPJ20/*Bdh1*^{Thy1-/-} female) mice per group. $P = 0.020$ (male, day 2, $t = 2.42$, $df = 46$), $p < 0.0001$ (male, day 3, $t = 4.47$, $df = 46$), $p =$ (continues on next page)

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0.00063 (male, day 4, $t = 3.67$, $df = 46$), $p = 0.013$ (hAPPJ20 female, day 2, $t = 2.61$, $df = 42$), $p < 0.0001$ (hAPPJ20 female, day 3, $t = 4.55$, $df = 42$), $p = 0.00050$ (hAPPJ20 female, day 4, $t = 3.78$, $df = 42$), $p = 0.00034$ (male, day 5, $t = 3.87$, $df = 46$), $p < 0.0001$ (male, day 13, $t = 4.38$, $df = 46$), $p = 0.0016$ (hAPPJ20 female, day 5, $t = 3.38$, $df = 42$), and $p = 0.0003$ (hAPPJ20 female, day 13, $t = 3.92$, $df = 39$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. Mice were given tamoxifen at four months old to knockout *Bdh1* in neurons.

DISCUSSION

While neurons are hypothesized to rely mainly on astrocyte-derived lactate (40) or direct glucose metabolism for energy (41, 42), we show that neurons can and must metabolize ketones for normal cellular bioenergetic functions, memory and survival, including under glucose-abundant conditions. In human neurons, physiologic levels of β HB are required for maximum acetyl-CoA generation, mitochondrial oxidation, and respiratory reserve. *In vivo*, mice with adult-induced knockout of *Bdh1* in *Thy1*-expressing cortical neurons die prematurely and display deficits in memory tasks, phenotypes that are further exacerbated in an Alzheimer's disease model. As such, these data reveal a previously unappreciated obligate requirement for β HB oxidation to AcAc by *Bdh1* in neuronal metabolism to support brain function, slow aging, and protect against neurodegeneration.

Neurons require *Bdh1* for physiologic energy metabolism

The constitutive function of ketone bodies, beyond their roles in fasting or specialized diets, remains understudied outside early development (9, 10). Our dual use of mitochondrial assays in human neurons *in vitro*, and cell-specific, adult-onset knockouts and longitudinal lifespan analyses *in vivo*, enabled us to uncover this requirement. Prior studies manipulating *Bdh1* or *Oxct1* used germline, whole-body, or liver-specific knockouts, which were confounded by developmental

effects, systemic or liver disease, or limited to early adulthood (43-49). By contrast, our Thy1-driven conditional model preserves normal systemic ketone levels and liver function, allowing us to dissociate β HB supply from its utilization within neurons.

The unclear extent of *in situ* ketogenesis in the brain may have obscured the constitutive requirement for ketone metabolism we observed. The liver generates nearly all circulating ketone bodies (2), readily measured via arteriovenous differences as hepatocytes lack Oxct1, the enzyme required for ketone oxidation (5). Assuming peripheral ketones are the brain's primary source, minimal arteriovenous differences in humans undergoing brain surgery (50) and in fed rats (51) argue against substantial brain ketone utilization in non-starved states. Brain uptake of ketone tracers correlates with circulating levels, which are low under basal conditions (52). However, *in situ* ketogenesis by astrocytes, as suggested by *in vitro* (6, 7) and model organism studies (8), and analogous to the lactate shuttle (40), could reconcile these findings. Clarifying the extent and function of brain ketogenesis remains an important direction for future research, with potential as a therapeutic target for neurodegenerative disease.

Recent studies show that acute β HB addition supports neural activity and boosts mitochondrial respiration (53, 54) in primary cultures, although the relative contributions from astrocytes versus neurons to β HB metabolism were not investigated. We found that β HB contributes to acetyl-CoA and TCA intermediates in human neurons *in vitro*, in a Bdh1-dependent manner. Loss of β HB metabolism to acetoacetate significantly decreases TCA metabolites, with the extent of reduction dependent on the relative availability of glucose and β HB, highlighting the potential to modulate brain energy via dietary fuels.

Notably, AcAc supplementation increased TCA metabolites and respiration more than did β HB. Since β HB oxidation depends on Bdh1-mediated conversion to AcAc, the stronger effect of

AcAc may reflect more efficient metabolism. Alternatively, AcAc may also have oxidation-independent signaling roles that regulate metabolism (4). These signaling effects may also contribute to the differing potency of AcAc and β HB, and is an important area for future investigation. Under constitutive conditions, blood concentrations of β HB and AcAc are similar (2). For our *in vivo* experiments we selected Bdh1 rather than Oxct1 (which would abrogate both β HB and AcAc oxidation) to moderately reduce overall ketone body oxidation in neurons, based on our observation of sensitive dose-responsive changes in metabolism, and because our *in vitro* data suggested that AcAc compensation for Bdh1 knockdown was incomplete (Fig. 2.3).

Ketone metabolism in AD pathophysiology

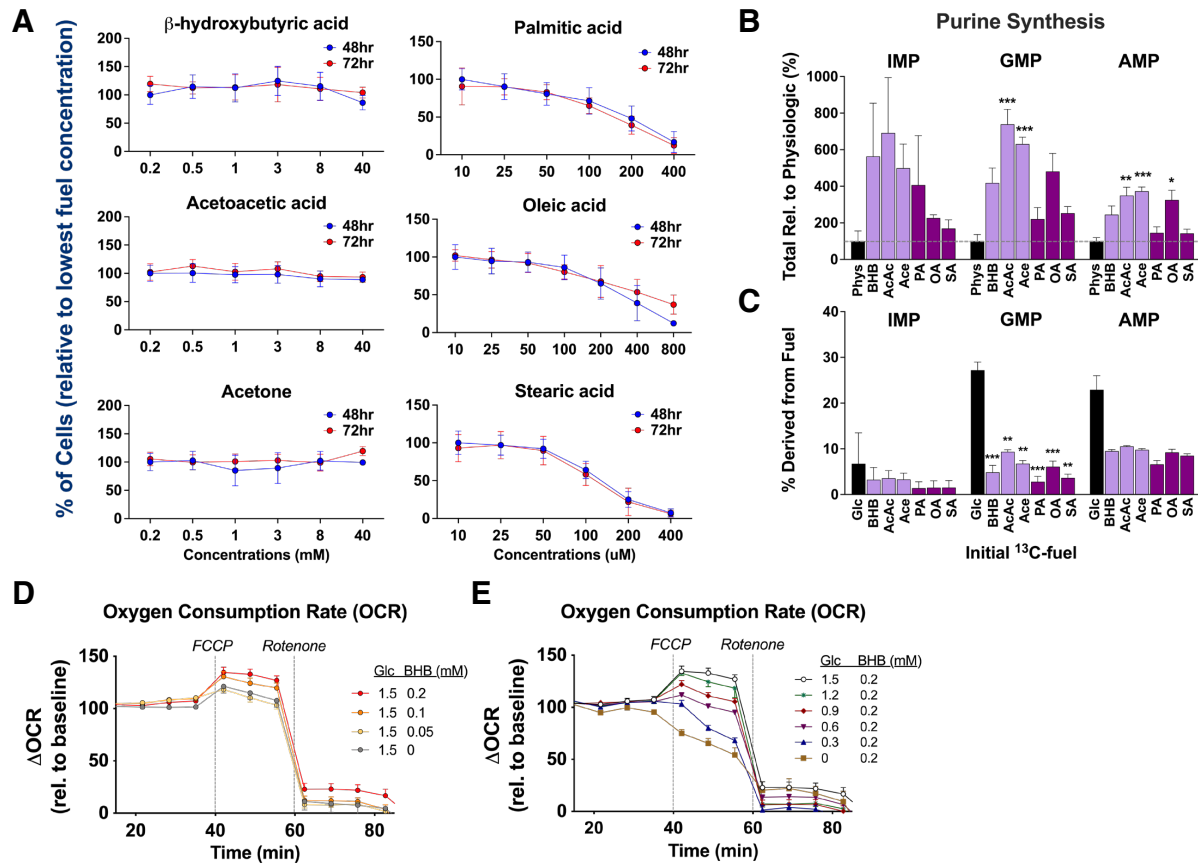
Our findings extend beyond normal physiology to implicate neuronal ketone metabolism in protection against neurodegeneration. While BDH1 mutations have not yet been linked directly to human disease, the gene lies within the 3q29 locus implicated in microdeletion and microduplication neurodevelopmental disorders (55, 56). BDH1 expression is also reduced in multiple brain cell types in AD (57). Moreover, polymorphisms in BDH1 are associated with progression from mild cognitive impairment to AD (58), supporting a potential role in disease susceptibility.

Our finding that BDH1 deficiency in neurons markedly increases the mortality of mutant APP (J20) mice supports that constitutive neuronal ketone metabolism may normally protect against AD pathophysiology. Considerable evidence supports that mutant APP can disrupt energy metabolism in mouse models and in patients (12, 59), while supplementation with ketones can improve function in AD mouse models (60, 61). As such, ketones may provide an alternate fuel source that bypasses mutant APP-induced energetic deficits. Importantly, we show here that this

is not only relevant to high-dose exogenous supplementation of ketones, but that the brain's intrinsic, constitutive ketone metabolism provides crucial endogenous resilience to AD deficits. Feeding a ketone ester to Alzheimer's mouse models has been shown to reduce seizure activity (62, 63) which is linked to memory deficits in humans with AD (64), but also a major cause of death in AD mouse models. Exacerbation of this seizure activity in the absence of constitutive neuronal BHB oxidation could be a mechanism of accelerated mortality in hAPPJ20/Bdh1^{Thy1-/-} mice. It is of keen interest to determine if the same or distinct mechanisms are responsible for β HB oxidation and BDH1 activity in improving health and survival during normal aging and in neurodegeneration models.

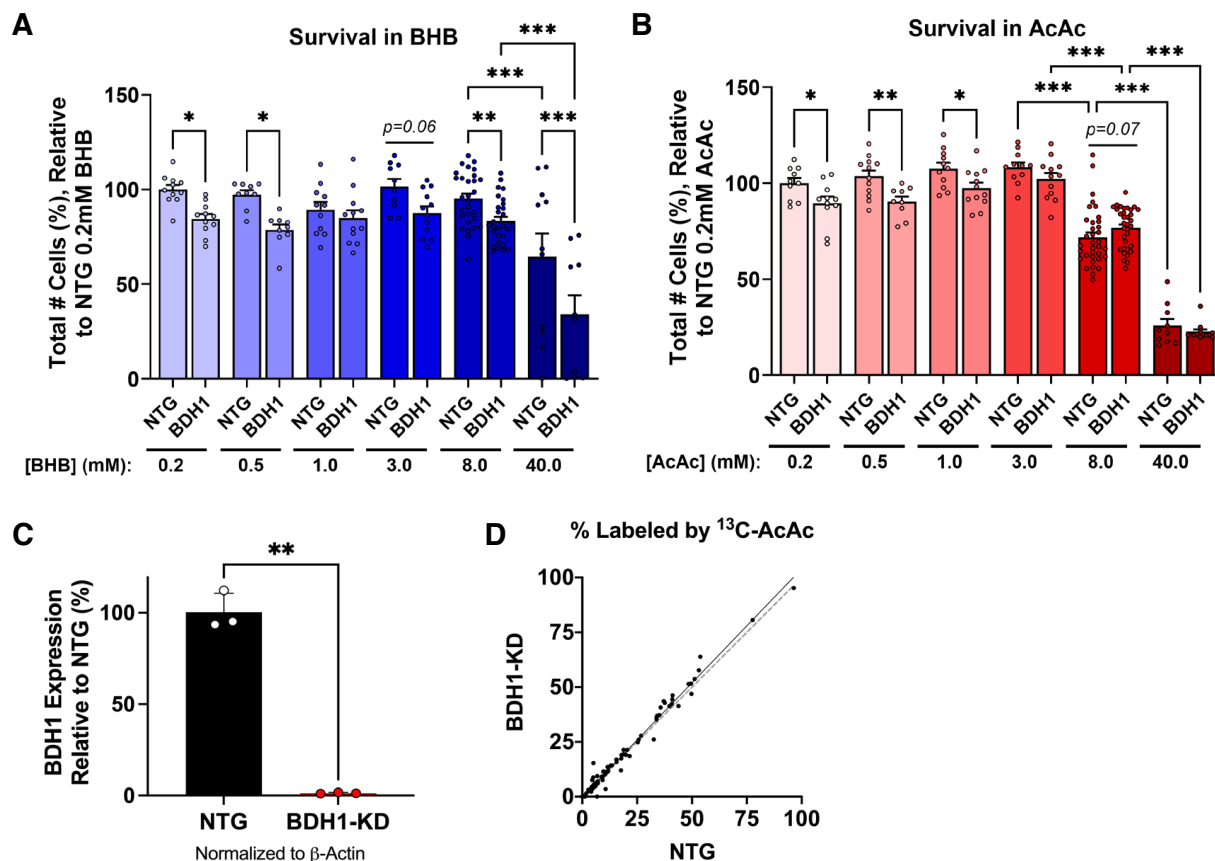
Altogether, our findings support the possibility that enabling or increasing constitutive ketone body metabolism in neurons could be protective against age-associated cognitive decline and AD. While various early clinical trials are investigating exogenously provided ketone bodies in AD (12, 65, 66), targeting endogenous ketogenic pathways may prove equally or more efficacious. Our findings illustrate the need to better understand glial ketogenesis, constitutive neuronal ketone oxidation, and the distinct roles of β HB, AcAc, and other non-glucose fuels in supporting normal neuronal function in aging and neurodegeneration.

SUPPLEMENTARY FIGURES



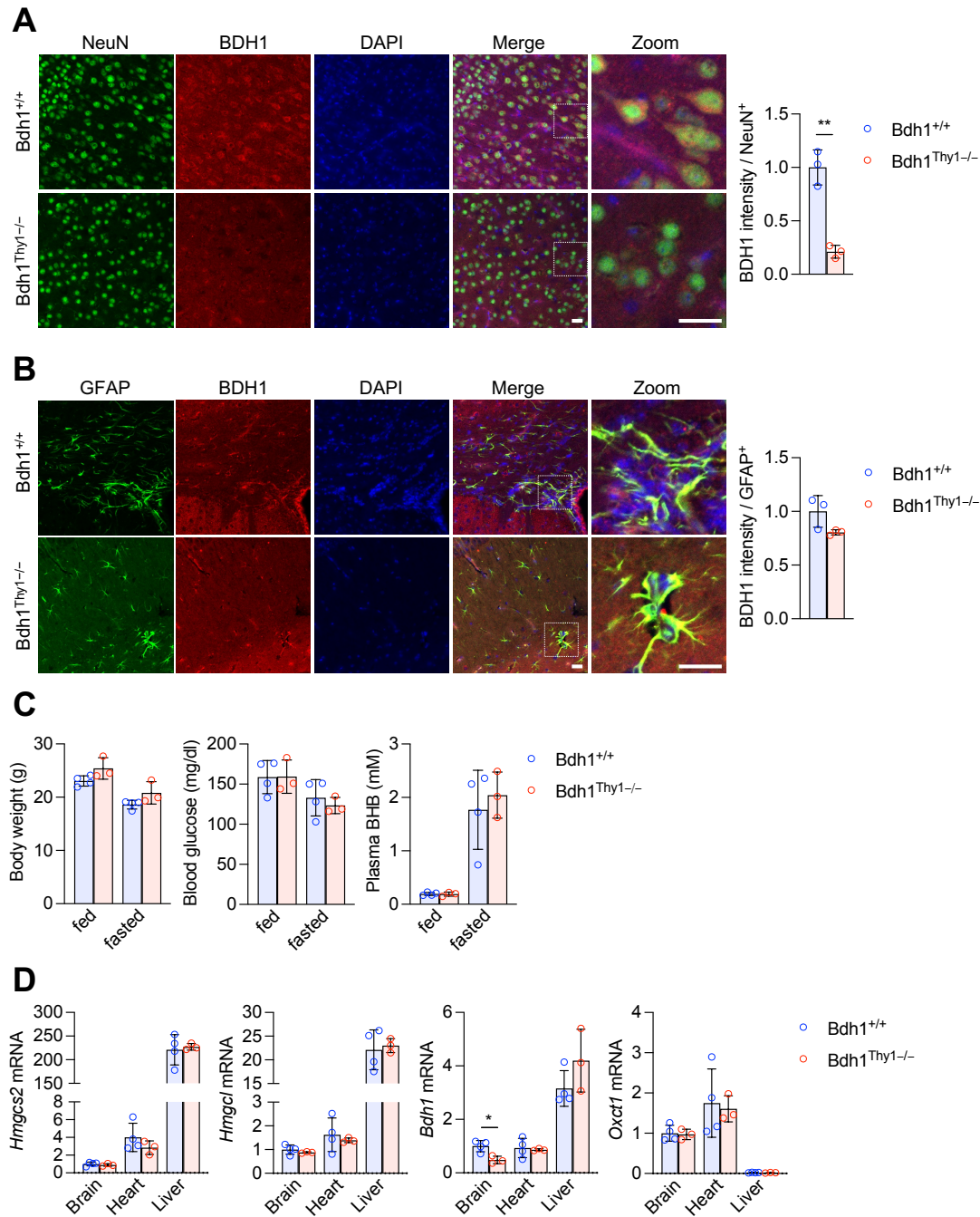
Extended Data Fig. 2.1. Human neurons are highly responsive to availability of lipid-derived fuel sources.

