

**Dihydromyricetin counteracts Alzheimer's Disease-like changes
induced by repeated social isolation**

Saki Watanabe¹, Jun Ni^{1,2}, Jalen Cai¹, Aijia Guo¹, Xuesi M Shao³, Jing Liang¹.

1. Titus Family Department of Clinical Pharmacy, Alfred E. Mann School of Pharmacy and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA 90033, USA.
2. Meinig School of Biomedical Engineering, Cornell University, Ithaca, NY 14850, USA.
3. Neurobiology, David Geffen School of Medicine, University of California Los Angeles, Los Angeles, CA, 90095, USA.

Corresponding author:

Jing Liang, MD, PhD

1985 Zonal Ave, PSC 504

Los Angeles, CA 90089

Tel: 323-442-3118

Email: jliang1@usc.edu

18 **Abstract**

19 Therapeutic avenues for Alzheimer’s disease (AD) are limited by the lack of animal models that
20 accurately mimic the disease’s natural progression. As social isolation increases the risk of AD by
21 50%, we developed a rodent SI model and refined it with SI withdrawal and re-SI (RSI) phases.
22 In this study, we found RSI mice to naturally exhibit AD-like pathological changes, including
23 astrocyte atrophy, GLUT1 reduction, Ca²⁺ imbalance, and increased phosphorylated tau (pTau),
24 and behavioral abnormalities, such as memory decline. Dihydromyricetin (DHM), a positive
25 allosteric modulator of γ -aminobutyric acid A receptors (GABA_ARs), counteracted these changes.
26 Our findings suggest that SI accumulation contributes to neuronal damage and highlight SI as a
27 significant factor in AD progression. This study also highlights the promise of DHM as a
28 therapeutic avenue to prevent such damages.

29

Introduction

Alzheimer's disease (AD) is a vital neurodegenerative cause of dementia, marked by cognitive decline, memory deficits, and impaired daily life abilities, along with neuropsychiatric symptoms (NPS) such as irritability, depression, or agitation.¹⁻⁴ The prevalence and burden of AD are continuously rising at a concerning faster rate than the growing elderly population.⁵ Its complex pathogenesis involves neuroinflammation,⁶⁻⁹ synaptic dysfunction,¹⁰⁻¹³ neurotransmitter imbalances,^{10,12,14,15} and the hallmark presence of tau neurofibrillary tangles (NFTs) and β -amyloid (A β) plaques.¹⁶⁻¹⁹ Despite extensive research, the precise causes, progression, and effective treatments of AD remain elusive.²⁰⁻²³ One challenge is the lack of suitable animal models replicating the disease's natural onset and progression.²⁴⁻²⁸

Social interactions and relationships, particularly those characterized by emotional support, play a pivotal role in mental and physical health, positively influencing biological functioning and healthy behaviors.²⁹⁻³³ On the contrary, social isolation (SI) has been associated with cognitive decline, memory loss, anxiety, depression, and a 50% increased risk of AD,³⁴⁻³⁸ suggesting that prolonged or repeated SI exacerbate these changes.³⁹⁻⁴² Rodents, like humans, are social creatures that thrive under group housing conditions and are recognized to display anxiety, stress, depression, cognitive decline, and seizures after SI conditions.⁴³⁻⁴⁶ Given these connections, we have established an SI mouse model, which consistently exhibits changes comparable to AD. Thus far, we have found that subjecting mice to four weeks of SI leads to anxiety-like behavior, memory impairment, reduced GABAergic transmission, downregulation of gephyrin expression (indicating the loss of clustering of γ -aminobutyric acid A receptors, GABA_ARs), neuroinflammation, mitochondrial dysfunction, and oxidative stress.⁴⁷⁻⁵¹ Encouragingly, administering dihydromyricetin (DHM, [(2R,3R)-3,5,7-trihydroxy-2-(3,4,5-trihydroxyphenyl)-2,3-

dihydrochromen-4-one]], a positive allosteric modulator of GABA_AR,⁵² effectively counteracted these deficits and neuropathological features in transgenic AD mouse models.⁵³ Collectively, these suggest that SI initially induces GABAergic impairment, while DHM facilitates the restoration of this transmission, consequently ameliorating downstream effects like mitochondrial dysfunction and neuroinflammation. However, the transition from these SI-induced changes to the onset of permanent AD-like damages remains unexplored. To bridge this gap, we investigated the timing of AD-like changes in repeated episodes of SI.

In this study, we introduce a repeated SI (RSI) phase into the SI mouse model. We sought to mimic the patterns of SI or loneliness in humans over a longer period, with the hypothesis that accumulation of SI will lead to progression from symptoms like cognitive impairments and neuropathological damages to AD. We then explored the therapeutic potential of DHM to prevent or ameliorate the progression.

Results

Repeated social isolation induces astrocyte atrophy, which is ameliorated by DHM administration.

Astrocytes are glial cells in the central nervous system (CNS) that play a multifaceted role in maintaining brain homeostasis. They provide structural support to neurons, regulate neurotransmitter and ion levels, manage energy metabolism, participate in the brain's immune defense, and establish tripartite synapses with GABAergic synapses, which are critical for cognitive function.^{54,55} By supporting these tripartite synapses, astrocytes actively contribute to the intricate network of neurotransmission.^{56–58} Consequently, astrocyte dysfunction is closely linked to the pathological features of AD.^{59,60} In our previous report, four weeks of SI induced astrocyte damage, demonstrated by increased glial fibrillary acidic protein (GFAP)-positive astrocyte

density, reduced astrocyte size, diminished morphological complexity, which were restored with DHM treatment.⁵¹ GFAP is a common marker for identifying and studying astrocytes, as they change GFAP expression and morphology in response to conditions such as inflammation, trauma, infections, or neurodegenerative diseases.^{61,62} Along with the diminished GABAergic transmission in our SI and AD models,^{47,53} we deduced that GABAergic tripartite synapses are likely to be involved in the SI-to-AD onset and progression.