(A) Survival curves of 21d human neurons incubated in lipid-based conditions for 48 or 72h, normalized to lowest fuel concentration at 48h. Conditions include metabolic reliance on ketones (β HBA (N=5-11 samples/condition/timepoint), AcAc (5-12, except 3 at 40mM), acetone (5-11, except 2 at 40mM)) or fatty acids (palmitic (10-12), oleic (4-12), stearic acid (7-10)). Data are means $\pm$ SD, from two experimental replicates. (B) Total metabolite change relative to physiologic, and (C) percent derivation (from ^{13}C -fuel) of purine-related species after 3d media incubation. Inosine monophosphate: IMP; guanosine monophosphate: GMP; adenosine monophosphate: AMP. N=3-6 samples/condition. Data are means $\pm$ SEM. (D) OCR changes in neurons incubated 3d with incremental changes in β HBA or (E) glucose availability. N=3-10 samples/condition. Data are means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ relative to physiologic condition by Brown-Forsythe and Welch one-way ANOVA with Dunnett's T3 multiple comparisons (B-C).



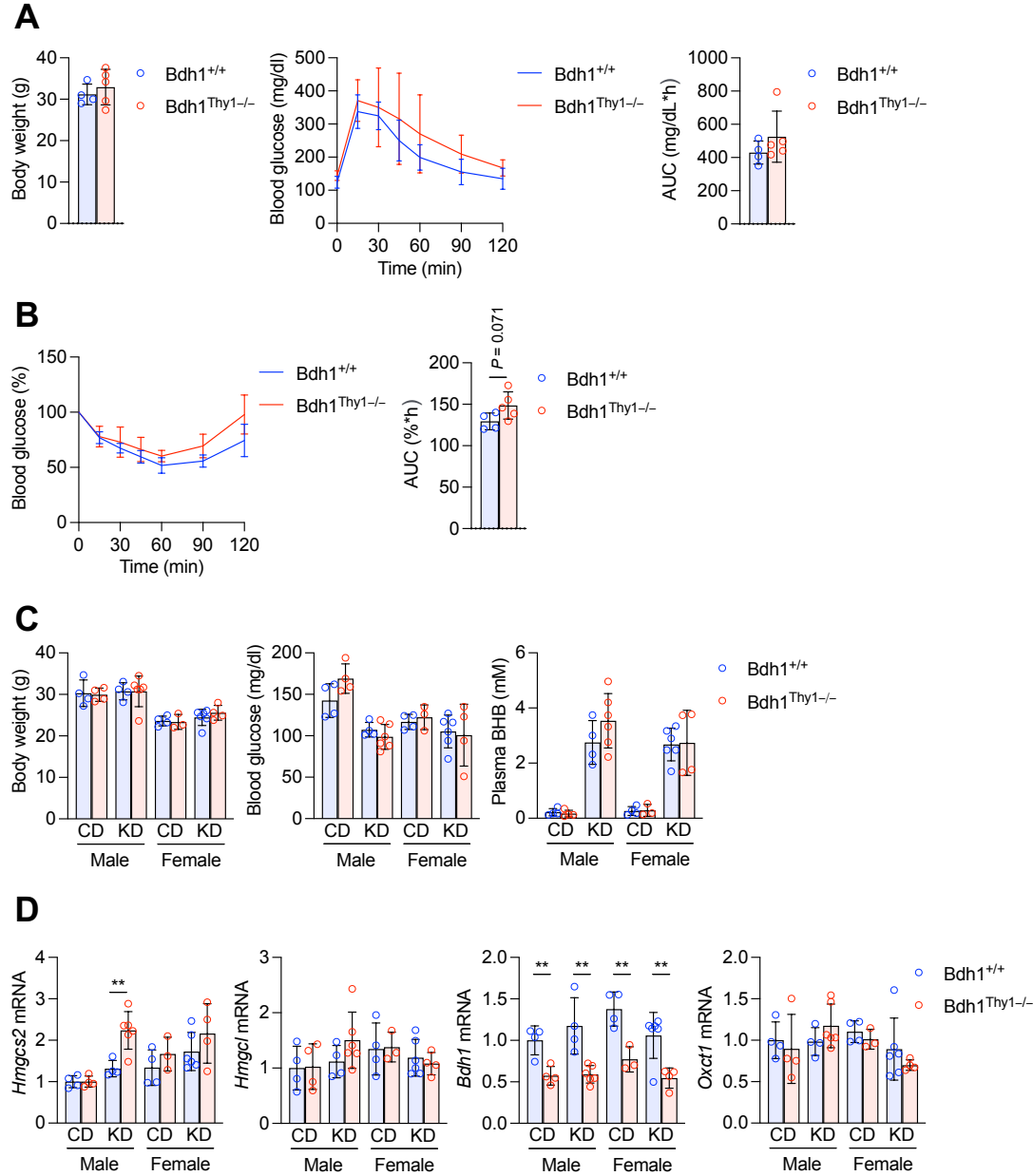
Extended Data Fig. 2.2. Impact of BDH1 knockdown on neuronal survival and fuel usage.

Survival curves of 21d NTG or BDH1 neurons incubated for 72hr under (A) β HBB-based or (B) AcAc-based metabolic conditions, normalized to NTG survival in 0.2mM ketone per experimental repetition. N=9-35 samples/condition, from two experimental replicates; data are means $\pm$ SEM. (C) Validation of *Bdh1* knockdown in BDH1 vs. NTG neurons for targeted metabolomics, normalized to β -Actin, confirming 99% knockdown. N=3 samples/genotype; means $\pm$ SD. (D) Regression plot of the BDH1 vs. NTG neuronal metabolome during metabolic reliance on AcAc. *Points*: metabolites; *values*: percent of metabolite containing carbons from ^{13}C -AcAc. N=115 metabolites; $y = 1.04x - 0.14$, $R^2 = 0.99$. *p < 0.05, **p < 0.01, and ***p < 0.001 by 2-way ANOVA with Tukey's multiple comparisons, select comparisons shown (A-B); or unpaired t-test with Welch's correction (C).



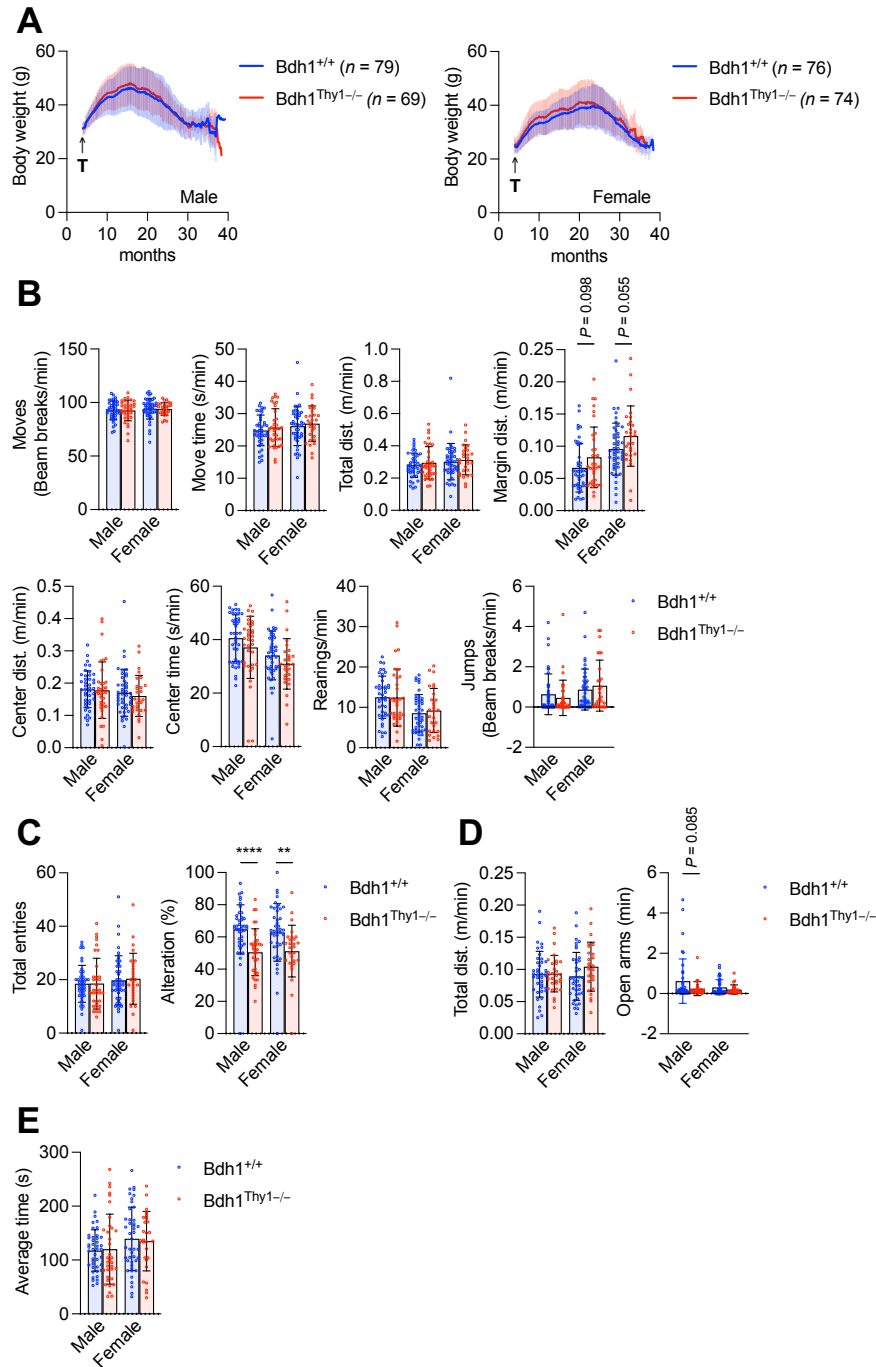
Extended Data Fig. 2.3. Validation of neuron- and organ-specific Bdh1 knockout in mice.

(A and B) Immunostaining of neurons (NeuN) (A) or astrocytes (GFAP) (B), BDH1, and nuclei (DAPI) in brain cortex sections of two-month-old mice harvested after 24 hours fasting and seven days after five consecutive days of tamoxifen injection. BDH1 fluorescent intensity was relatively quantified in cells (N=30 per mouse) that were positive for NeuN (A) or GFAP (B). Scale bar, 25 μ m. (A and B) N=3 (Bdh1^{+/+} male) and 3 (Bdh1^{Thy1-/-} male) mice per group. P = 0.0014 (NeuN, t = 7.85, df = 4) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (C) Body weight, blood glucose, and plasma β HB levels before and after 24 hours fasting. (D) qPCR of genes involved in ketone metabolism after 24 hours fasting. (C and D) N=4 (Bdh1^{+/+} male) and 3 (Bdh1^{Thy1-/-} male) mice per group. P = 0.0015 (Bdh1, brain, t = 3.67, df = 5) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD.



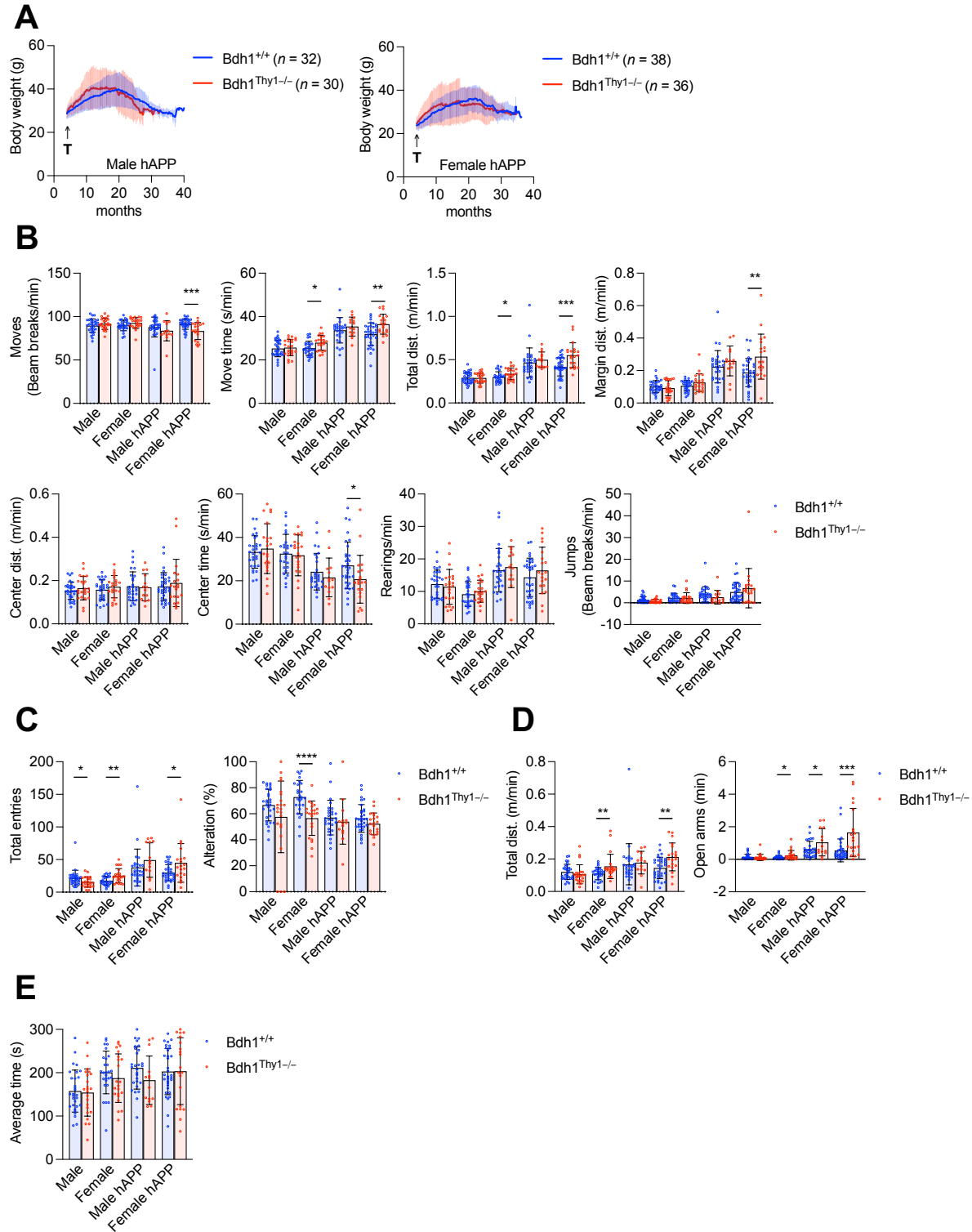
Extended Data Fig. 2.4. Metabolic characterization of neuronal *Bdh1*-KO mice.

(A) Body weight, glucose tolerance test and the area under the curve (AUC) of nine-month-old mice measured 15 days after five consecutive days of tamoxifen injection. (B) Insulin tolerance test and AUC of nine-month-old mice measured 19 days after five consecutive days of tamoxifen injection. (A and B) N=4 ($Bdh1^{+/+}$ male) and 5 ($Bdh1^{Thy1-/-}$ male) mice per group. Data are means $\pm$ SD. (C) Body weight, blood glucose, and plasma β HB levels of four- to five-month-old mice after seven days control diet (CD) or ketogenic diet (KD). Both diets were fed three days after five consecutive days of tamoxifen injection. (D) qPCR of genes involved in ketone metabolism in the brain. (C and D) N=4 ($Bdh1^{+/+}$ male CD), 4 ($Bdh1^{Thy1-/-}$ male CD), 4 ($Bdh1^{+/+}$ male KD), 6 ($Bdh1^{Thy1-/-}$ male KD), 4 ($Bdh1^{+/+}$ female CD), 3 ($Bdh1^{Thy1-/-}$ female CD), 6 ($Bdh1^{+/+}$ female KD), and 4 ($Bdh1^{Thy1-/-}$ female KD) mice per group. $P = 0.0053$ (*Hmgcs2*, male, KD, $t = 3.78$, $df = 8$), $p = 0.0061$ (*Bdh1*, male, CD, $t = 4.13$, $df = 6$), $p = 0.0038$ (*Bdh1*, male, KD, $t = 4.02$, $df = 8$), $p = 0.0080$ (*Bdh1*, female, CD, $t = 4.27$, $df = 5$), and $p = 0.0087$ (*Bdh1*, female, KD, $t = 3.45$, $df = 8$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD.



Extended Data Fig. 2.5. Behavioral characterization of aged $Bdh1^{Thy1-/-}$ mice.