The hippocampus serves essential functions in memory formation, emotional regulation, spatial navigation, and learning.⁶³ This region is particularly susceptible to early-stage damage in AD, characterized by rapid tissue loss as one of the initial pathological features.^{63,64} Notably, the hippocampus maintains a unique capacity for neurogenesis, but this capacity progressively declines as AD advances, especially in the dentate gyrus.^{64,65} Therefore, the dentate gyrus emerges as a region of interest for investigating early-stage AD-like changes in our novel RSI model. To understand how RSI affects astrocyte health and whether RSI exacerbates these changes, we examined for GFAP-positive astrocytes in the dentate gyrus (DG) using our novel RSI model (**Fig. 1**).

We found that astrocyte size decreased from 153.0 μm^2 to 108.1 μm^2 ($p \leq 0.0001$), while two weeks of daily oral 2 mg/kg DHM treatment restored its size to 138.0 μm^2 ($p \leq 0.0001$, **Fig. 1D**). In the same region, astrocyte density increased from 269.0/mm² in control to 543.0/mm² ($p=0.0013$) in RSI mice and brought back to 282.9/mm² with DHM treatment ($p=0.0002$) (**Fig. 1A, E**). Astrocyte morphology and complexity, as defined by Sholl analysis, were diminished in RSI compared to control at 10, 15, 20, 25, and 30 μm^2 from the nucleus ($p=0.0176$, 0.0003, 0.0002, 0.0002, 0.0290, respectively), and increased with DHM treatment at 10, 15, and 20 μm^2 from the nucleus ($p \leq 0.0032$, 0.00001, 0.0036, respectively) (**Fig. 1B, C, F**). Compared to our previous findings after four weeks

98 of SI,⁵¹ RSI induced more severe changes in astrocytes, characterized by a reduction in astrocytes
99 size and signs of atrophy. Our results suggest that multiple episodes of SI have a more detrimental
100 impact on astrocyte health, impairing astrocyte ability to participate in tripartite synapses. DHM
101 was able to restore health in astrocytes, indicated by reduced GFAP expression and restoration of
102 astrocyte size and morphology.

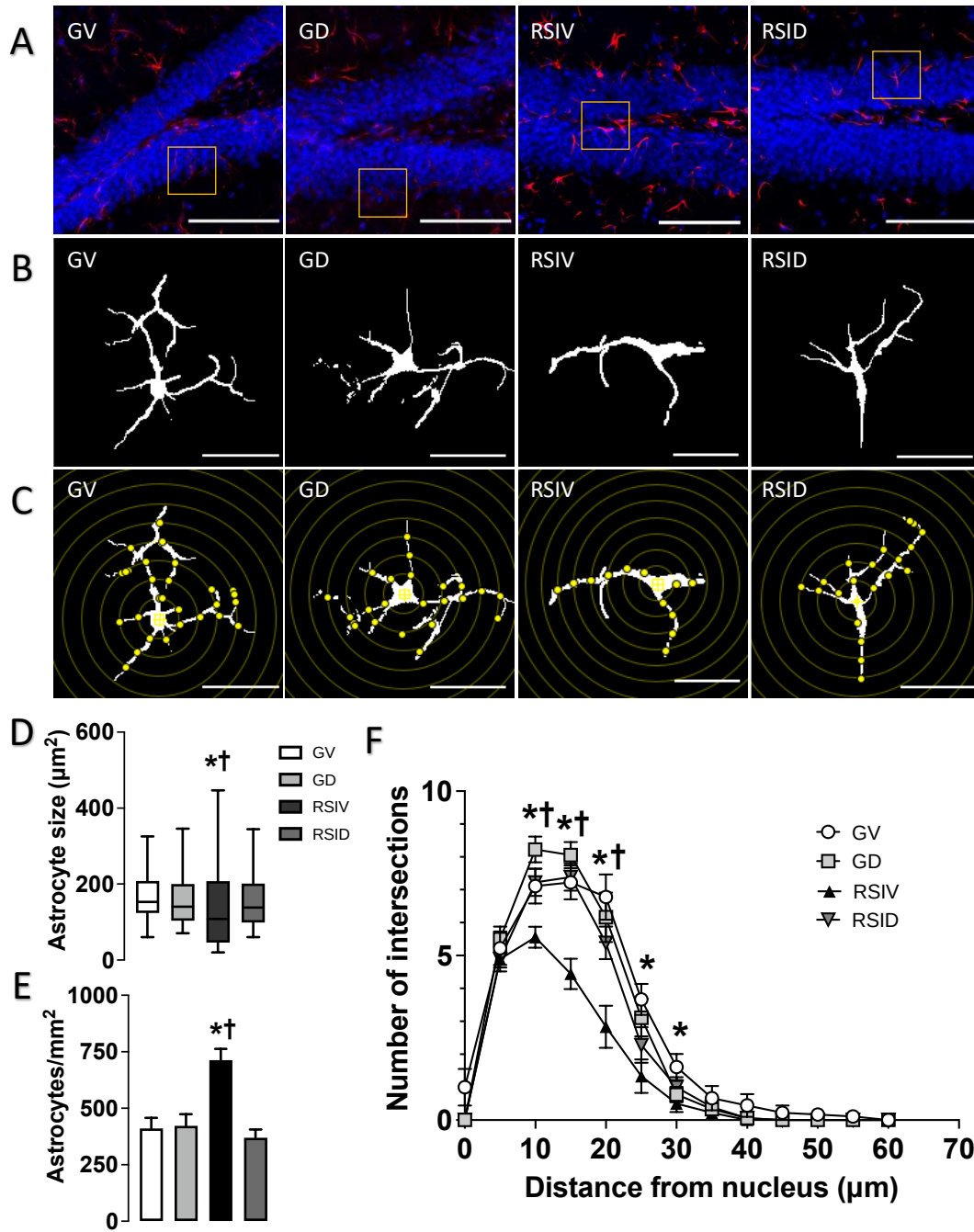


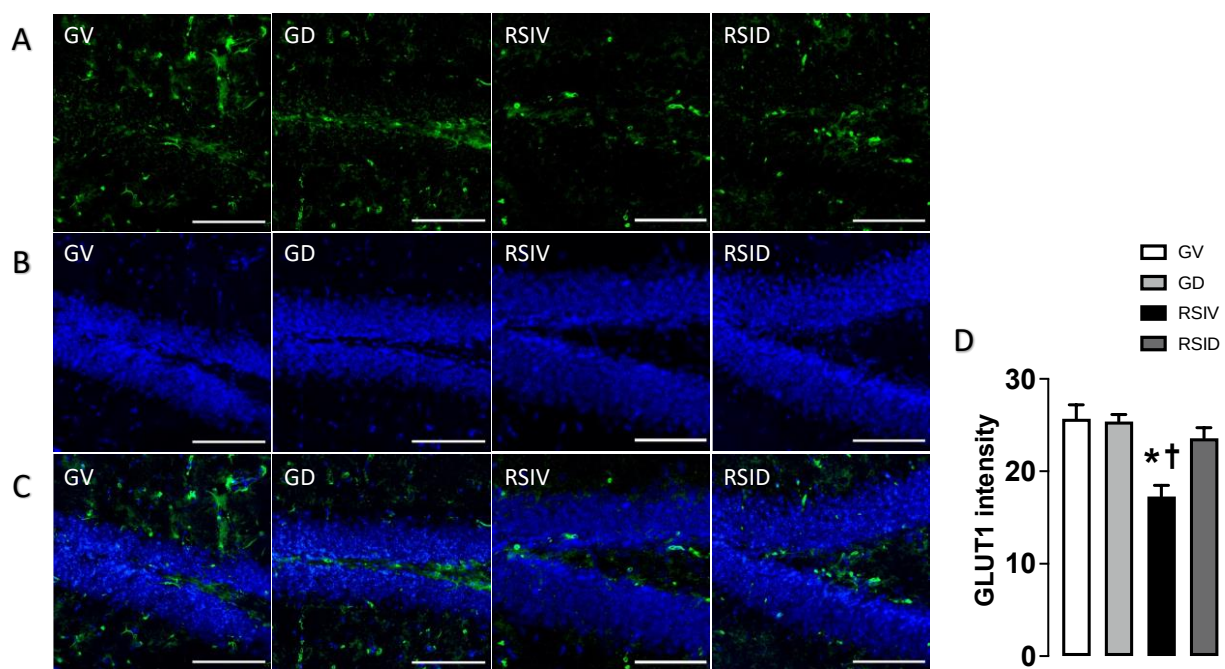
Fig. 1. Astrocytes in hippocampus. (A) Representative images of astrocytes in the dentate gyrus of mice. Blue=DAPI, nucleus. Red=GFAP, astrocytes. Yellow square indicates area of representative astrocyte used in morphological (Sholl) analysis. Scale bar=100 μm . (B) Binary representation of single astrocyte used for Sholl analysis. Scale bar=20 μm . (C) Representative

result of Sholl analysis. Yellow circles indicate steps and yellow dots represent intersections. Scale bar =20 μm . (D) Astrocyte size in μm^2 . Data shown as box-and-whisker plot, where center line is the median, limits are the interquartile range (IQR), and whiskers are the minimum and maximum. (E) Number of astrocytes in the dentate gyrus per mm^2 . Bars represent mean $\pm$ SEM. Two-way ANOVA with Holm-Sidak's multiple comparison. Kruskal-Wallis one-way ANOVA on ranks with multiple comparisons, Dunn's method. (F) Quantification of morphological complexity. Error bars represent SEM. Two-way repeated measures ANOVA. *; $p \leq 0.05$ vs GV. †; $p \leq 0.05$ vs RSIV. n=5-6 per group. Three astrocytes were randomly selected and morphologically analyzed per group. GV = group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated SI with vehicle treatment. RSID = repeated SI with DHM treatment.

Repeated social isolation reduces GLUT1 expression, which is normalized after DHM administration.

Previously, we have reported metabolic dysfunction in four-week SI mice, demonstrated by reduced ATP levels, altered electron transport chain expression and function in the mitochondria, and increased oxidative stress.⁴⁷⁻⁴⁹ These changes are also noted in AD, in addition to significantly decreased glucose metabolism.⁶⁶ Put together, we questioned whether this impaired metabolism was due to an overall reduction in glucose availability and therefore loss of energy and astrocyte function. Glucose transporter 1 (GLUT1) is a member of the glucose transporter family responsible for glucose uptake across the blood brain barrier (BBB) and into astrocytes.^{67,68} Because neurons do not express GLUT1,^{68,69} measuring GLUT1 levels can provide insights into glucose distribution and mobilization in astrocytes after RSI.

130 Immunohistochemical stains presented lower GLUT1 intensity in the dentate gyrus ($17.27 \pm$
131 1.206) of RSI mice compared to the control (25.68 ± 1.513 , $p=0.0004$) (**Fig. 2**). DHM restored
132 GLUT1 expression (23.58 ± 1.145 , $p=0.0035$) in RSI mice. While decreased GLUT1 levels are
133 not a direct measure of glucose concentration and metabolism, our results suggest that fewer
134 transporters are available for astrocytes to uptake glucose. Less glucose availability impacts
135 mitochondrial function, supporting our previous findings of SI-induced metabolic damage.⁴⁷⁻⁴⁹



137 **Fig. 2. GLUT1 expression in dentate gyrus.** Representative image of (A) GLUT1, green; (B)
138 DAPI, blue; and (C) merged GLUT1 and DAPI. Scale bar=100 μ m. (D) Quantification of GLUT1
139 intensity represented as mean gray value. Bars represent mean $\pm$ SEM. Two-way ANOVA with
140 Holm-Sidak's multiple comparison. *, $p \leq 0.05$ vs GV. †; $p \leq 0.05$ vs RSID. n=4-6 per group. GV =
141 group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated
142 SI with vehicle treatment. RSID = repeated SI with DHM treatment.

Repeated social isolation disrupts Ca^{2+} homeostasis, which is restored by DHM administration.