(A) Body weight curves. (B) Open field test at 21 months old. N=42 ($Bdh1^{+/+}$ male), 35 ($Bdh1^{Thy1-/-}$ male), 44 ($Bdh1^{+/+}$ female), and 28 ($Bdh1^{Thy1-/-}$ female) mice per group. Data are means $\pm$ SD. (C) Y-maze at 21 months old. N=43 ($Bdh1^{+/+}$ male), 35 ($Bdh1^{Thy1-/-}$ male), 44 ($Bdh1^{+/+}$ female), and 28 ($Bdh1^{Thy1-/-}$ female) mice per group. $P < 0.0001$ (alteration, male, $t = 4.16$, $df = 76$) and $p = 0.0069$ (alteration, female, $t = 2.78$, $df = 70$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (D) Elevated plus maze at 24 months old. N=40 ($Bdh1^{+/+}$ male), 30 ($Bdh1^{Thy1-/-}$ male), 40 ($Bdh1^{+/+}$ female), and 26 ($Bdh1^{Thy1-/-}$ female) mice per group. Data are means $\pm$ SD. (E) Rotarod at 21mo. N=43 ($Bdh1^{+/+}$ male), 35 ($Bdh1^{Thy1-/-}$ male), 44 ($Bdh1^{+/+}$ female), and 28 ($Bdh1^{Thy1-/-}$ female) mice per group. Data are means $\pm$ SD.



Extended Data Fig. 2.6. Behavioral characterization of aged *Bdh1*^{Thy1-/-} mice on a hAPPJ20 background.

(A) Body weight curves. (B) Open field test at 12 months old. N=30 (*Bdh1*^{+/+} male), 23 (*Bdh1*^{Thy1-/-} male), 27 (*Bdh1*^{+/+} female), 22 (*Bdh1*^{Thy1-/-} female), 28 (hAPPJ20/*Bdh1*^{+/+} male), 14 (hAPPJ20/*Bdh1*^{Thy1-/-} male), 33 (hAPPJ20/*Bdh1*^{+/+} female), and 20 (hAPPJ20/*Bdh1*^{Thy1-/-} female)

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mice per group. $P = 0.00060$ (moves, hAPPJ20 female, $t = 3.66$, $df = 51$), $p = 0.011$ (move time, female, $t = 2.64$, $df = 47$), $p = 0.0020$ (move time, hAPPJ20 female, $t = 3.25$, $df = 51$), $p = 0.49$ (total dist., female, $t = 2.02$, $df = 47$), $p = 0.00024$ (total dist., hAPPJ20 female, $t = 3.95$, $df = 51$), $p = 0.0027$ (margin dist., hAPPJ20 female, $t = 3.16$, $df = 51$), and $p = 0.043$ (center time, hAPPJ20 female, $t = 2.07$, $df = 51$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (C) Y-maze at 12 months old. $N=30$ ($Bdh1^{+/+}$ male), 23 ($Bdh1^{Thy1-/-}$ male), 27 ($Bdh1^{+/+}$ female), 22 ($Bdh1^{neu-/-}$ female), 28 (hAPPJ20/ $Bdh1^{+/+}$ male), 14 (hAPPJ20/ $Bdh1^{Thy1-/-}$ male), 33 (hAPPJ20/ $Bdh1^{+/+}$ female), and 20 (hAPPJ20/ $Bdh1^{Thy1-/-}$ female) mice per group. $P = 0.046$ (total entries, male, $t = 2.05$, $df = 51$), $p = 0.0032$ (total entries, female, $t = 3.11$, $df = 46$), $p = 0.013$ (total entries, hAPPJ20 female, $t = 2.59$, $df = 50$), and $p < 0.0001$ (alteration, female, $t = 4.31$, $df = 46$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (D) Elevated plus maze at 12 months old. $N=30$ ($Bdh1^{+/+}$ male), 23 ($Bdh1^{Thy1-/-}$ male), 27 ($Bdh1^{+/+}$ female), 22 ($Bdh1^{Thy1-/-}$ female), 28 (hAPPJ20/ $Bdh1^{+/+}$ male), 14 (hAPPJ20/ $Bdh1^{Thy1-/-}$ male), 33 (hAPPJ20/ $Bdh1^{+/+}$ female), and 20 (hAPPJ20/ $Bdh1^{Thy1-/-}$ female) mice per group. $P = 0.0094$ (total dist., female, $t = 2.71$, $df = 47$), $p = 0.0022$ (total dist., hAPPJ20 female, $t = 3.23$, $df = 51$), $p = 0.043$ (open arms, female, $t = 2.08$, $df = 47$), $p = 0.042$ (open arms, $t = 2.01$, $df = 40$), and $p = 0.00060$ (open arms, hAPPJ20, female, $t = 3.66$, $df = 51$) by unpaired two-tailed t-test assuming Gaussian distribution. Data are means $\pm$ SD. (E) Rotarod at 12 months old. $N=30$ ($Bdh1^{+/+}$ male), 23 ($Bdh1^{Thy1-/-}$ male), 27 ($Bdh1^{+/+}$ female), 22 ($Bdh1^{Thy1-/-}$ female), 28 (hAPPJ20/ $Bdh1^{+/+}$ male), 14 (hAPPJ20/ $Bdh1^{Thy1-/-}$ male), 33 (hAPPJ20/ $Bdh1^{+/+}$ female), and 20 (hAPPJ20/ $Bdh1^{Thy1-/-}$ female) mice per group. Data are means $\pm$ SD.

METHODS

Neuronal differentiation and maturation

These studies were conducted in human WTC11 induced pluripotent stem cells (iPSCs) expressing doxycycline-inducible neurogenin 2 (NGN2) and inducible CRISPRi (dCas9-KRAB) machinery, obtained from Martin Kampmann (UCSF). iPSCs were cultured in StemFlex media (Gibco A3349401) on Matrigel-coated (1:120 in DMEM/F12; Corning 354277, Gibco 11320033) culture flasks. Differentiation of the iPSCs was divided into 2 parts: pre-differentiation and maturation. Pre-differentiation occurred over 3d of culture in pre-differentiation media; maturation occurred over 18-22d in neuronal differentiation media. Pre-differentiation media was based in Knockout DMEM/F12 (Gibco 12660012), with 1X NEAA (Gibco 11140050), 1X N2 supplement (Gibco 17502001), 10 ng/mL NT-3 (StemCell 78074.1), 10 ng/mL BDNF (StemCell 78005.1), and 1 μ g/mL of laminin (Gibco 23017015). On the first day of pre-differentiation, iPSCs were passaged onto Matrigel-coated flasks with pre-differentiation media containing doxycycline (2 μ g/mL) and ROCK inhibitor (ROCKi; 10 μ M). Media was refreshed on the third day of pre-differentiation to remove ROCKi.

On the fourth day, pre-differentiated neurons were passaged into 6- or 96-well plates with neuronal differentiation media and doxycycline (2 μ g/mL), at densities of 500,000 or 10,000-12,000 cells/well, respectively. Plates were pretreated for 2d with 1X poly-L-ornithine (Sigma-Aldrich P4957) and coated for 1d with 5 μ g/mL laminin. Neuronal differentiation media was based in 1:1 DMEM/F12 (Gibco 11320033) and Neurobasal A media (Gibco 10888022), with 1X NEAA, 0.5X GlutaMAX (Gibco 35050061), 0.5X N2 supplement, 0.5X B27 supplement (Gibco 17504001), 10 ng/mL NT-3, 10 ng/mL BDNF, and 1 μ g/mL of laminin. After 2d, media was refreshed to remove doxycycline and then refreshed every 7d to feed the neurons.

Minimal base media for metabolic restriction

All metabolic conditions were created in an absolute minimal media. The media was based in Neurobasal A -glucose -pyruvate (Gibco A2477501), with 1X NEAA (Gibco 11140050), 0.375X GlutaMAX (Gibco 35050061), 0.5X N2 supplement (Gibco 17502001), 0.5X B27 supplement (Gibco 17504001), 10 ng/mL NT-3 (StemCell 78074.1), 10 ng/mL BDNF (StemCell 78005.1), and 1 µg/mL laminin (Gibco 23017015).

Timescale targeted metabolomics in ketogenic conditions

WTC11 human neurons were differentiated in 6-well plates, matured for 20d, then incubated in metabolic media prior to metabolite extraction. On day 20, media was changed to one of the following metabolic conditions, with 4 replicates each:

- [1] Physiologic – 1.5mM glucose (Sigma-Aldrich G6152), 0.2mM pyruvate (Sigma-Aldrich P5280), 0.2mM BHB (Sigma-Aldrich H6501), 1mM lactate (Sigma-Aldrich L7022)
- [2] Ketogenic – 0.5mM glucose, 0.2mM pyruvate, 3mM BHB
- [3] Extreme ketogenic – 10mM BHB only

Conditions were run in duplicate with [U-13C] versions of either glucose (Cambridge Isotope Labs CLM-1396) or BHB (Cambridge Isotope Labs CLM-3853). All conditions were formulated in previously-described minimal base media. For Acetyl-CoA measurements, an additional condition of 1.5mM glucose, 0.2mM pyruvate, 10mM BHB, and 1mM lactate was assayed. After 4, 24, or 48h of metabolic media incubation, cellular metabolites were extracted via ammonium acetate wash (150mM, pH 7.4) at 4C and incubation with 80% methanol for 30min at -80C, followed by centrifugation at 14,000 rpm for 10min at 4C. Metabolite supernatants were dried in a Labconco CentriVap prior to quantification at the UCLA Metabolomics Core.

Fatty acid conjugation

For all fatty acid-based metabolic medias, fatty acids were first resuspended in 100% ethanol and slowly added to 10% fatty acid-free BSA (Roche 03117057001) in minimal base media at 37C, for a final concentration of 2.5mM (palmitate) or 5mM (oleate, stearate). Solutions were vortexed vigorously and incubated for >30min at 37C. Control conditions with 10% BSA, ethanol, or both in base media were also included.

Survival assays

Human neurons were differentiated in 96-well plates, matured for 18 days, then incubated for 48 or 72h in metabolic media prior to assaying for cell death. On day 18, media was changed to metabolic conditions formulated in minimal base media, with increasing doses of BHB (Sigma-Aldrich H6501), AcAc (Sigma-Aldrich A8509), Acetone (Fisher Scientific A18-4), palmitate (Sigma-Aldrich P0500), oleate (Sigma-Aldrich O1008), or stearate (Sigma-Aldrich S4751).

After media incubation, cells were incubated with Calcein (Invitrogen C3099) and Hoechst (Invitrogen H3570) at 37C for 15min prior to imaging on the Thermo Scientific CellInsight CX7 microscope. Images were analyzed in CellProfiler to determine survival via colocalization of Calcein and Hoechst. Counts were normalized to average survival in the lowest concentration (at 48h, for fuel assays; in NTG1, for BDH1 assays) per experimental replicate and combined to generate dose curves.

Targeted metabolomics

Human neurons were differentiated in 6-well plates, matured for 18d, then incubated for 3d in metabolic media prior to metabolite extraction. On day 18, media was changed to one of the following metabolic conditions, with 4-6 replicates each:

- [1] Basal (unrestricted) – 21.25mM [U-13C]-glucose and 2.5mM pyruvate
- [2] Physiologic – 1.5mM glc and 0.2mM BHB, with [U-13C]-glc or -BHB
- [3] Physiologic variant – 1.5mM glc and 0.2mM AcAc, with [U-13C]-glc or -AcAc
- [4] Ketone reliance – 3mM or 8mM [U-13C]-BHB, -AcAc (Supelco 77988), or -acetone (Cambridge Isotope Labs CLM-1334)
- [5] Fatty acid reliance – 50μM [U-13C]-palmitate (Sigma-Aldrich 605573), -oleate (Cambridge Isotope Labs CLM-460), or -stearate (Sigma-Aldrich 605581)
- [6] Competition – 3mM glc, BHB, and AcAc; run in triplicate with [U-13C] per fuel

All conditions were formulated in previously-described minimal base media; basal condition contained extra GlutaMAX to a final concentration of 1.125X. After 72h of media incubation, cellular metabolites were extracted and quantified at the UCLA Metabolomics Core. Values were normalized to corresponding physiologic condition per tracer run.

Seahorse

WTC11 human neurons were differentiated in Seahorse XFe96/XF Pro Cell Culture Microplates (Agilent 103792-100) as previously described, matured for 18d, then incubated for 3d in metabolic media prior to Seahorse assay. On day 18, media was changed to one of the following metabolic conditions:

- [1] Physiologic – 1.5mM glc and 0.2mM BHB
- [2] BHB variants – 1.5mM glc and varying doses of BHB
- [3] Glucose variants – 0.2mM BHB and varying doses of glucose
- [4] Ketone reliance – 8mM BHB, AcAc, or acetone
- [5] Fatty acid reliance – 50μM palmitate, oleate, or stearate

After 72h of metabolic media incubation, all conditions were remade in minimal media, but with Seahorse XF DMEM (Agilent 103575-100) as the base media instead of Neurobasal A. Plates were incubated in CO₂-free incubator at 37C for 1-2h prior to loading into Seahorse XF96 Extracellular Flux Analyzer. Seahorse sensor cartridges (Agilent 103792-100) were calibrated per Agilent instructions. Oxygen consumption rate (OCR) was measured at baseline and again after sequential injection of DMSO (Sigma-Aldrich D2438), FCCP (carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone, Sigma-Aldrich C2920), rotenone (Sigma-Aldrich R8875), and DMSO. OCR signals were normalized to average baseline reading per replicate.

Generation of BDH1 knockdown cell line

The dual-guide RNA expression plasmid pJR103 (Addgene plasmid #187242) was used as the lentiviral transfer vector. The following protospacer sequences were cloned into pJR103: for the non-targeting control (NTG1), 5'-GGGCTAAGGGGCCGTGTACT-3' and 5'-GGGACGAAGCAGTCCGAGCG-3'; for BDH1 knockdown (BDH1-KD), 5'-GCGCAGGAGTGCTGGTGGAG-3' and 5'-GGCGTGTAGAAGCGTCCGGG-3'.

Lentivirus was produced by transient transfection of HEK293T cells (ATCC CRL-11268). Cells were seeded to reach 80–95% confluence after 24h. For each well of a 6-well plate, 1 µg of pJR103 transfer plasmid and 1 µg of third-generation packaging plasmid mix [equal ratio of pRSV-Rev (Addgene plasmid #12253), pMDLg (Addgene plasmid #12251), pCMV-VSV-G (Addgene plasmid #8454)] were diluted in 200 µL OPTI-MEM (Thermo Fisher Scientific 31985070). TransIT-Lenti transfection reagent (Mirus Bio MIR 6600) was added at 6 µL per well and incubated at RT for 10min before being added dropwise to HEK293T cells.

Viral supernatants were harvested at 48h and 72h post-transfection, filtered through a 0.45 µm PVDF syringe filter (Millipore Sigma SLHV033RB), and mixed with one-quarter volume of

Lentivirus Precipitation Solution (Alstem VC125). The mixture was incubated at 4 °C for 24h, followed by centrifugation at $1,500 \times g$ for 30min at 4 °C. The supernatant was carefully aspirated, and the pellet centrifuged again for 5min at $1,500 \times g$ to remove residual liquid. The viral pellet was resuspended in 100 μ L sterile PBS (UCSF media core).

For transduction, the WTC11 iPSCs were infected with concentrated lentivirus at the time of passaging. 48h post-infection, cells were passaged into fresh medium containing 1 μ g/mL puromycin (Sigma Aldrich P9620) and selected for two passages until greater than 95% of the population was marker-positive, as assessed by flow cytometry. Cells were then passaged once in puromycin-free medium to allow recovery prior to subsequent differentiation experiments.

Confirmation of BDH1 knockdown

Total RNA was isolated from cells collected at multiple timepoints following lentiviral transduction. RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN 74134) and reverse-transcribed using the High-Capacity RNA-to-cDNA™ Kit (Thermo Fisher Scientific 4387406) in a 20 μ L reaction following the manufacturer's protocol. Quantitative PCR was performed using TaqMan™ Gene Expression Assays (Thermo Fisher Scientific 4444557) on a QuantStudio 5 Real-Time PCR System (Applied Biosystems). Reactions were carried out in technical triplicate. The TaqMan™ assay for BDH1 (Hs00366297_m1) was used to quantify target gene expression, with ACTB (β -actin; Hs99999903_m1) as the endogenous control. Relative BDH1 expression was calculated using the comparative Ct ($\Delta\Delta$ Ct) method, normalizing to ACTB. Expression levels were compared to those of cells transduced with non-targeting guide (NTG1) controls at day 0 to determine knockdown efficiency.

Mouse strains, housing, and husbandry

All mice were maintained in accordance with the guidelines set forth by the National Institutes of Health, and all experimental protocols were approved by the Buck Institute Institutional Animal Care and Use Committee (IACUC). The Buck Institute is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). Mice were maintained in a specific pathogen-free barrier facility under a 6:00 am to 6:00 pm light cycle. The facility maintains a temperature of 68-72°F and an ambient humidity level exceeding 30%. All mice were maintained in groups of up to five mice per cage, unless otherwise specified in procedures described below. The cages were furnished with wooden bedding, a nestlet, and a house, and were changed every two weeks. The diet was provided in a recess of the metal wire lid situated at the upper portion of the cage and changed every two weeks, and the wire lid was changed with the diet. Water was provided in a bottle. *Bdh1*^{fl/fl} mice were previously published (22). Thy1-CreERT2 (SLICK-H) mice (Strain #:012708) (35) and APP/J20 mice (Strain #034836-JAX) (39) were obtained from the Jackson Laboratory. The age of mice at the time experiments were undertaken is presented in the figures. Knockout of *Bdh1* was induced by intraperitoneal tamoxifen injections (25 mg/ml corn oil, 2.5 mg per 25 g of body weight) on five consecutive days. The lifespan cohorts began with four months old mice after tamoxifen injections and followed them until death; the healthspan involved behavioral testing at the described ages.