Another crucial element in neurotransmission is calcium ion (Ca^{2+}) signaling. Ca^{2+} functions as a second messenger in various signaling pathways, influencing critical processes such as synaptic plasticity, membrane excitability, and synaptogenesis.⁷⁰ In healthy and resting conditions, neurons maintain a pronounced and heavily regulated concentration gradient between intracellular and extracellular environments.⁷⁰ Given its role in numerous physiological functions and high concentration gradient, even subtle deviations in Ca^{2+} homeostasis can be harmful.⁷¹ Notably, aging neurons exhibit documented alterations in Ca^{2+} balance, rendering them susceptible to metabolic and cellular stress, which can contribute to the onset or progression of neurodegenerative diseases, including AD.⁷² In fact, Ca^{2+} homeostasis is closely associated with astrocyte function^{73–75} as well as many pathological hallmarks of AD, including abnormal synaptic activity, apoptosis, deposition of amyloid- β , and tau hyperphosphorylation.⁷⁶ The endoplasmic reticulum (ER), a dynamic and multifunctional organelle, plays a central role in maintaining Ca^{2+} homeostasis and serves as the primary hub for storage and release of Ca^{2+} .⁷⁷ One of the receptors responsible for releasing Ca^{2+} from the ER is the 1,4,5-triphosphate receptor (IP3R).⁷⁶ In association with Ca^{2+} release, IP3Rs are involved in synaptic plasticity and memory consolidation, where alterations in their expression and function are implicated in Ca^{2+} signaling dysfunction in AD models.⁷⁸ To determine whether Ca^{2+} homeostasis is disrupted under RSI, in association with astrocyte atrophy, we examined IP3R expression and Ca^{2+} levels in the hippocampus (**Fig. 3**). IP3R intensity was increased after RSI (29.62 ± 2.014) compared to control (17.65 ± 1.141 , $P \leq 0.0001$), and restored after DHM treatment (24.34 ± 1.339 , $P = 0.0387$). Similarly, RSI induced an increase in total Ca^{2+} levels in the hippocampus (**Fig. 3E**), averaging at a concentration of 1.468 ± 0.03074 mM compared to the average observed in the control (1.302 ± 0.02649 mM,

P=0.0037). Two weeks of DHM administration reduced total Ca^{2+} concentration to 1.343 ± 0.02845 mM (P=0.0277). These results imply that RSI induces a disruption in Ca^{2+} homeostasis, demonstrated by ER alterations in its Ca^{2+} channel expression and an overall increase of total Ca^{2+} concentration, while DHM can retain proper homeostasis.

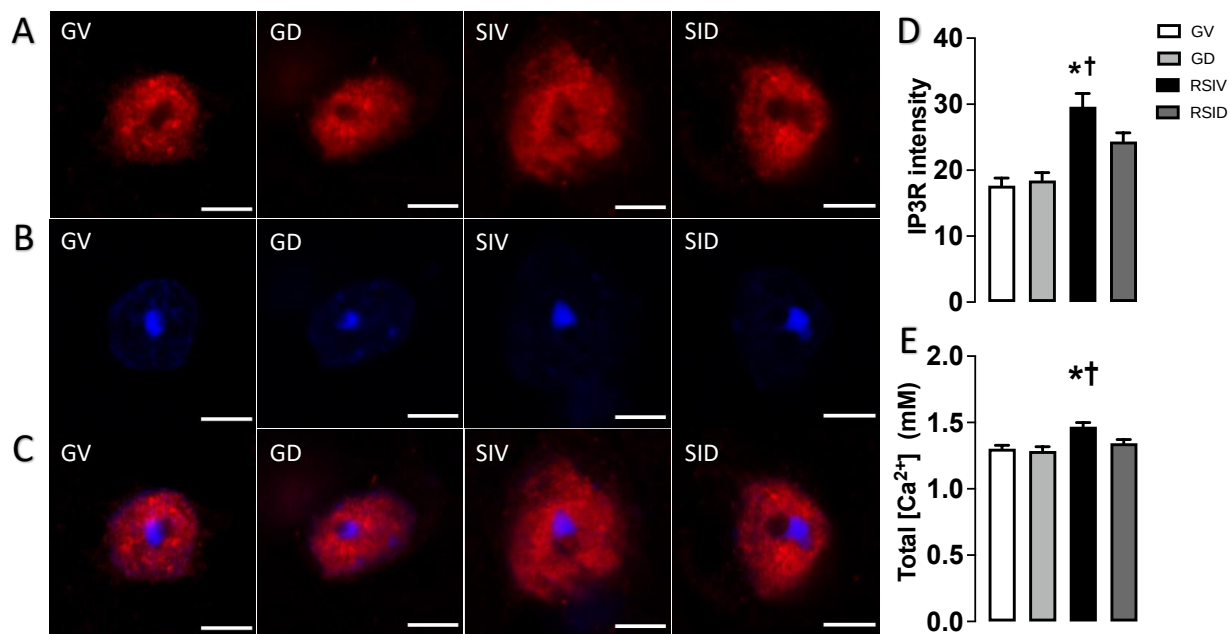


Fig. 3. IP3R and Ca^{2+} levels in hippocampus. Representative images of (A) IP3R, red; (B) DAPI, blue; and (C) merged IP3R and DAPI. Scale bar=20 μm . (D) Quantification of IP3R intensity represented as mean gray value. (E) Total Ca^{2+} concentration. Bars represent mean $\pm$ SEM. Two-way ANOVA with Holm-Sidak's multiple comparison. *, p<0.05 vs GV. †; p<0.05 vs RSID. n=4-6/group. Five cells were randomly selected and analyzed per group. GV = group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated SI with vehicle treatment. RSID = repeated SI with DHM treatment.

Repeated social isolation increases pTau(S404) levels, which are normalized by DHM administration.

In a healthy brain, the tau protein is natively unfolded and functions to stabilize microtubule structure; in AD, however, these proteins are found to be hyperphosphorylated, aggregated, and thereby unable to properly support microtubule and neuronal structure.⁷⁹ While such neurofibrillary tangles (NFTs) of phosphorylated tau (pTau) are widely accepted hallmarks of AD,^{18,19} little is known about the onset or progression of the change in tau regulation.

To further evaluate our RSI model as a model of natural AD onset and progression, we examined changes in pTau levels for potential hyperphosphorylations. Moreover, to gain more insight into the changes in tau phosphorylation patterns, we decided to examine pTau levels via four common phosphorylation sites: T181, T217, T231, and S404 (**Fig. 4**).⁸⁰ We found that pTau expression was increased only at the S404 phosphorylation site in RSI mice (1.464 ± 0.1242) compared to control (0.9165 ± 0.04013 , $p \leq 0.0001$) (**Fig. 4A**). DHM was able to reduce these levels (1.009 ± 0.07387 , $p=0.0007$) in RSI mice. Expression levels did not differ across all treatment groups at phosphorylation sites T181, T217, and T231 (**Fig. 4B, C, D**). Thus, we decided to further investigate the histological changes of pTau(S404) expression in the dentate gyrus.

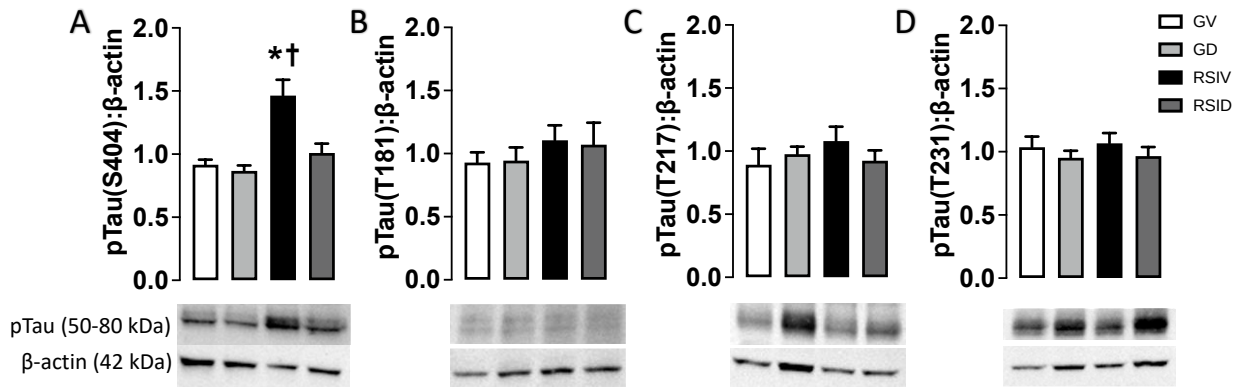


Fig. 4. pTau levels in hippocampus. Quantification (top) and representative western blots (bottom) of pTau (A) S404, (B) T181, (C) T217, and (D) T231 in the hippocampus with β -actin as the loading control. Band order corresponds to the bars above. Bars represent mean $\pm$ SEM. Two-way ANOVA with Holm-Sidak's multiple comparison. *, $p \leq 0.05$ vs GV. †, $p \leq 0.05$ vs RSID. GV = group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated SI with vehicle treatment. RSID = repeated SI with DHM treatment.

pTau(S404) intensity was higher in RSI mice (35.98 ± 2.819) compared to control (26.39 ± 1.353 , $p=0.0094$), and modestly reduced with DHM treatment (29.11 ± 1.020 , $p=0.0548$) (**Fig. 5A-C**). RSI mice presented with a higher number of pTau(S404) particles ($228.7 \pm 63.01/\text{mm}^2$) compared to control ($44.35 \pm 13.38/\text{mm}^2$, $p=0.0236$) and reduced with DHM treatment ($63.12 \pm 27.19/\text{mm}^2$, $p=0.0300$) (**Fig. 5D**). The average size of these particles was larger in RSI mice ($25.23 \pm 2.234 \mu\text{m}^2$) compared to control ($12.55 \pm 3.198 \mu\text{m}^2$, $p=0.0421$) and to RSI mice receiving DHM treatment ($12.59 \pm 3.050 \mu\text{m}^2$, $p=0.0221$) (**Fig. 5E**). Our results indicate the impact of RSI on tau phosphorylation patterns that mirror AD-like changes, and the ability of DHM to resist this pattern change.