Mouse diets and feeding

Unless otherwise indicated, mice were fed the Buck Institute standard vivarium chow with 18% protein, 6% fat, and 44% carbohydrates (2918; Teklad). The customized diets from Envigo contained the following macronutrient content per calorie: Control (CD) with 10% protein, 13%

fat, and 77% carbohydrate (TD.150345); KD with 10% protein and 90% fat (TD.160153). Both diets had similar micronutrient content, fiber, and preservatives on a per-calorie basis, had similar fat sources, and were always provided ad libitum.

Immunofluorescence staining

The brains were fixed with 4% PFA and subsequently sectioned at a thickness of 30 μ m using a microtome. The brain sections were blocked with 10% normal donkey serum and 0.5% triton-X. The sections were incubated with primary antibodies, mouse anti-NeuN (1:250) (ab104224; Abcam), or mouse anti-GFAP (1:250) (G3893; Sigma), and rabbit anti-BDH1 (1:250) (15417-1-AP; Proteintech), overnight at 4C. Subsequently, the sections were incubated with secondary antibodies, anti-mouse Alexa Fluor 488-conjugated (1:500) (A-21202; Invitrogen) and anti-rabbit Alexa Fluor 555-conjugated (1:500) (A-31572; Invitrogen) and mounted with a solution containing DAPI. The slides were photographed using a Zeiss LSM 700, and the resulting images were subsequently quantified using the ImageJ software.

Blood glucose and plasma BHB measurements

Blood glucose was measure via distal tail-snip by an AimStrip Plus Blood Glucose Meter (37231; Germaine Laboratories). Blood was obtained via distal tail-snip (10-40 μ L) or, if the animal was being euthanized for tissue collection, by cardiac puncture immediately following euthanasia. Blood was collected into lithium-heparin coated microvettes (16.443.100; Sarstedt), and plasma separated by centrifugation at 1500 x g for 5 min at 4C. Plasma was frozen at -20C until thawed for assay. We confirmed that freeze-thawing had no effect on assay results. We measured BHB using the Stanbio Chemistry Beta-Hydroxybutyrate LiquiColor Reagent (2440-

058; Stanbio), run using 3 μ L samples in duplicate. We found it critical to subtract baseline absorbance of sample-enzyme mixture prior to adding catalyst, to account for sample hemolysis.

Glucose and insulin tolerance tests

For the glucose tolerance test, mice were fasted overnight and received an intraperitoneal injection of glucose (2 g/kg body weight). For the insulin tolerance test, the mice were fasted for four hours before being injected intraperitoneally with insulin (0.75 U/kg body weight).

Quantitative PCR (qPCR)

RNA was isolated by Quick-RNA MicroPrep Kit (R1051; Zymo Research), cDNA synthesis was carried out with Superscript cDNA Synthesis Kit (1708891; BioRad), and qPCR was performed using iTaq Universal SYBR Green Supermix (1725121; BioRad) in a BioRad CFX96 Real-Time System. Gene expression analyses were normalized to the Rplp0 housekeeping gene.

Primers;	Rplp0:	Fw_GCTTCGTGTTACCAAGGAGGA,
		Rv_GTCCTAGACCAGTGTTCTGAGC;
	Hmgcs2:	Fw_TGCTATGCAGCCTACCGCAAGA,
		Rv_GCCAGGGATTTCTGGACCATCT;
	Hmgcl:	Fw_GCACTTTGCCAAAGCAGGTGAAG,
		Rv_CGGAAAGCATGTCGATCAGCCT;
	Bdh1:	Fw_ AGGCTGTGACTCTGGATTTGGG,
		Rv_CTGGATGGTTCTCAGTCGGTCA;
	Oxct1:	Fw_GAGCGACAGTTCCTTTCTGGTG,
		Rv_TCCCATAACCCTGTGCTGGTGTA.

Lifespan endpoints

The endpoints for a healthy lifespan were based on the NIA Interventions Testing Program (<https://www.nia.nih.gov/research/dab/interventions-testing-program-itp>), with additional

rigorous criteria developed in collaboration with the Buck Vivarium. The goal of the euthanasia criteria was to relieve and prevent any suffering without unnecessarily reducing healthy lifespan. Indications for euthanasia involved obvious discomfort, impending death, systemic signs of unwellness, or any condition that was likely a harbinger of impending discomfort or death. Benign conditions of aging, even if abnormal in young mice, did not prompt euthanasia unless they were associated with these criteria or thought likely to lead to them over days or weeks. All mice were weighed weekly and monitored with increasing frequency up to five times a week after 24 months old. Specific indications for euthanasia of aged mice included the following:

Moribund

BCS 2 or less

Poor general health

Poor mobility

Any obvious discomfort

Discoordination, ataxia, or abnormal gait

Progressive weight loss to > 15% below normal body weight

Unexplained weight gain > 15% above prior body weight

Wounds that do not heal after two weeks of treatment, or worsen despite treatment

Development of new wounds while on treatment for other wounds

Full-thickness wounds that expose underlying fascia or muscle, wounds > 2cm in any dimension, or any wound that is obviously causing discomfort

Rectal prolapse, penile prolapse, or paraphimosis

Suspected tumor associated with other signs of systemic unwellness (wound, weight loss, etc.) Suspected tumor > 2cm or ulcerated

Healthspan study

All behavioral testing was performed in consultation with the Buck Mouse Phenotyping Core, incorporating expert recommendations as to type, sequence, and timing of the various behavioral tests. A key factor was their suitability for use with old mice, compatibility for performing multiple tests on the same animals, and suitability for repeat measurements over time. All tests were carried out during normal daytime hours, in normal room light except where noted.

Novel object recognition

The novel object recognition test was conducted in white plastic boxes with dimensions of 40 x 40 x 30 cm (length x width x height). On the first day of the study, the subjects were allowed to acclimate to the testing environment for five minutes. On the second day, the subjects were given a 10-minute period to become familiar with two identical objects (plastic boxes or bottles) and then a 10-minute test in which they were presented with one known and one novel object (one box and one bottle). On the second day of the experiment, a video camera was utilized to document the experimental procedures. The familiarization period and novel object test were conducted six hours apart. The time spent at both the novel and known objects were recorded.

Open field

The open field apparatus (Tru Scan for Open Field Activity Monitoring; Coulbourn Instruments) consisted of Tru Scan test arenas (25 x 25 x 40 cm) (length x width x height). Mice were recorded for 10 min, in dark conditions.

Y-maze

The Y-maze apparatus was constructed of three arms, each measuring 35 x 5 x 20 cm (length x width x height). These arms radiated out from a central platform, which was shaped like

a triangle with sides measuring 5 cm. The room was maintained at a low light level. Each mouse was positioned in the center of the maze, oriented toward one of the arms, and permitted to explore the maze freely for eight minutes. The trials were recorded using a video camera.

Elevated plus maze

The elevated plus maze apparatus consists of two platforms joined in a plus shape. Each platform is 74 cm x 5 cm, with a 5 cm square region where they meet. One platform is flat and open. The other platform has 20 cm high walls. The entire apparatus is 62 cm above the floor. A computer tracking system tracked mouse movement through the apparatus, reporting both distance moved and dwell time. The room was kept at low light. Mice were permitted to explore for 5 min.

RotaRod

The RotaRod was performed with a RotaRod apparatus (47650; Ugo Basile), using a two-day protocol. Day 1 involved a training run at fixed 10 rpm. Days 2 involved three data runs. The data runs on day 2 used steady rod acceleration from 5-40 rpm over 5 min. The time was stopped at 5 min or when the mouse fell. Mice were removed if they rotated around the rod twice.

Barnes maze

The Barnes maze consists of a 110 cm diameter round platform, elevated 80 cm from the floor, and 40 holes (each 5 cm in diameter). All holes were opened except for one target hole that led to an escape box located under the platform. Visual cues and white noise were employed to encourage the mice to expeditiously locate the escape box. Testing was conducted in three phases. Adaptation (Day 0): The mice were permitted to explore the platform for a period of 60 seconds. Any mouse that was unable to locate the escape box was guided and allowed to remain in that location for an additional 60 seconds. Learning Acquisition (Days 1–4): Over the course of four

consecutive days, the mice were trained to locate the target and enter the escape box within 180 seconds. Each day, the mice underwent two trials. If they did not locate the target or escape box within 180 seconds, their primary latency was recorded as 180 seconds. Probe (Days 5 and 13): On Days 5 and 13, a single 90-second trial was conducted to measure primary latency. If the mice did not locate the target within 90 seconds, their latency was recorded as 90 seconds.

Quantification and statistical analysis

In vitro data are presented as the mean $\pm$ standard error (SEM) and *in vivo* data are presented as mean $\pm$ standard deviation (SD). Unless otherwise specified in the figure legend, metabolomics and Seahorse datasets were analyzed by Brown-Forsythe and Welch one-way ANOVA with Dunnett's T3 multiple comparisons to compare conditions, or 2-way ANOVA with Dunnett's multiple comparisons for changes over time. Survival assays were analyzed by unpaired two-tailed t-test with Welch's correction. For rodent behavioral tests, p-values were calculated by unpaired two-tailed t-test under the assumption of Gaussian distribution. For lifespan experiments, Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests were used to calculate p-values. Statistical calculations were conducted using GraphPad Prism 10 (GraphPad Software).

Data Availability, Materials & Correspondence

All data are available in the manuscript or the supplementary materials. All data, code, and materials used in the analysis will be made available to any researcher for purposes of reproducing or extending the analyses. Correspondence and material requests should be addressed to Ken Nakamura and John C Newman.

CHAPTER 3: Sex and *APOE* Genotype Jointly Modify Memory After Brain Irradiation

ABSTRACT

The *APOE*ε4 allele is the strongest genetic risk factor for Alzheimer's disease (AD), the most common neurodegenerative disease associated with memory dysfunction. Whether *APOE* genotype influences cognitive resilience in response to exogenous exposures is poorly understood. Using genetic mouse models of neurodegeneration, we tested whether *APOE* genotype influences memory function after exposure to brain irradiation, a common cancer therapy. Neurobehavioral testing of mice indicates that *APOE3* and *APOE4* genotypes impact memory after brain irradiation in opposing ways and in a sex-dependent manner. While *APOE3* mice demonstrated worsened memory function after brain irradiation, in *APOE4* mice, brain irradiation was associated with improved memory function, particularly in females. These behavioral differences did not correlate with ApoE, amyloid beta, and total or phosphorylated tau. Interestingly, the addition of mutant APP had similar, non-additive effects as radiation on worsening memory in *APOE3* mice, and also blocked the protective effects of radiation on behavior in *APOE4* mice, suggesting that mutAPP could act downstream of both E4 and radiation to impact memory. Our findings underscore the importance of considering genetic predisposition to neurodegeneration and sex as key factors influencing memory responses to exogenous exposures such as brain radiation.

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of progressive cognitive decline with age. The apolipoprotein E type 4 allele (*APOE*ε4) is the most prevalent genetic risk factor for AD, present in ~20-25% of the population and 60-70% of patients with AD (67-69). Amyloid precursor protein (*APP*), a gene that can also be mutated in AD, undergoes proteolytic processing to give rise to amyloid-beta plaques (70). The risk for developing AD is *APOE* gene dose-dependent, with a stronger effect in females than males (68, 71). Females with one or two copies of *APOE*ε4 develop more severe negative changes in hippocampal volume and memory performance than *APOE*ε4-carrying males (72, 73).

Genetic variants that influence the risk of developing AD could serve as biomarkers of prognostication for other age-related diseases associated with memory dysfunction, especially if there are shared pathophysiologic mechanisms. Cancer is a common co-morbidity in aging, and individuals receiving various types of cancer therapy can develop progressive deficits in short-term memory and other cognitive domains (74). Both systemically-delivered cancer drug therapy and brain-focused radiation therapy can be associated with side effects impacting memory (75-77). For some cancer survivors, memory dysfunction can be irreversible and impact long term quality of life. Unfortunately, this complication is poorly understood and challenging to predict, prevent, or manage.

While limited retrospective clinical studies suggest an association of *APOE*ε4 with worse cognitive function in patients with cancer (78), it is not proven whether genetic susceptibility to neurodegeneration—via *APOE*ε4 or other genes—influences the risk of cognitive decline after cancer therapy. Additionally, it is unknown whether sex interacts with genetic risk to influence memory in a radiation exposure setting. Defining such interactions could open new avenues for

personalizing protective strategies for millions of individuals living with cancer to mitigate impacts to cognitive function.

We used an AD mouse model homozygous for either human ApoE3 or ApoE4 knocked in (KI), with or without mutant App^{NL-G-F} (Swedish, Iberian and Arctic mutations associated with familial AD)(79), to investigate whether AD genetic risk factors and sex influence cognitive function after brain irradiation, a common treatment for brain tumors. Brain irradiation dosing is localized to the brain only, in contrast to cancer drug therapies that circulate systemically and frequently impact multiple organs concurrently.

Age-matched female and male mice were exposed to brain irradiation and underwent comprehensive neurobehavioral testing and histopathological analyses of their brains. These studies demonstrate that *APOE* genotype and sex together significantly influence the trajectory of memory function after brain irradiation, providing a key insight into how AD risk interacts with exogenous stressors. These findings point to major opportunities for improving prognostication and preserving memory function in patients with cancer.

RESULTS

***APOE* genotype impacts memory**

Age-matched 3-5-month-old mice homozygous for either human *APOE3* or human *APOE4* were generated and randomized to no radiation or whole brain irradiation groups (5 Gy/dose x 2 consecutive daily doses). Control and irradiated mice underwent neurobehavioral testing at one month post-irradiation (**Fig. 3.1A**). At baseline, in the absence of radiation, spatial memory was significantly worse in homozygous *APOE4* versus *APOE3* mice in both females and males, as

indicated by increased entries into the aversive zone during active place avoidance (APA) testing (**Fig. 3.1B, Extended Data Fig. 3.1A/B**). This baseline difference is consistent with the negative effect of *APOE4* on memory function.

***APOE3* mice develop worsened memory after brain irradiation**

Brain radiation impacted memory function in *APOE3* mice, as irradiated *APOE3* mice (female and male) demonstrated a greater number of entrances into the aversive zone on multiple testing days (**Fig. 3.1C/F**) and on probe (**Fig. 3.1D/G**) and reinstatement (**Fig. 3.1E/H**). Irradiated *APOE3* mice of both sexes also spent more time in the aversive zone than unirradiated *APOE3* mice (**Fig. 3.1 I/L**) and males in particular avoided it for less time on both probe and reinstatement (**Fig. 3.1J/K/M/N, Extended Data Fig. 3.1E**). Interestingly, pair-wise comparison across each of these memory metrics indicated that the relative adverse effect of radiation on memory was greater in *APOE3* male mice than in female mice (**Extended Data Fig. 3.1A/C/E**). These data indicate susceptibility to memory dysfunction after radiation in *APOE3* mice.

***APOE4* mice maintain or improve memory after brain irradiation**

Consistent with previous findings, in unirradiated mice, *APOE4* is associated with worse memory function as compared to *APOE3* mice, regardless of sex. Interestingly, at one month post-irradiation, spatial memory testing indicated that in contrast to irradiated *APOE3* mice, irradiated *APOE4* mice maintained spatial memory better than unirradiated *APOE4* mice (**Fig. 3.1C-N**). Grouped analyses of unirradiated and irradiated *APOE4* mice (female and male) identified a significant trend of preserved spatial memory function in irradiated mice through fewer entrances into the aversive zone on probe (**Extended Data Fig. 3.1B**)

This effect, measured in multiple metrics, was particularly notable in female *APOE4* mice (**Fig. 3.1D/E vs. 3.1G/H**). Irradiated female *APOE4* mice also spent less time in the aversive zone (**Fig. 3.1I**). Two-way ANOVA of genotype and radiation in female mice indicated a significant interaction for *APOE* genotype and radiation on percentage of time spent in the aversive zone, number of entrances on probe, number of entrances on reinstatement, and maximum time of avoidance on probe and reinstatement (**Fig. 3.1J/K**).

At one month post-irradiation, memory performance metrics also distinguished *APOE* genotype and radiation effects in male mice. In contrast to male *APOE3* mice whose memory worsened after brain irradiation, irradiated male *APOE4* mice maintained or improved their memory performance (**Fig. 3.1F-H,L-N**), and similarly showed significant *APOE* genotype and irradiation interactions favoring radiation on number of entrances on probe (**Fig. 3.1G**), maximum time of avoidance on probe (**Fig. 3.1M**), and maximum time of avoidance on reinstatement (**Fig. 3.1N**). Thus, *APOE4* mice of both sexes demonstrated improved memory measured after irradiation.

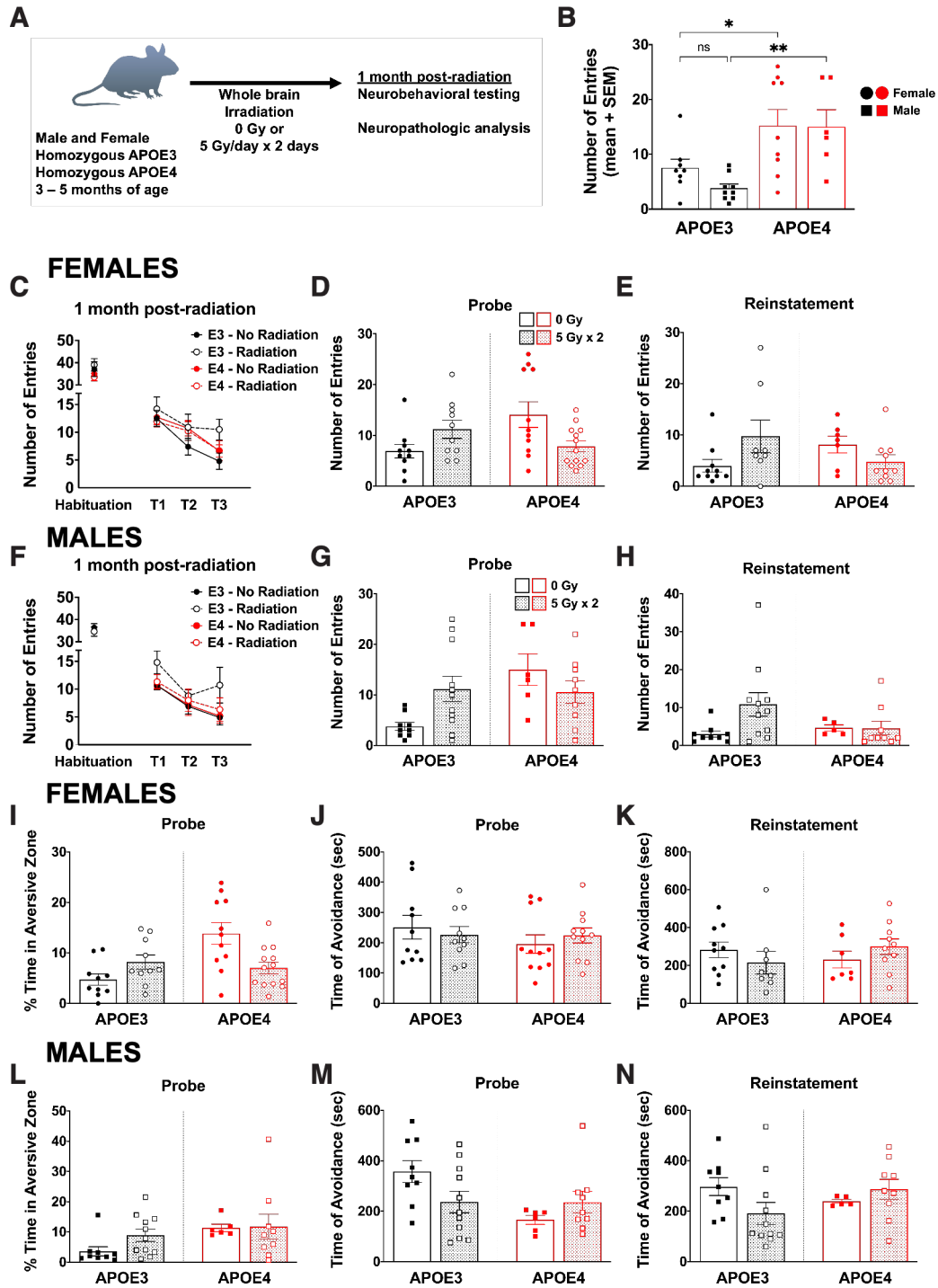


Figure 3.1. Brain irradiation impacts memory in a genotype- and sex-dependent manner. (A) Experimental schema for brain irradiation, neurobehavioral testing, and histological analyses. Female and male APOE3 and APOE4 mice underwent Active Place Avoidance (APA) testing at 1 month after brain irradiation. (B) Number of entrances into the aversive zone in females vs. males without radiation (genotype effect, $p=0.002$). * $p < 0.05$, ** $p < 0.01$ (C/F) Number of entries into the aversive zone across habituation, training (continues on next page)

(continues from previous page)

(T1-T3), **(D/G)** probe (**D**: genotype x radiation, $p=0.004$; **G**: $p=0.016$), and **(E/H)** reinstatement (**E**: genotype x radiation, $p=0.03$) in females (C-E) and males (F-H). **(I/L)** Percent time spent in the aversive zone (**I**: genotype x radiation, $p=0.0016$), and time of zone avoidance on **(J/M)** probe (**J**: genotype x radiation, $p=0.03$) and **(K/N)** reinstatement (**K**: genotype x radiation, $p=0.03$) for females (I-K) and males (L-N). 2-way ANOVAs with Fisher's LSD post-hoc test. Bars indicate mean and s.e.m.

Non-memory behavioral domains are similar between unirradiated & irradiated *APOE* mice

In addition to APA testing, we assessed whether *APOE*, sex, and/or radiation impact global neurologic function in mice. These assays included the open field test (OF), elevated plus maze (EPM), visual cliff (VC), and hotplate tests, each of which test behaviors such as anxiety, vision, and pain response that rely on neurological domains beyond memory (**Extended Data Fig. 3.2**). Total movement measured on the open field activity test was comparable between *APOE3* and *APOE4* mice of both sexes (**Extended Data Fig. 3.2C-D, G-H**) and not significantly changed after irradiation. The hot plate and visual cliff tests also indicated no differences attributable to either *APOE* genotype or radiation (**Extended Data Fig. 3.2K-L/O-P/Q-R/T-U**), suggesting that *APOE* and brain radiation interactions localize specifically to memory function and do not globally impact neurologic function.

Brain irradiation impacts hippocampal APOE levels

Given the *APOE* genotype-specific effects of radiation on memory, we next examined if radiation differentially impacts APOE3 and E4 levels. Brain samples from irradiated and control mice were collected at either 1- or 4-months post-radiation to screen for longitudinal changes in protein expression, and co-stained for human ApoE (ab52607, 1:1000), GFAP (MAB3402, 1:1000), and MAP2 (ab5392, 1:1000) for colocalization in astrocytes and neurons, respectively (**Fig. 3.2B**). There were no significant effects of radiation on ApoE levels in astrocytes (**Fig.**

3.2C/E, Extended Data Fig. 3.3C) or neurons (Fig. 3.2D/F, Extended Data Fig. 3.3D) at both 1 month (Fig. 3.2) and 4 months (Extended Data Fig. 3.3) after radiation, although significant interactions were detected between radiation and genotype in males at 1 month, and a significant genotype effect in neurons at 4 months (Extended Data Fig. 3.3D). We also compared total brain levels of APOE between APOE3 and APOE4 mice via immunoblotting and similarly found no significant changes in total APOE one month after 3 Gy/day for two days (6 Gy total) or 5 Gy/day for two days (10 Gy total) doses of irradiation in females & males (Extended Data Fig. 3.3A-B).

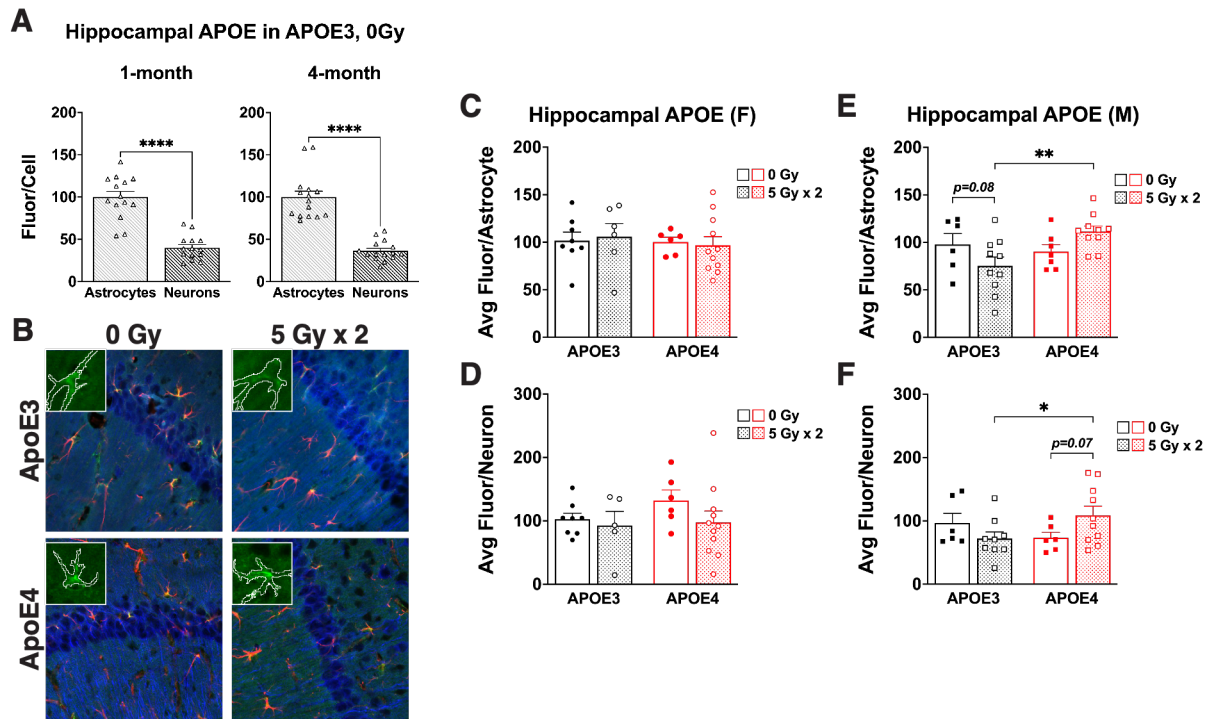


Figure 3.2. Effect of genotype and brain irradiation on hippocampal neuronal and astrocytic APOE. Immunohistochemical analyses of brain samples obtained from irradiated (5 Gy x 2) or control (0 Gy) *APOE3* (N=7-8) and *APOE4* (N=7-10) mice, 1 month after radiation. Samples were co-stained for human ApoE (green), GFAP (red), and MAP2 (blue) and hippocampal regions were imaged via confocal microscopy. **(A)** Comparison of relative ApoE intensity in hippocampal astrocytes versus neurons in control mice at 1 and 4 months post-radiation. **(B)** Representative images with insets showing ApoE in astrocytic masks, and analysis of average cellular ApoE intensity for **(C,E)** astrocytes and **(D,F)** neurons in females (C-D) and males (E-F) 1 month after radiation. Intensity of all cells was averaged per FOV (plotted). 2-way ANOVAs with Tukey's multiple comparisons. Bars indicate mean and s.e.m. * $p < 0.05$, ** $p < 0.01$.

Mutant APP mice maintain or improve memory after brain irradiation

Considering that *APOE4* influences both the accumulation of amyloid beta (80-83) and behavioral response to radiation (**Fig. 3.1**), we next investigated the interplay between *APOE*, radiation and amyloid beta. Specifically, we investigated how mutant App^{NL-G-F} (mutAPP) impacts the effects of radiation on memory, as a function of *APOE* genotype. Mice harboring mutant *APP* with either the *APOE3* (APOE3/APP) or *APOE4* (APOE4/APP) backgrounds were irradiated—identical to the above-described *APOE* mice—at 3- to 5- months of age and underwent comprehensive neurobehavioral testing at one month post-irradiation. To facilitate comparison, data for APOE3 and APOE4 mice (first shown in **Fig. 3.1**) are co-plotted as controls against corresponding APP groups.

At one month, unirradiated APOE3/APP mice demonstrated worse memory at baseline compared to their unirradiated APOE3 counterparts during APA testing, consistent with the effect of *APP* and similar to the effects of radiation alone on APOE3 mice (**Fig. 3.3A-B/F**). We found sex-dependent differences in APP's effects: in females, APP was associated with worse initial learning (**Fig. 3.3A**) whereas in males, APP had no effect on the initial learning phase (**Fig. 3.3E**). This difference between sexes persisted in probe and reinstatement tests (**Fig. 3.3B-D/F-H**).

When treated with brain irradiation, APOE3/APP mice were resistant to the effects of radiation and actually demonstrated a non-significant trend toward improved memory function on some measures in the female APOE3/APP mice, such as number of entries on reinstatement and percentage of time in the aversive zone (**Fig. 3.3C-D/G-H**). Indeed, the combination of radiation and mutAPP was not worse than either alone in APOE3 mice, raising the possibility that radiation and mutAPP converge on the same pathway to worsen memory.

In this genetic background, sex also influenced memory post-radiation. In contrast to female ApoE3/APP mice, male ApoE3/APP mice demonstrated worse memory function after brain irradiation in reinstatement and probe (**Fig. 3.3G-H**), similar to male APOE3 mice. Two-way ANOVA identified a significant impact of radiation alone on male memory function, and unlike in females, no significant interaction between APP and radiation. In mice over-expressing human APP with the Swedish mutation, female mice had greater amyloid plaque load than male mice, indicating a sex difference in APP-mediated amyloid pathology (84). APP had a greater effect on female mice than males in our study, and the interaction between APP and radiation in female mice may reflect the stronger impact of APP in females.

We next examined the interaction between APP and radiation on an APOE4 background. Adding mutAPP to APOE4 had no effect on memory performance in either female or male mice (**Fig. 3.3I-P**). However, mutAPP blocked the protective effects of radiation on memory in APOE4 mice (**Fig. 3.3I-P**), meaning that memory deficits were comparable in APOE4/APP mice with or without radiation, further supporting that radiation may converge on a shared pathway with APOE4 and mutAPP to impact memory. This was observed in multiple metrics, including number of entries on probe and reinstatement and the percent time spent in the aversive zone (**Fig. 3.3J-L/N-P**). No significant interaction was identified between APP and radiation in the ApoE4 background, unlike the interaction measured in the APOE3 background. Therefore, the protective capacity of radiation against APOE4 was no longer present when mutant APP was also present, suggesting that radiation has opposing interactions with APOE4 and mutant APP.

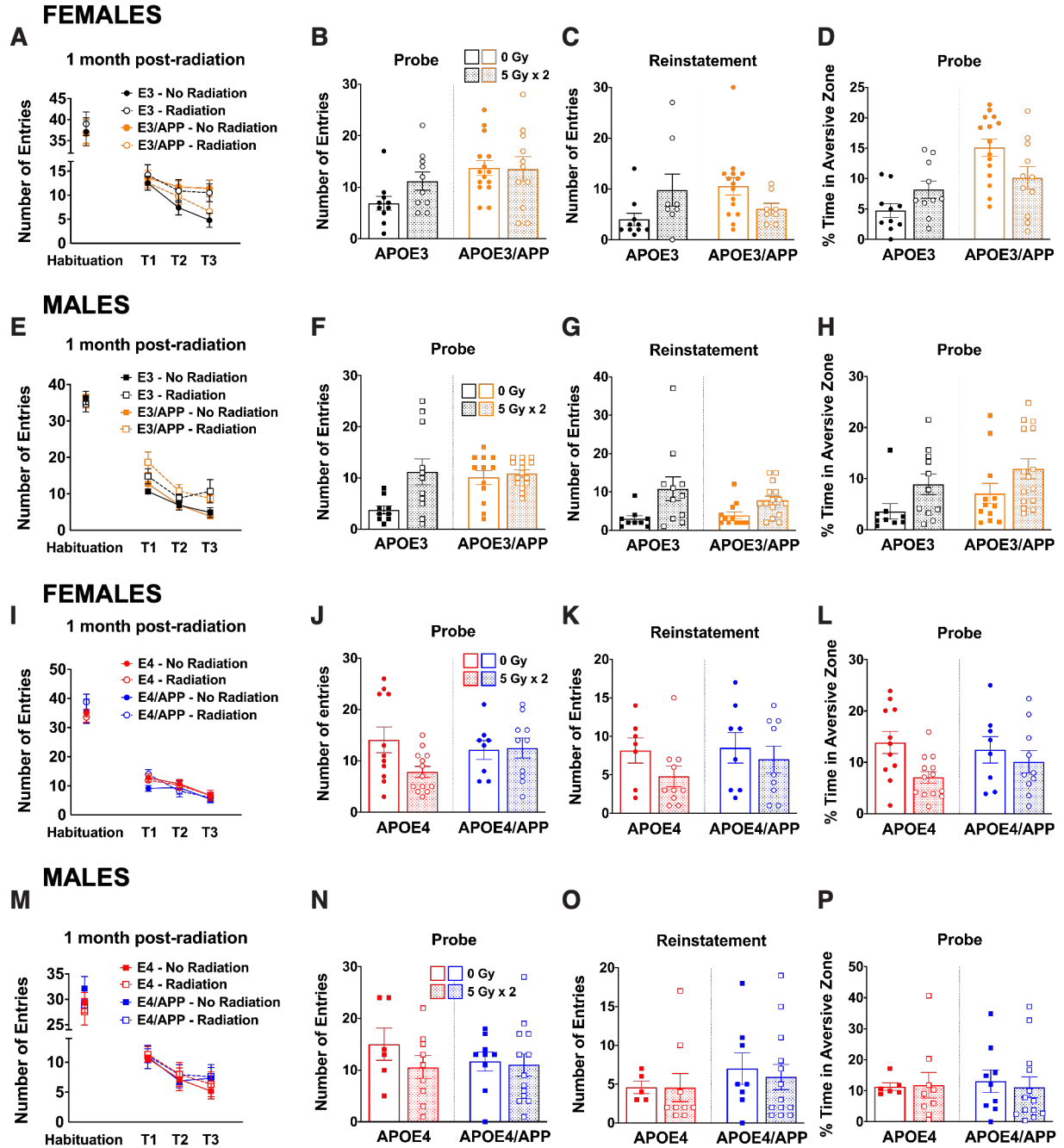


Figure 3.3. Brain irradiation impacts memory in APP mutant mice. Female and male APOE3/APPKI and APOE4/APPKI mice underwent Active Place Avoidance (APA) testing at 1 month after brain irradiation, in tandem with APOE3/APPWT (A-H) and APOE4/APPWT (I-P) counterparts. (A/E/I/M) Number of entries into the aversive zone across habituation, training, (B/F/J/N) probe (F: radiation effect $p=0.009$, APP $p=0.05$, radiation x APP $p=0.03$; J: radiation x APP $p=0.09$), and (C/G/K/O) reinstatement (C: radiation x APP $p=0.01$; G: radiation $p=0.002$), and (D/H/L/P) percent time spent in aversive zone on probe (D: APP $p=0.0002$, APOE x radiation $p=0.008$; H: radiation $p=0.01$; L: radiation $p=0.03$). 2-way ANOVAs with Fisher's LSD post-hoc test.