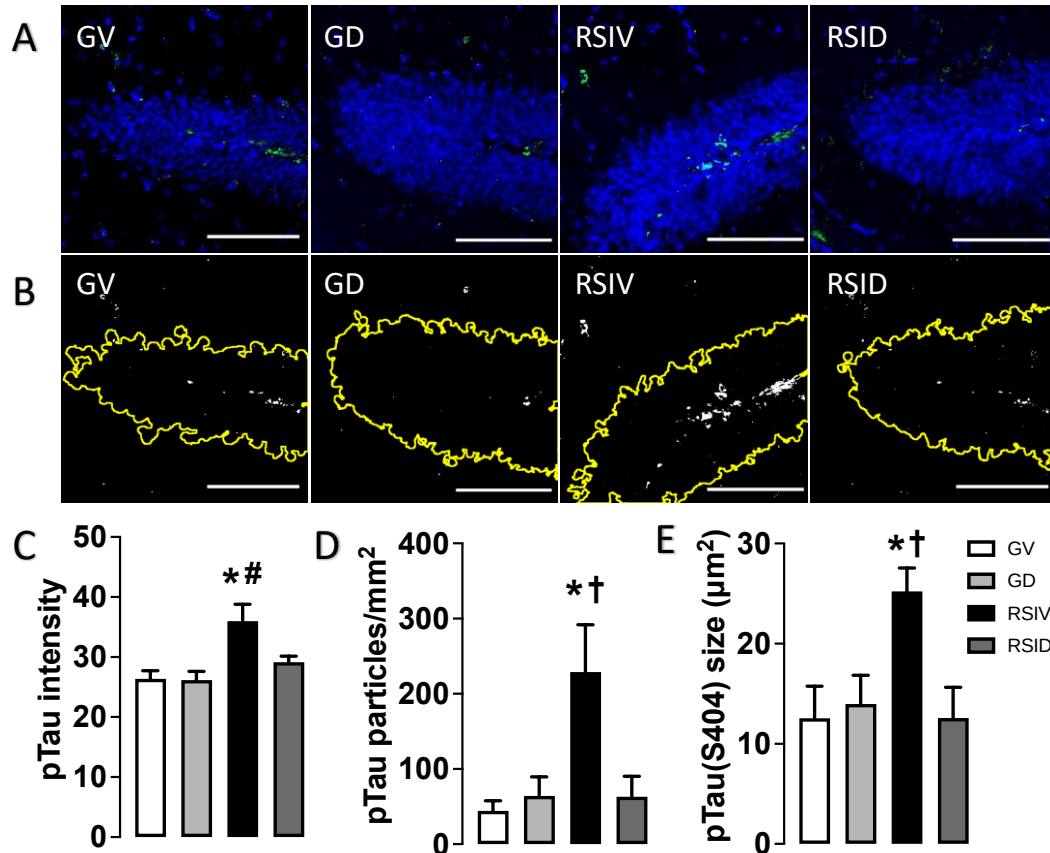


Fig. 5. pTau (Ser404) in dentate gyrus. (A) Representative images of pTau(S404) in the dentate gyrus of mice. Blue=DAPI, nucleus. Green=pTau(S404). Scale bar=100 µm. (B) Binary representation of pTau(S404) particles in the dentate gyrus. Yellow outline indicates the analyzed ROI. (C) Normalized intensity of pTau(S404) fluorescence in the dentate gyrus, represented as mean gray value. (D) Number of pTau(S404) particles per mm². (E) pTau(S404) size in µm². Bars represent mean ± SEM. Two-way ANOVA with Holm-Sidak's multiple comparison. *; $p \leq 0.05$ vs GV. †; $p \leq 0.05$ vs RSID. #; $p \leq 0.10$ vs RSID. GV = group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated SI with vehicle treatment. RSID = repeated SI with DHM treatment.

Repeated social isolation impairs memory, sociability, and weight maintenance, which are improved by DHM administration.

In addition to cellular and molecular studies, we assessed for AD-like behavioral and physical changes in RSI mice, starting with their recognition memory as an overall measure of cognition. Mice are naturally curious creatures that tend to spend more time exploring unfamiliar or novel objects.⁸¹ Utilizing this concept under the novel object recognition (NOR) test, mice under RSI exhibited lower recognition memory (**Fig. 6A**) as they spent less time exploring the novel object ($44.21 \pm 4.210\%$) compared to control ($72.80 \pm 2.394\%$, $p \leq 0.0001$). Two weeks of daily oral DHM treatment improved their memory by roughly 22% ($66.37 \pm 2.572\%$, $p \leq 0.0001$), suggesting that accumulation of social deprivation and loneliness lead to cognitive challenges, while DHM prevents or restores them.

In addition to cognitive impairment, a large fraction of individuals with AD display behavioral and psychological symptoms of dementia (BPSD), also known as neuropsychiatric symptoms (NPS), including agitation, irritability, apathy, aggression, depression, disinhibition, and appetite changes.^{5,82,83} To determine general social behavioral changes after RSI, we conducted a social interaction test, where two stranger mice were allowed to freely interact in an open, neutral, and novel arena.⁸⁴ RSI mice spent considerably less time socially interacting with the other mouse ($5.268 \pm 0.9645\%$, **Fig. 6B**) compared to control ($16.58 \pm 2.130\%$, $p = 0.0038$). DHM treatment increased this ratio in RSI mice by over twofold ($12.45 \pm 1.287\%$, $p = 0.0472$), indicating behavioral changes induced by RSI and the ability of DHM to improve sociability.

As we have reported mitochondrial dysfunction without significant weight change in our previous studies in four-week SI mice,^{47–49} we questioned whether RSI can impact weight change. While

RSI mice did not display changes in appetite as measured by weekly food intake (data not shown), their weight gain was noticeably higher compared to their age-matched control ($10.58 \pm 0.6932\%$, $P=0.0081$, **Fig. 6C**). RSI mice that received DHM treatment exhibited less weight gain ($11.16 \pm 0.4428\%$, $P=0.0355$). These findings demonstrate the phenotypic impact of RSI on metabolism, and the ability of DHM to slow dramatic weight gain even under conditions of SI and loneliness.

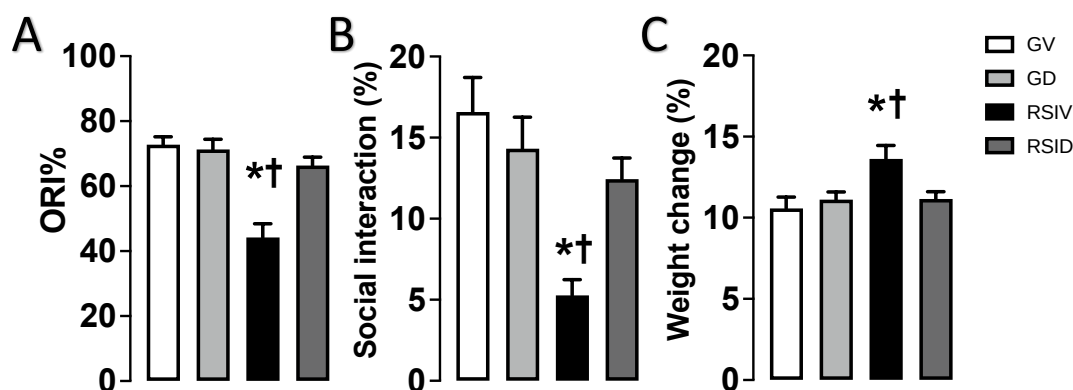


Fig. 6. Cognitive and social changes. (A) Cognition of mice represented as percentage of novel object recognition (ORI%). (B) Fraction of time (%) that pair of mice spent socially interacting with each other. (C) Change in weight (%) between the first and last day of the 10-week experimentation period. Bars represent mean $\pm$ SEM. Two-way ANOVA with Holm-Sidak's multiple comparison *; $p \leq 0.05$ vs GV. †; $p \leq 0.05$ vs RSID. $n=11-12$ /group. GV = group housed with vehicle treatment. GD = group housed with DHM treatment. RSIV = repeated SI with vehicle treatment. RSID = repeated SI with DHM treatment.