Irradiation does not significantly alter amyloid- β plaque deposition in the mouse brain

Given the observed behavioral changes in memory, we next investigated whether brain irradiation plays a role in altering neuropathological hallmarks of Alzheimer's disease. Brain samples from irradiated and control mice were collected at either one or four months post-radiation and quantified for amyloid- β ($a\beta$) plaque deposition via DAB (3,3'-diaminobenzidine) immunohistochemistry using 82E1 antibody. At the 1-month timepoint, percent area of $a\beta$ plaque trended upwards with radiation in the cortex and thalamus of APOE3 and APOE3/APP males (**Fig. 3.4C-D**), as well as in APOE3 and E4 females (**Fig. 3.4A-B**), although not to significant levels.

Plaque deposition was not expected to reach significantly high levels at 1 month following radiation, given that mice in this cohort ranged from 4- to 6-months of age. This was supported by the relatively low signal visualized during DAB staining (**Fig. 3.4E**). We thus collected samples from additional cohorts at 4 months post-radiation, when the mice ranged from 7- to 9-months old. As expected, $a\beta$ plaque deposits were increased in the cortex of APOE3/APP and particularly APOE4/APP mice (**Extended Data Fig. 3.4A/C**) when compared to all other cohorts. Radiation did not significantly affect plaque deposits in any genotype (**Extended Data Fig. 3.4A-D**).

To investigate potential changes in the composition of deposits, we also co-stained for beta-pleated sheet structures within amyloid- β plaque cores (ThioS) versus immature peptides (82E1). The level of 82E1 radiation from each core was scored and averaged per mouse, revealing an increase in immature peptides for APOE3/APP and particularly APOE4/APP mice compared to their AppWT counterparts (**Fig. 3.4F-G**). There was also a significant increase in irradiated APOE4/APP mice, suggesting a compositional change after radiation exposure.

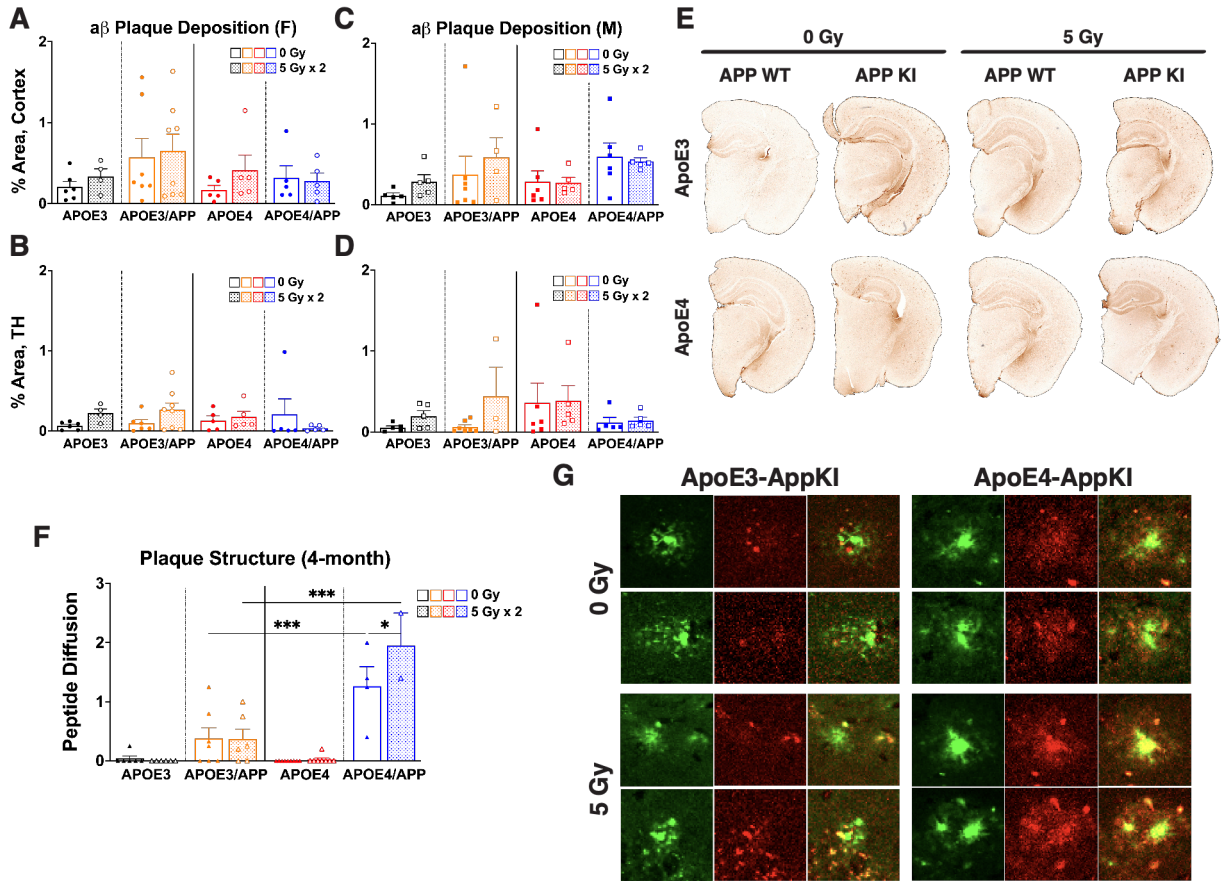


Figure 3.4. Brain amyloid- β plaque deposition and structure following brain irradiation. DAB (3,3'-Diaminobenzidine) conducted on brain samples from irradiated (5 Gy x 2) or control (0 Gy) mice, 1 month after radiation. Samples were quantified for percent area of amyloid- β plaque deposition via 82E1 antibody in the (A,C) cortex and (B,D) thalamus (TH) of *ApoE3-AppWT* (N=9-11), *ApoE3-AppKI* (N=12-14), *ApoE4-AppWT* (N=10-11), and *ApoE4-AppKI* (N=10-11) mice and stratified by sex (A-B, females; C-D, males). Representative images shown in (E). (F) Analysis of plaque composition, 4 months after radiation via co-stain for mature $\alpha\beta$ plaques (Thioflavin S, green) and immature peptides (82E1, red) in *ApoE3-AppWT* (N=6 per group), *ApoE3-AppKI* (N=6-7), *ApoE4-AppWT* (N=8-9), and *ApoE4-AppKI* (N=2-4) mice. Samples were imaged with confocal microscopy and scored for magnitude of 82E1 signal surrounding ThioS+ cores (0=no signal; 3=significant signal). Intensity scores were averaged across FOVs per mouse, with representative images shown in (G). 2-way ANOVAs with Tukey's multiple comparisons.

We performed anatomically-focused immunohistochemical screens of microglia, total tau, and phospho-tau in hippocampal sections of mice 1 and 4 months after brain irradiation. At both timepoints, there was a significant effect of radiation on number of microglia, exhibited through

microglial decreases across many genotypes (**Extended Data Fig. 3.5A-B**). Radiation did not significantly alter hippocampal tau at either timepoint, quantified by immunohistochemical analyses (**Extended Data Fig. 3.5C-D**), nor total brain tau in either sex, quantified by immunoblot (**Extended Data Fig. 3.5E-F**). Total brain phospho-tau also was not significantly altered by increasing levels of radiation, as quantified by immunoblot (**Extended Data Fig. 3.5G-H**). Finally, we quantified cortical phospho-tau (AT8) deposition via DAB at the 4 month timepoint and observed significant increases after radiation in the cortex of APOE4/APP mice (**Extended Data Fig. 3.5I**), but not in any other genotypes.

DISCUSSION

Brain irradiation is a critical and often life-saving therapy for various brain tumors. It is frequently associated with cognitive changes, particularly in the case of whole-brain radiotherapy, that can persist in long term cancer survivors (74, 85, 86). These cognitive changes vary in severity but often have a lasting impact on quality of life. Understanding susceptibility to such changes is essential for optimizing cancer therapy and developing strategies to preserve cognitive function. Currently, there are no known biomarkers to predict cognitive function after cancer therapy.

Given the established role of ApoE4 in predisposing to cognitive decline and neurodegeneration in AD and other neurodegenerative conditions (87), as well as the prevalence of genotypes with or without ApoE3 and ApoE4 (approximately 50-70% E3/E3, 20-25% E3/E4, 2-5% E4/E4) (3, 21), it is relevant to assess potential interactions between ApoE and the effects of brain radiation in humans. Recent advances in anti-AD therapies, many of which target $\text{A}\beta$, tau, or ApoE4 (88, 89), raise the important question of whether these therapies may also be applicable to other forms of cognitive dysfunction.

Using animal models of AD, we investigated whether human *APOE* variants influence memory after brain irradiation and evaluated whether brain pathologic AD markers correlate with functional change. Mice received a whole-brain radiation dose of 10 Gy, representing a low cumulative dose that a broad range of patients might receive for brain radiotherapy. Unlike high radiation doses typically concentrated on tumors, the dosing used in this study is not associated with the severe and typically late-onset brain injuries of radionecrosis or brain atrophy that produce gross structural abnormalities (90).

Prior studies investigating the effects of brain irradiation in animal models have described mixed, and in some ways, conflicting results. In C57BL/6J mice, low-dose brain irradiation did not impact memory or brain levels of amyloid precursor protein (APP), amyloid- β ($a\beta$), tau, or phosphorylated tau (p-tau) (91). In swine, low-dose brain irradiation was associated with reduced hippocampal p-tau and APP (92). One study comparing neurobehavioral function in APOE2, APOE3, and APOE4 mice found that APOE4 mice performed worse than other APOE genotypes after brain irradiation (93).

The impact of radiation on the adverse cognitive effects of mutant APP and $a\beta$ has also been unclear. Interestingly, one study evaluating hemibrain irradiation in a mouse model with human APP and a mutant human presenilin gene found that at eight weeks post-irradiation, irradiated mice performed better in the Morris maze compared to unirradiated mice and had significantly fewer $a\beta$ plaques in their brains (94), suggesting that radiation could actually protect against mutant APP and $a\beta$ under certain conditions. Another study in 3xTg-AD mice found that hemi-brain irradiation reduced both $a\beta$ plaque burden and tau levels (95). In 5XFAD mice analyzed four days after low-dose brain irradiation, $a\beta$ accumulation was unchanged, but synaptic

degeneration and neuronal loss were ameliorated compared to unirradiated mice (96). These studies suggest that radiation can reduce $\text{a}\beta$ levels in the brain in some contexts, but not universally.

Our data show that radiation has *APOE* genotype-specific effects, with memory function worsening in an E3 background and improving in an E4 background. Although we observed that radiation protected against the adverse cognitive effects of ApoE4, these improvements were no longer present in ApoE4 mice with mutAPP. Our findings thus support that protective effects of radiation may depend on *APOE* genotype, and we speculate that discrepant findings in the effects of radiation on mutAPP may relate to differences between human and mouse ApoE.

Interestingly, although we failed to see an impact of radiation on decreasing plaques, we did observe a difference in the morphology of $\text{a}\beta$ plaques specifically in mutAPP mice on an ApoE4 background. However, the radiation protection against memory loss occurred in ApoE4 mice without $\text{a}\beta$ plaques, indicating that the protective effects occur independent of amyloid- β accumulation. Similarly, we did not find changes in ApoE or tau levels, or accumulation of p-tau that could explain the effects of radiation on cognition. We did find that radiation decreased the number of hippocampal microglia, consistent with prior findings (97). However, this occurred similarly across genotypes and hence is unlikely to underlie the ApoE genotype-specific differential impact of radiation on cognition.

The finding that mice with the combination of ApoE3 and radiation exhibited memory deficits comparable to ApoE4 animals suggests that radiation induces pathological processes that overlap with those driven by ApoE4. However, because radiation improves performance in ApoE4 animals, radiation is unlikely to act as a simple, additive ApoE4-like insult. Rather, radiation may engage a pathway that is differentially regulated in ApoE3 and ApoE4 backgrounds, producing toxicity in ApoE3 animals but a compensatory or protective response in ApoE4 animals.

Interestingly, memory function following brain irradiation differed between female and male mice. Female APOE4 mice exhibited more pronounced memory improvement at one month post-irradiation compared to male APOE4 mice. Similarly, in APOE3/APP mice, females showed an improvement in memory function at one month post-irradiation, whereas males did not. These findings suggest that in both genetic Alzheimer's disease (AD) models, females harboring AD risk factors experience memory improvement after radiation, highlighting sex as a significant independent variable influencing the effect of radiation on memory.

Sex-dependence of memory function and neuropathology has been documented in multiple mouse models. In the APP/PS1 (APPSwe/PS1dE9) mouse model, females exhibit faster decay of long-term potentiation and a higher burden of $\text{a}\beta$ plaques and activated microglia in the brain (98). Furthermore, sex dependence is also genotype-specific for APOE. For example, androgens protect against APOE4-induced cognitive decline in female mice, but enhance spatial learning and memory in female APOE3 mice (99). In APP/PS1/tau triple-transgenic mice, females display more severe brain pathology, including amyloid plaques and neuroinflammation, as well as worse spatial memory than males (100). The sex-dependent mechanisms impacting cognitive function in these genetic contexts may also operate to impact cognition after brain irradiation.

Potential clinical relevance

Our findings (**Fig. 5**) suggest that memory effects may occur at specific radiation dose thresholds for different APOE genotypes and sexes after whole-brain irradiation. Additional considerations warrant further study, including in patients. For example, partial-brain irradiation targeting specific brain subregions may influence the interaction between APOE genotype, sex, and radiation response. Importantly, the impact of brain radiation on the E3/E4 genotype remains

unknown, and we did not investigate the E2 genotype, which is distinct from E3 and E4 in terms of Alzheimer's disease risk. These variables may have significant clinical implications for individuals receiving brain-directed cancer therapies and warrant further investigation.

Neurodegeneration is also strongly age-dependent, with the risk of cognitive dysfunction increasing with advancing age. In this study, we irradiated mice at 3 to 5 months of age to assess the effects of brain radiation in a model prior to the full onset of AD. The $\text{A}\beta$ plaque burden in the mice was negligible due to their relatively young age. It is plausible that the interaction between radiation and APOE genotype may also be age-dependent, with older mice—who exhibit greater underlying brain pathology—potentially experiencing different memory outcomes after radiation exposure compared to the younger mice studied here.

Currently, there are no established biomarkers to predict cognitive risk following cancer therapy, which limits clinicians' ability to assess risk and implement adaptive strategies. Our findings (**Fig. 5**) underscore the importance of considering genetic predisposition to neurodegeneration and sex as key factors influencing memory response to exogenous exposures such as brain radiation. By exploring these intersections, we may uncover insights that translate into clinical strategies for mitigating cognitive risks associated with cancer therapy.

We investigated brain irradiation as a common cancer treatment-related exposure, one that thousands of patients in North America undergo daily. By identifying cognitive risk factors such as APOE genotype and sex, treatments might be tailored to individuals, potentially preserving—or even improving—cognitive function. However, this strategy requires further validation through human studies.