Discussion

Our present data demonstrate evidence linking repeated social isolation and loneliness with neurosynaptic and neurometabolic damage that contribute to cognitive and behavioral impairments mirroring AD. After incorporating withdrawal and re-social isolation phases in our SI model, we observed significant pathological and cognitive changes reminiscent of AD, including astrocyte atrophy, GLUT1 reduction, Ca^{2+} imbalance, increased pTau, memory decline, and behavioral abnormalities. These findings are consistent with emerging literature that suggests social isolation as a contributing factor in the pathogenesis of AD.^{85–90} Remarkably, our results also demonstrate that DHM, a non-allosteric modulator of GABA_ARs, counteracted these pathological changes and symptoms. Our data suggest that DHM has a neuroprotective effect, offering a promising therapeutic avenue for preventing or ameliorating the neurodegenerative damages associated with AD through GABAergic modulation.

Astrocytes oversee key functions in the CNS, such as metabolism, blood brain barrier integrity, immune responses, and neurotransmission at synapses.^{62,91,92} Notably, they contribute to neurotransmission and cognitive processes by enwrapping pre- and postsynaptic terminals to form tripartite synapses.⁵⁶ At these tripartite synapses, in addition to structural support, astrocytes modulate neurotransmission through calcium ion (Ca^{2+}) release, neurotransmitter reuptake, gliotransmitter release (such as ATP), and long-term potentiation.^{55,56} Astrocyte processes are highly dynamic in size, motility, and morphology, enabling them to execute these functions and regulate synaptic strength and synaptic elimination.⁹³ These functions, however, are compromised under pathological conditions and CNS insults, where astrocytes undergo reactive astrogliosis.⁶¹ The most commonly utilized marker of reactive astrogliosis is glial fibrillary acidic protein (GFAP), which is usually undetectable in healthy astrocytes, with a few exceptions such as in the

hippocampus.⁹⁴ Reactive astrocytes are not involved in the formation of tripartite synapses and are thought to participate in synaptic elimination, as evidenced by the correlation between synaptic loss and increased numbers of GFAP-positive astrocytes.^{95–98}

Therefore, our findings of increased GFAP-positive astrocytes (**Fig. 1A, E**) indicate that RSI leads to astrogliosis and astrocyte proliferation, resulting in reduced synaptic support and potential synaptic elimination. As synaptic loss is a major correlate of cognitive function and AD,^{99–101} this reduction in synaptic support and density is likely contributing to the cognitive and social behavioral deficits that we observed in this study (**Fig. 6A, B**) along with our previous findings after four-weeks of SI.^{48,51,102} Astrocytes are considered to contribute early to AD along with neuroinflammation and oxidative stress,¹⁰³ both of which we have observed in our previous studies with four-week SI.^{48,50} However, compared to a single episode of four-week SI, astrocytes after RSI exhibited more atrophic morphology, with significantly smaller size and complexity (**Fig. 1B-D, F**). Evidence suggest that astrocytes display degeneration, atrophy, and senescence in the brains of patients with AD as well as in in vivo models.^{103–105} Thus, the difference between single SI and RSI may be attributed to chronic reactive astrogliosis and eventual atrophy, which likely contributes to the observed cognitive deficits and progression of AD.

In addition to the loss of synaptic support, reactive astrocytes have been shown to alter their metabolic phenotype.¹⁰⁶ In the four-week SI model, we observed disrupted metabolism in mice, evident from reduced ATP levels and dysfunctional electron transport chain complexes in the mitochondria.^{47–49} Metabolic changes in the astrocytes after SI have not been investigated until this study. One marker, GLUT1, is consistently implicated in neurodegeneration, including AD,¹⁰⁷ where even small deficits in GLUT1 can deprive cells of nutrients and energy.¹⁰⁸ Under RSI, we found a reduction in GLUT1 expression in the hippocampus (**Fig. 2**), demonstrating a change in

the metabolic profile of astrocytes, with reduced glucose uptake through GLUT1. This reduction likely contributes to mitochondrial impairment,^{48,49} astrocyte atrophy (**Fig. 1**), and metabolic dysfunction, including a more pronounced increase in body weight (**Fig. 6C**). While unintentional weight loss is often associated with AD and linked to poorer disease outcomes,^{109–111} it is essential to acknowledge the emerging recognition of mid-life obesity as a risk factor for AD.^{112–115} Notably, four weeks of SI did not significantly affect the mice's weight gain, indicating a degree of adaptability to a single four-week episode of SI. However, this adaptability may take on a chronic nature when subjected to repeated episodes of SI, ultimately disrupting metabolic homeostasis, and increasing susceptibility to weight gain in mice. Hence, weight gain observed in RSI mice, which were approximately 20 weeks old at the time of sacrifice, may be associated with the increased risk of mid-life obesity and therefore AD.

Disruption of Ca^{2+} homeostasis affects various aspects of neuronal signaling and health, including synaptic plasticity.⁷⁶ Astrocytes themselves modulate a diverse array of Ca^{2+} signals, playing a pivotal role in regulating long-term memory in the hippocampus.¹¹⁶ The maintenance of the high Ca^{2+} gradient heavily relies on ATP-dependent mechanisms, the mitochondria, and the ER, and thus any disruption in this delicate balance can impact copious downstream pathways.^{71,117} Based on our present findings of elevated Ca^{2+} levels and IP3R expression (**Fig. 3**), RSI disrupts Ca^{2+} homeostasis which could lead to a plethora of downstream signaling disruptions. Although these findings do not specify how exactly Ca^{2+} is imbalanced, such as the extra- vs intracellular calcium concentration, it still demonstrates a general change in Ca^{2+} homeostasis, potentially related to astrophic astrocyte and reduced GABAergic transmission.

In the present study, we have identified increased tau phosphorylation (**Fig. 4, 5**), a well-accepted pathological hallmark of AD, in the hippocampus of RSI mice. While hyperphosphorylation at

332 sites T181, T217, and T231 are commonly observed in NFTs of AD,¹¹⁸ phosphorylation patterns
333 at earlier stages or disease onset are not well documented. Few studies have suggested S404
334 hyperphosphorylation to be an early phenomenon of AD. One study found that phosphorylation at
335 sites S396-404 was increased in mild AD cases with early pTau aggregates sustaining a well-
336 preserved neuronal soma.¹¹⁹ An in vitro mutational study with human tau found that
337 phosphorylation at S396 and S404 is primarily responsible for the functional loss of tau-mediated
338 tubulin polymerization, suggesting their role in initiating destabilization of microtubules.¹²⁰ After
339 RSI, we found mice hippocampus to express increased pTau at site S404 (**Fig. 5**), but not in T181,
340 T217, and T231 (**Fig. 4**), supporting the possibility that increased phosphorylation at S404 is an
341 early altered posttranslational modification of tau related to AD onset.