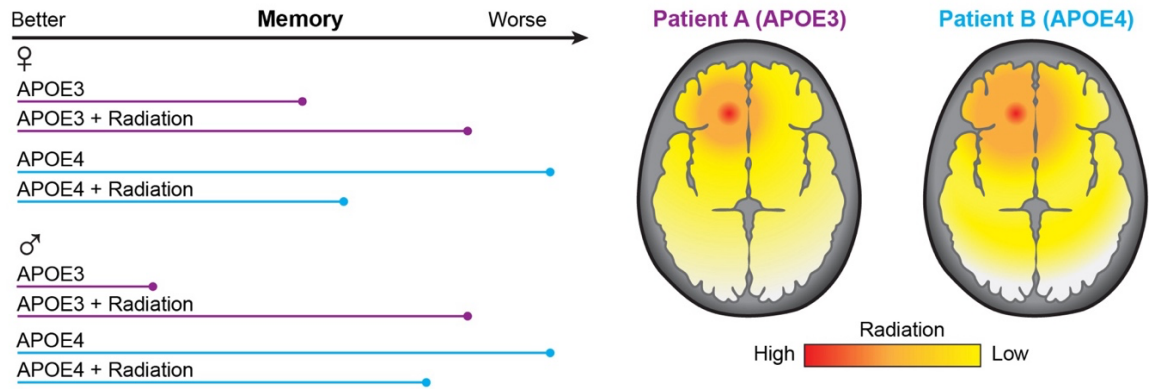
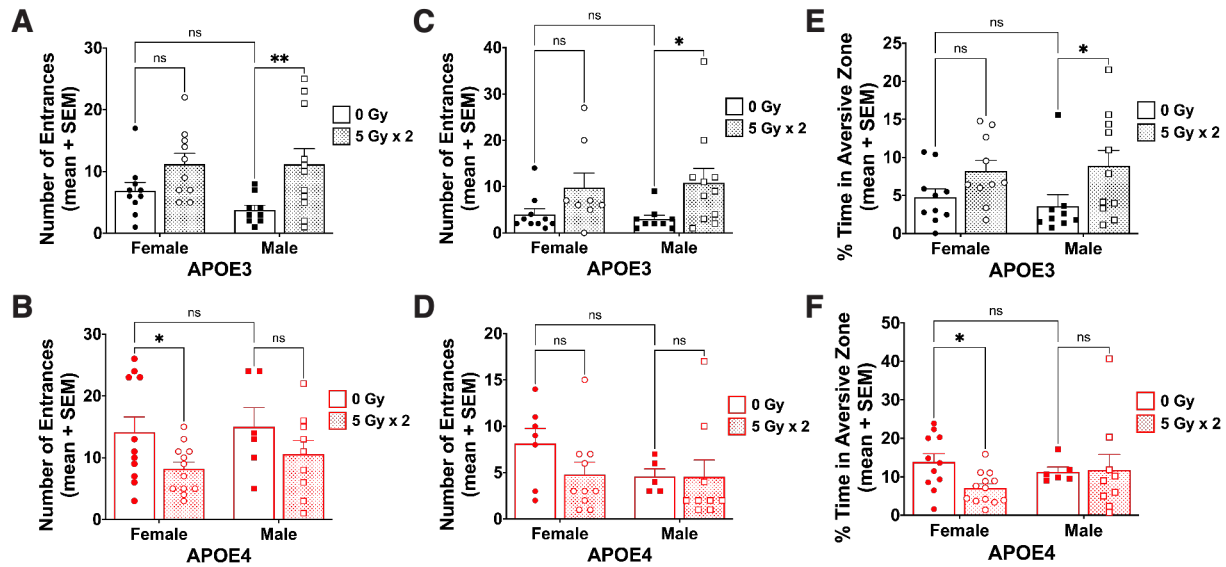


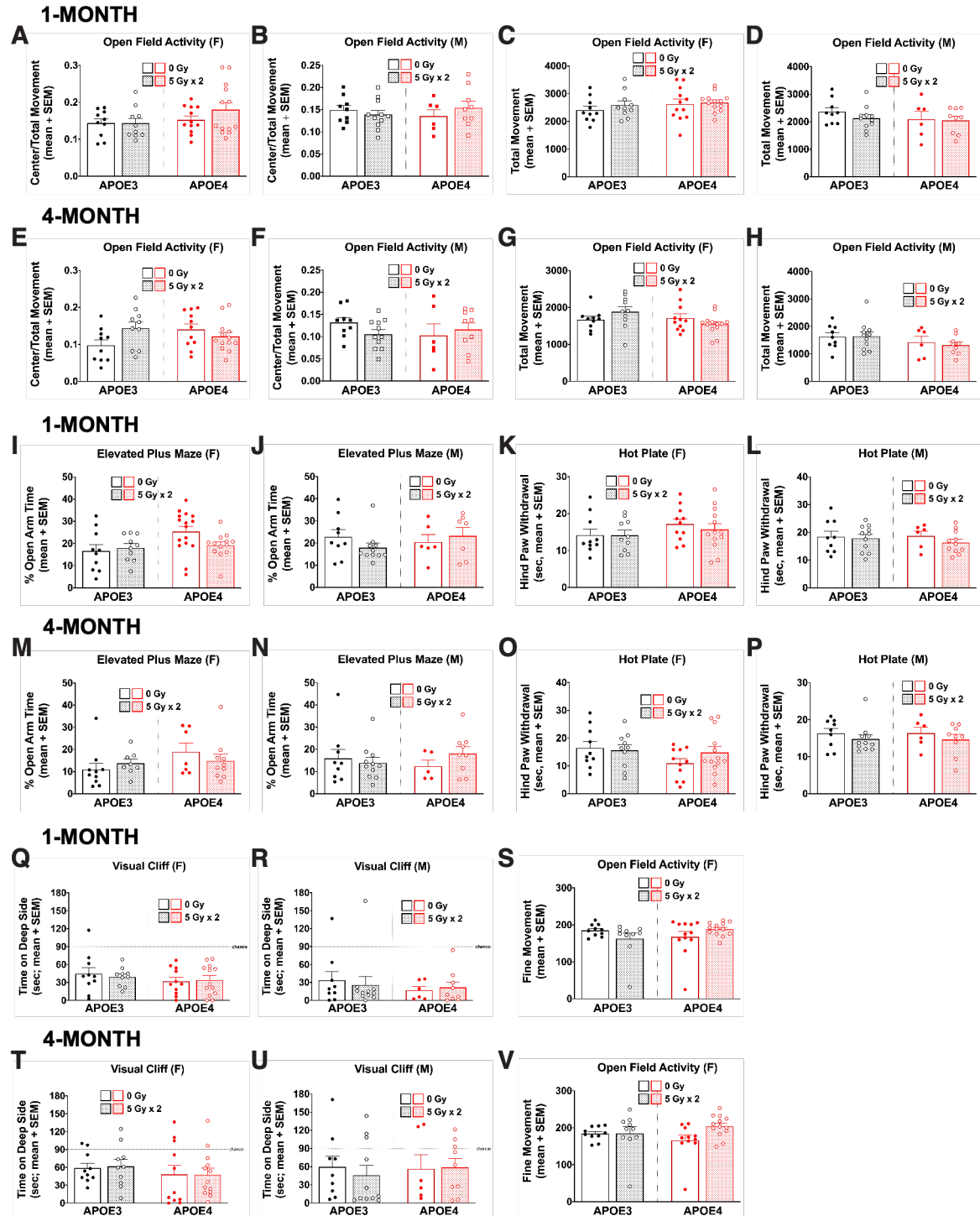
Figure 3.5. Relative effects of APOE genotype, sex, and radiation on memory and the subsequent radiation treatment approaches for APOE3 and APOE4 individuals. Lines indicating memory function are based on memory function data in Figure 3.1.

SUPPLEMENTARY FIGURES



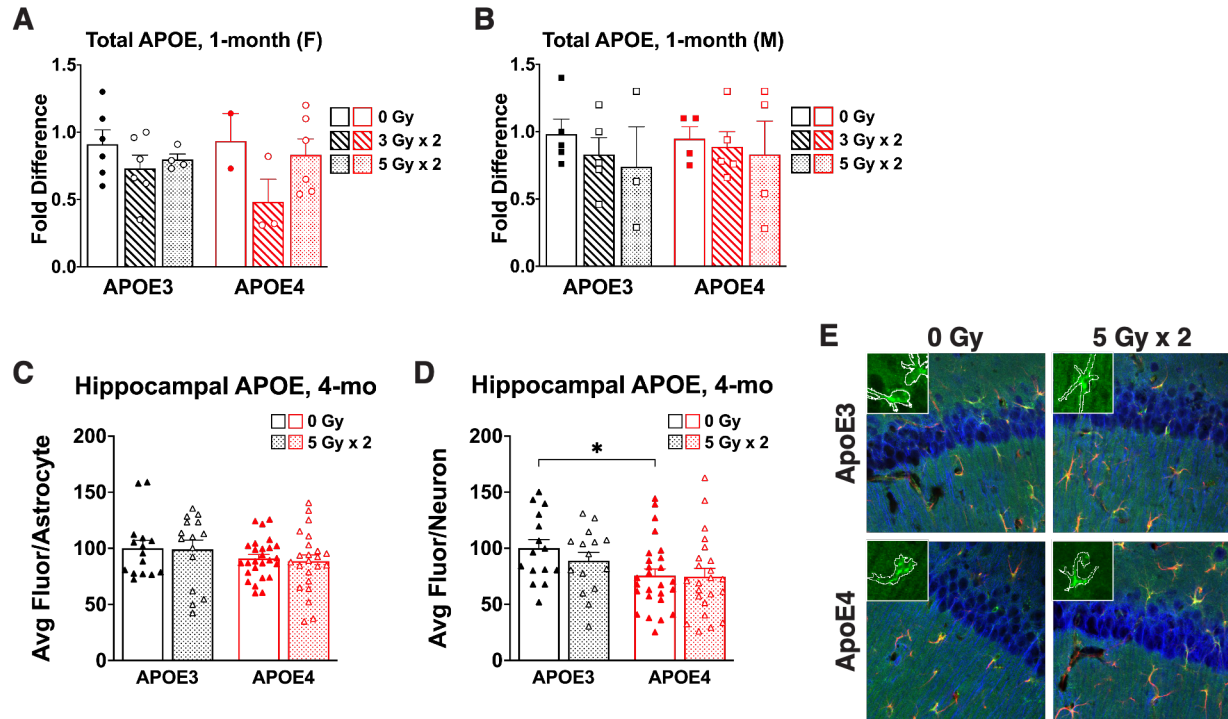
Extended Data Fig. 3.1. Effect of brain irradiation on memory in male versus female APOE3 and APOE4 mice.

Female and male APOE3 and APOE4 mice underwent Active Place Avoidance (APA) testing at 1 month after brain irradiation. Number of entrances into the aversive zone on (A/B) probe (A: radiation effect, $p=0.03$; B: radiation $p=0.03$) and (C/D) reinstatement (C: radiation $p=0.008$), and (E/F) percent time spent in aversive zone (E: radiation $p=0.009$) for APOE3 (A/C/E) and APOE4 (B/D/F) male and female mice. 2-way ANOVAs with Fisher's LSD post-hoc test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



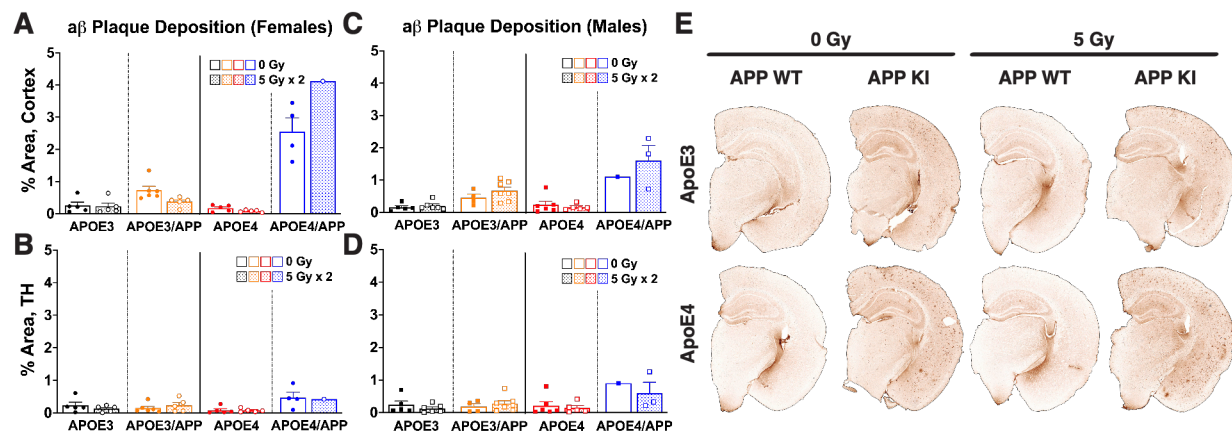
Extended Data Fig. 3.2. Short- and long-term effect of brain irradiation on global neurologic function in APOE3 and APOE4 mice.

Female (F) and male (M) APOE3 and APOE4 mice underwent (A-H, S, V) Open Field, (I-J, M-N) Elevated Plus Maze, (K-L, O-P) Hot Plate, and (Q-R, T-U) Visual Cliff testing at 1 month (A-D, I-L, Q-S) and 4 months (E-H, M-P, T-V) after brain irradiation to examine short- and long-term impact on global neurologic function. S: APOE x radiation $p=0.06$; E: APOE x radiation $p=0.02$; V: APOE x radiation $p=0.04$. 2-way ANOVAs with Fisher's LSD post-hoc test.



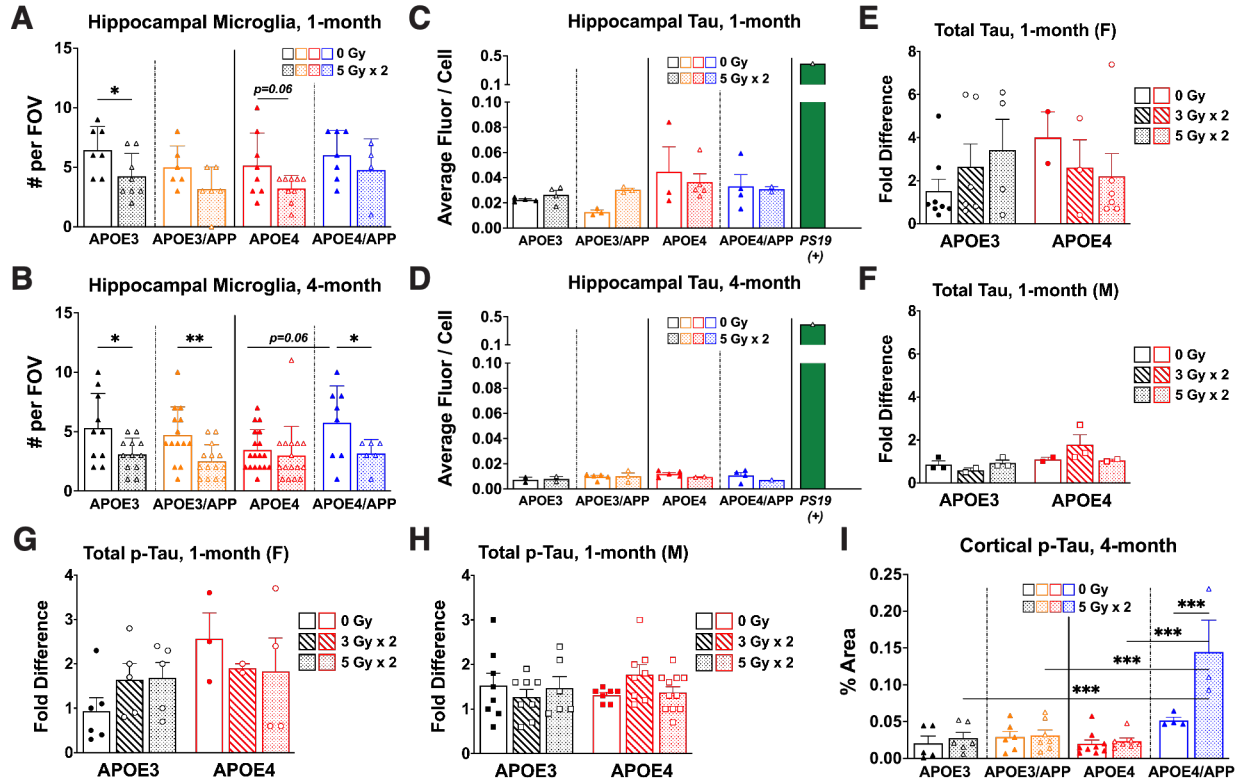
Extended Data Fig. 3.3. Long-term effect of brain irradiation and genotype on brain APOE levels.

Western blot comparison of total brain APOE in *APOE3* or *APOE4* (A) female and (B) male mice, 1 month after whole-brain radiation (3 Gy x 2 or 5 Gy x 2 doses). Immunohistochemical analyses were also conducted on brain samples obtained from irradiated (5 Gy x 2) or control (0 Gy) *APOE3* (N=5 per group) and *APOE4* (N=8-9) mice, 4 months after radiation. Samples were co-stained for human ApoE (green), GFAP (red), and MAP2 (blue), and hippocampal regions were imaged and analyzed for average cellular ApoE intensity in (C) astrocytes and (D) neurons. Intensity of all cells was averaged per FOV (plotted). Representative images in (E). 2-way ANOVAs with Tukey's multiple comparisons.



Extended Data Fig. 3.4. Long-term effect of brain irradiation on amyloid- β plaque deposition.

DAB (3,3'-Diaminobenzidine) conducted on brain samples obtained from irradiated (5 Gy x 2) or control (0 Gy) mice, 4 months after radiation. Samples were quantified for percent area of amyloid- β plaque deposition via 82E1 antibody in the (A,C) cortex and (B,D) thalamus (TH) of *ApoE3-AppWT* (N=10-11), *ApoE3-AppKI* (N=10-12), *ApoE4-AppWT* (N=10-11), and *ApoE4-AppKI* (N=4-5) mice and stratified by sex (A-B, females; C-D, males). Representative images per genotype in (E).



Extended Data Fig. 3.5. Effects of brain irradiation on microglia and tau pathology across APOE and APP genotypes.

Immunohistochemical analyses of hippocampal brain sections obtained from irradiated (5 Gy x 2) or control (0 Gy) mice, 1 month [*E3-AppWT* (N=4/group), *E3-AppKI* (N=3/group), *E4-AppWT* (N=4-5), *E4-AppKI* (N=2-4)] or 4 months [*E3-AppWT* (N=2-6), *E3-AppKI* (N=3-7), *E4-AppWT* (N=2-9), *E4-AppKI* (N=1-4)] after brain irradiation. Sections were co-stained for microglia (Iba1), tau (HT7), and neurons (MAP2) and imaged via confocal microscopy. Number of microglia per FOV (**A-B**) and average tau fluorescence per hippocampal neuron per mouse (**C-D**) were quantified and compared to values from a PS19 positive control mouse (green). Western blots were conducted to quantify (**E-F**) total tau and (**G-H**) phospho-tau in female and male mouse brains, 1 month after radiation (3 Gy x 2 or 5 Gy x 2 doses). (**I**) DAB (3,3'-Diaminobenzidine) was conducted on brain samples obtained from irradiated (5 Gy x 2) or control (0 Gy) mice, 4 months after radiation. Samples were quantified for percent area of p-tau via AT8 antibody in the cortex of *ApoE3-AppWT* (N=5-6), *ApoE3-AppKI* (N=6-7), *ApoE4-AppWT* (N=7-9), and *ApoE4-AppKI* (N=3-4) mice. 2-way ANOVAs with Tukey's multiple comparisons.

METHODS

Mice breeding, brain irradiation

All animal work was conducted under an institutionally approved IACUC protocol (AN197760-00). Mice were generated using a humanized model of AD maintained on a C57BL/6 background. Using the variants of APO E (E3 and E4) expressed, and the presence (+) or absence (−) of APP, animals included in this study were female and male mice having genotypes: E3/APP+, E3/APP−, E4/APP+, and E4/APP−. Mice were randomized to control or treatment groups (0 Gy whole brain radiation consisting of two consecutive daily doses of 5 Gy for a total dose of 10 Gy). Whole brain irradiation was performed as previously described (101). Between the ages of 3 and 5 months, mice were anesthetized (80 mg/kg ketamine and 10 mg/kg xylazine; Sigma, USA) intraperitoneally, with eyes and body protected using lead shielding, while their whole brains were sham-irradiated (0 Gy) or irradiated (5 Gy/day) for 2 consecutive days using a small animal cesium irradiator (J.L. Shepherd & Associates, San Fernando, CA, USA). For all behavioral testing, histological analyses, and biochemical assays, experimenters were blinded to the genotype and treatment of mice. For behavioral testing the testing order of mice was randomized.

Tissue Collection

Following behavioral tests, mice were euthanized with CO₂ and perfused with phosphate-buffered saline (PBS). Brains were harvested and bisected (in a brain matrix) into hemispheres with a microtome blade. The right hemispheres were snap-frozen in dry ice and stored at −80 °C, while the left hemispheres were fixed in cold 4% PBS–paraformaldehyde (PFA) and stored at 4 °C. Lysates were prepared by sonicating the frozen tissues in a mixture of

radioimmunoprecipitation assay buffer (Cat. No. 89901, Thermo Scientific, USA) and protease/phosphatase inhibitor cocktail (100:1, Cat. No. 5872S, Cell Signaling Technology, USA), followed by centrifugation. Supernatants were stored at -80°C for Western blot analysis. Fixed tissues were kept in PFA overnight, then transferred into 0.002% PBS–azide solution (stored at 4°C for no longer than 2 weeks), and finally transferred to 30% PBS–sucrose 24 h prior to frozen sectioning at $30\text{ }\mu\text{m}$ on a sliding knife microtome. Brain sections were stored in cryoprotectant solution at -20°C until used for immunohistochemical staining.

Immunohistochemical analyses

Hippocampal brain sections were selected, rinsed twice with PBS and twice with PBS-Tx (1X PBS w/ 0.2% Triton X-100), and incubated in blocking solution (5% goat serum and 0.2% Triton-X in 1X PBS) for 1 h at room temperature with gentle agitation. Sections were then incubated in primary antibodies diluted in blocking solution overnight at 4°C with gentle agitation. Primary antibodies included rabbit anti-ApoE (ab52607, 1:1000), mouse anti-GFAP (MAB3402, 1:1000), chicken anti-MAP2 (ab5392, 1:2000), rabbit anti-Iba1 (Wako #019-19741, 1:2000), mouse anti-HT7 (Thermo #MN1000, 1:500), and mouse anti-82E1 (IBL #10326, 1:500).

The following morning, brain sections were rinsed three times with PBS-Tx prior to incubation in fluor-conjugated secondary antibodies diluted in blocking solution for 2 h at 4°C . Secondary antibodies included goat anti-mouse Alexa Fluor 488 (A32723, 1:500), goat anti-rabbit Alexa Fluor 488 (A32731, 1:500), goat anti-mouse Alexa Fluor 594 (A32742, 1:500), and goat anti-rabbit Alexa Fluor 594 (A32740, 1:500). Sections were rinsed twice with PBS-Tx and twice with PBS prior to mounting with Vectashield antifade mounting medium with DAPI and imaging on a Zeiss LSM880 microscope. Resulting images were analyzed via ImageJ and CellProfiler.

For quantification of amyloid- β plaques and phosphorylated tau aggregates, hippocampal brain sections were selected and rinsed 3 times with 1X dPBS and 3 times with 1X TBS prior to quenching endogenous peroxidase activity with buffer (10% MeOH and 3% H_2O_2 in 1X TBS) for 5 min at RT. Sections were subsequently washed 3 times with 1X TBS before blocking in TBS++++ [1X TBS with 10% (v/v) FBS, 3% (w/v) fatty-acid-free BSA, and 1% (w/v) glycine; Triton X-100 added prior to use to 0.4% (v/v) final concentration] for 75 min at RT with gentle agitation. After block, sections were incubated in primary antibody [mouse anti-82E1 (IBL #10326, 1:500) or mouse anti-AT8 (Thermo #MN1020, 1:500)] diluted in TBS++++ overnight at 4 °C with gentle agitation. The following morning, sections were washed 3 times with 1X TBS before incubation in horse anti-mouse secondary antibody (VECTASTAIN Elite ABC-HRP Kit, PK-6102; 1:300 dilution in TBS++++) for 2 h with gentle agitation. ABC complex was prepared with 1:300 dilution of buffers A and B in TBS++++ and incubation at RT for 30 min, light-protected. Sections were washed 3 times with 1X TBS prior to 2 h incubation in ABC complex mixture at RT with gentle agitation, followed by 3 washes in 0.1 M Tris buffer (pH 8.0). 1X DAB solution was prepared using 50X DAB stock and 0.1 M Tris buffer, with 10 min incubation prior to development with 1:10,000 30% H_2O_2 . Sections were immediately transferred into DAB reaction for 2 min (AT8) or 4.5 min (82E1) prior to 2 washes in Tris, 2 washes in TBS, and 2 washes in PBS. Sections were mounted with Permount, imaged on Leica Versa slide scanner, and analyzed via FIJI and CellProfiler.

Immunoblotting analyses

Protein concentrations in lysates were determined using the Pierce BCA Protein Assay Kit (Cat. No. 23227, Thermo Scientific, USA). Equal amounts of protein (20–30 μg) were denatured

with reducing agent, separated on precast NuPAGE 4–12% polyacrylamide gels (Cat. No. NP0329BOX, Thermo Fisher Scientific, USA) and run in NuPAGE MES SDS Running Buffer (Cat. No. NP0002, Bio-Rad, USA) at 200 V for 50 min using a Bio-Rad PowerPac Basic Electrophoresis Power Supply. Protein bands were transferred to a Trans-Blot Turbo Mini 0.2 μ m PVDF membrane (Cat. No. 1704156, Bio-Rad, USA). The membrane was cut horizontally to separate target proteins from loading controls, and both sections were blocked for 1 h in 5% bovine serum albumin (BSA) prepared in Tris-buffered saline with Tween-20 (TBS-T). Membranes were probed with the following primary antibodies: phospho-tau (Ser202, Thr205) (1:1000, Cat. No. MN1020, Thermo Fisher Scientific, USA), tau (1:1000, Cat. No. MN1020, Thermo Fisher Scientific, USA), apolipoprotein E (1:1000, Cat. No. 178479, Millipore, USA), and α -tubulin (1:2000, Cat. No. 2144, Cell Signaling Technology, USA). Detection was performed using appropriate secondary antibodies: HRP-linked rabbit IgG (1:2000, Cat. No. 7074, Cell Signaling Technology, USA), HRP-linked mouse IgG (1:2000, Cat. No. 7076, Cell Signaling Technology, USA), and goat anti-mouse IgG (1:5000, Cat. No. 12-349, Millipore, USA). Proteins were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Cat. No. 34096, Thermo Fisher Scientific, USA). Images were captured using a Bio-Rad ChemiDoc Touch system (Bio-Rad Laboratories, USA). All immunoblots represent at least three independent experiments.

Neurobehavioral testing

Mice in both cohorts were exposed to multidimensional behavioral profiling to generate datasets that best represented animals' visual acuity and acrophobia, sensory function and nociceptive sensitivity, as well as exploratory drive and cognition. Since anxiety (elevated plus maze test), impaired sensory function (hot plate test), visual function (visual cliff test), and motor

activity (open field test) can affect memory-related behaviors, the adopted battery of behavioral approaches strengthens the validity of the cognitive assessments (active place avoidance test) amidst other confounding variables. All behavioral tests were carried out at Gladstone Institute, UCSF, by a third party blind to the experimental group. Active Place Avoidance behavior was monitored with overhead camera connected to a computer system with commercial tracker software (Noldus, Virginia, USA)) capable of measuring parameters of interest automatically. Averaged group performance scores on each test and timepoint were compared between unirradiated control mice and each experimental group.

Elevated Plus Maze: Anxiety behavior was assessed in a 10-minute session on an elevated plus maze apparatus (Kinder Scientific, California, USA) consisting of two open arms (without walls, 15” long, 2” wide) and two closed arms with 6.5” walls and 2” x 2” intersection. The entire maze was elevated 30.5” above the ground. Time spent and distance traveled in each arm was recorded.

Open Field Test: Locomotor behavior was assessed in 15-minute sessions in a 40 x 40 cm acrylic arena and data was analyzed by Activity Monitor 7 software (Med Associates, Vermont, USA). The arenas were housed in sound attenuating chambers under dim lighting (~20 lux). Total activity and center/total movement ratio was measured.

Active Place Avoidance: Spatial learning and memory were assessed using an active place avoidance apparatus (Noldus, Virginia, USA) which consisted of a 40 cm circular arena which rotated at one revolution per minute throughout the testing. The procedure was completed across six days with one 10-minute trial per day. On the first day of testing, mice were habituated to the

rotating arena and were allowed to freely explore in the absence of the shock. On days 2-4, training was performed with a 60-degree section of the circular arena designated as the shock zone and fixed in the room. During this phase, each entry into the shock zone resulted in a 500ms, 0.2mA shock; shocks were delivered every 1.5 seconds until the animal left the zone. On day 5, a probe trial was conducted with the shock turned off to assess pure spatial memory. And, finally, on day 6, a reinstatement session was conducted with shock delivery similar to the training phase.

Visual Cliff: Visual acuity was assessed using a visual cliff apparatus which consisted of two 21 x 42 cm areas (a shallow and a deep side) and a 16 cm wall surrounding the arena. The “shallow” side of the arena was covered with a checked laminated pattern on the floor and walls (2 x 2 cm). The “deep” side was made of uncovered clear Plexiglas and was suspended 101cm above the floor to create the illusion of a cliff. Testing was conducted across two days. On the first day, animals were placed on a 5 x 7.5 cm block which was elevated 2.5 cm above the Plexiglas floor and straddled the shallow and deep sides of the arena. Animals were then allowed to choose which side they preferred to step off the block across four separate trials with an intertrial interval of ~15 minutes. On the second day of testing, the animals were allowed to freely explore the arena for 180 seconds with time spent in each of the areas recorded.

Hot Plate: Thermal nociception was measured using a hot plate (Maze Engineers, Illinois, USA) set to 52 degrees Celsius. Hindpaw withdraw latency was measured with a maximum exposure time of 45 seconds.

CHAPTER 4: CONCLUSION

The work presented in this dissertation examines how memory function is preserved or lost in aging and Alzheimer's disease, approaching this question from two different directions: (1) the intrinsic metabolic requirements of neurons, and (2) the genetic underpinnings that shape the brain's response to injury. Though methodologically distinct, these two studies arrive at a consistent conclusion: cognitive resilience is governed by a multitude of therapeutically actionable factors, including neuronal energy metabolism, APOE genotype, and biological sex.

Chapter 2 established that neuronal ketone oxidation is not merely an adaptation to starvation, but a constitutive requirement for normal brain function. In human neurons, physiologic concentrations of β HB were necessary for maximal acetyl-CoA production and mitochondrial respiratory reserves, and acetoacetate proved a more effective oxidative substrate than β HB itself. In mice, adult-onset deletion of neuronal *Bdh1* shortened lifespan and impaired memory even under standard dietary conditions, and these deficits were markedly exacerbated on an Alzheimer's disease background. Together, these findings highlight an obligate role of neuronal ketone oxidation in sustaining brain function and potentially protecting against neurodegeneration, revealing a metabolic vulnerability that endogenous ketones may be leveraged to combat.

Chapter 3 showed that inherited risk for neurodegeneration determines the cognitive outcome of brain irradiation, and that it does so in opposite directions depending on APOE genotype. APOE3 mice developed worse memory after irradiation, whereas APOE4 mice maintained or improved memory, an effect heightened in females specifically. The introduction of mutant APP overrode the genotype-specific responses, worsening memory in APOE3 and abolishing the protective effect of APOE4. These results indicate that radiation engages pathways

differentially regulated by APOE genotype, and that genetic risk and sex together shape the cognitive response to cancer therapy.

Taken together, these two studies reinforce a shared theme. In each, a factor that might be viewed as purely deleterious—loss of a metabolic enzyme, or exposure to ionizing radiation—produced outcomes that differed depending on genetic and physiological context. Adult-onset neuronal ketone deficiency was more harmful to aging females on a wild-type background, but on an APP background, the phenotype worsened substantially and shifted to affect males more. Brain irradiation was harmful or protective depending on APOE genotype and sex, but such effects were masked by the introduction of a mutAPP background. In both cases, amyloid background emerged as a decisive modifier and sex differences recurred throughout, underscoring the importance of studying both factors in models of cognitive aging.

Several limitations qualify these conclusions and warrant further investigation. Both studies rely on mouse models that approximate but do not reproduce human disease, and the human APOE isoforms and amyloid mutations used here may interact with mouse physiology in ways that differ from a human context. The mechanisms linking *Bdh1* loss and radiation exposure to memory impairment remain incompletely defined—for example, the contribution of glial ketogenesis, the signaling versus energetic roles of ketones, and the interactive pathway through which APOE genotype converts radiation insult into harm or benefit. The radiation studies were also limited to whole-brain exposure in young animals with minimal amyloid burden, painting an incomplete picture of how dose, plaque load, and age may modify the observed interactions. Addressing these unknowns will require finer mechanistic dissection and validation in human-relevant contexts.

Despite the limitations, these findings point toward a future in which cognitive risk can be both predicted and therapeutically targeted. If constitutive neuronal ketone metabolism protects the aging and neurodegenerating brain, then enhancing that metabolism—through diet, exogenous ketones, or pharmacological targeting of endogenous pathways—may offer a strategy against cognitive decline, particularly in individuals predisposed to glucose hypometabolism. If APOE genotype and sex predict the cognitive consequences of cancer therapy, then genotyping may help guide personalized strategies by anticipating neurological side effects and mitigating harmful outcomes. In both cases, the broader message is the same: the resilience of memory in aging and Alzheimer’s disease is shaped by factors we can measure and, increasingly, hope to modify.

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