342 The observed tau hyperphosphorylation is likely connected to the other findings in this study.
343 Senescent or atrophic astrocytes are suggested to contribute to tau hyperphosphorylation (NFTs),
344 and glutamate excitotoxicity,¹⁰⁵ the first pathology observed in the present study with RSI, and the
345 latter implicated in decreased GABAergic transmission from our previous studies using four-week
346 SI and AD transgenic models.^{47,53} Moreover, GLUT1 reduction is associated with
347 hyperphosphorylation of tau and increased NFT in human brains,¹²¹ further suggesting that RSI-
348 induced GLUT1 reduction might be associated with the increased pTau(S404). Additionally,
349 disruptions in IP3R expression have been shown to disrupt Ca²⁺ signaling, leading to tau
350 hyperphosphorylation, memory deficits, and A β accumulation.⁷⁸ This disruption in Ca²⁺
351 homeostasis can also result in altered tau phosphorylation patterns along with its ability to bind to
352 microtubules.^{76,117}

353 Our findings hold particular significance in the context of AD, as we uncovered key AD-like
354 changes in our RSI mouse model. DHM mitigated all RSI-induced changes, including astrogliosis,

GLUT1 expression, Ca^{2+} homeostasis, tau hyperphosphorylation, cognitive and social behavioral deficits, and weight change. Aa DHM functions as a positive allosteric modulator (PAM) of GABA_ARs,⁵² our results strongly suggest that GABAergic signaling dysfunction serves as an upstream mechanism driving the SI-to-AD-like pathology. While GABAergic dysfunction is consistently demonstrated in AD,^{122–126} it has received less clinical and research attention than its glutamatergic and cholinergic neurotransmitter counterparts. Restoring GABAergic signaling mitigated SI-induced downstream effects, pointing to GABA_ARs as a potential target for AD prevention or early treatment. Our present study highlights the potential of targeting GABAergic signaling as an avenue for intervention in AD, offering new insights into the mechanisms linking SI-induced changes to AD-like pathology.

In this study, we found RSI to demonstrate robust AD-like changes, and DHM to be effective at restoring these changes. Our present findings further underscore the significance of consistent, long-term social connectedness in maintaining proper cognitive, brain, and metabolic health. Our research contributes to our understanding of AD onset and progression and offers insights into potential interventions for mitigating the impacts of SI, including targeting GABA_ARs. We highlight the cognitive, behavioral, synaptic, and metabolic impact of repeated social isolation; the utility of our novel RSI model as a potential model of natural AD onset/progression; as well as the potential of DHM and GABAergic targets to mitigate RSI-induced impacts. This work sheds light on the significance of societal connectedness in the context of natural AD onset and opens avenues for further research and potential therapeutic development.

Methods

377 *Animals*

378 All animal experiments were performed according to the protocols approved by the University of
379 Southern California (USC) Institutional Animal Care and Use Committee (IACUC), and all
380 methods were carried out in accordance with relevant guidelines, regulations, and
381 recommendations. Six-week-old male and female C57BL/6 mice (Charles River Laboratories,
382 Hollister, CA) were housed in the vivarium under a 12 h light/dark cycle with direct bedding and
383 free access to food and water. C57BL/6 mice are one of the most commonly utilized mice strains
384 that have been shown to increase tau phosphorylation along with worsened cognition when crossed
385 with a common mouse model of tauopathy (rTg4510) and after seizure induction.^{127,128} Animals
386 were habituated to the vivarium for 2 d before beginning experimentation and randomly allocated
387 to housing and treatment conditions. Three mice were allocated in each cage for group housing.

388 For the RSI groups, each mouse was singly housed in opaque cages and minimally handled. Mice
389 were experimentally housed for a total of ten weeks and weighed weekly. During the last two
390 weeks, mice were orally administered either sucrose (vehicle) or DHM (2 mg/kg) via agars daily.
391 Euthanization and tissue collections were conducted at the University of Southern California
392 (USC) under approved protocols.

393 Groups were randomly separated as follows for a total of 10 weeks prior to sacrifice:

- 394 1. GV: 10-week group housing with vehicle treatment
- 395 2. GD: 10-week group housing with DHM treatment
- 396 3. RSIV: 4-week SI, 2-week SI withdrawal, and 4-week re-SI with vehicle treatment
- 397 4. RSID: 4-week SI, 2-week SI withdrawal, and 4-week re-SI with DHM treatment

Percent weight change was calculated as $(m_i - m_f)/(m_i + m_f) \times 100$, where m_i and m_f represent initial and final weight of the 10-week experimentation period.

Treatment preparation

DHM (HPLC purified $\geq 98\%$, Master Herbs Inc., Pomona, CA) 2 mg/kg was prepared in 3% agar cubes with 5% sucrose as described previously^{48–51,102}. In short, 3% agar was dissolved in $\sim 90^\circ\text{C}$ water, then mixed with 5% sucrose or DHM + 5% sucrose until cooled and solidified. Agar was cut into cubes of 0.5 x 0.5 x 0.5 cm and one cube per mouse was administered as treatment. Both vehicle and DHM cubes were administered orally once a day for the last two weeks during the dark period of the 12-hour light/dark cycle with minimal disturbance to the mice. Complete consumption of the respective treatment was observed each day.

Novel Object Recognition (NOR)

We conducted the NOR test according to previous reports⁸¹. In short, this task is based on the tendency of rodents to explore a novel object for a longer period of time compared to a familiar one. On day one, each mouse was familiarized with the empty open field (40 cm L $\times$ 40 cm W $\times$ 35 cm H) for 5 mins. On day two, they were subjected to a 5-min exploration session of two identical, symmetrically placed objects. 24 h later, they were subjected to a 3-min retention session, where they were allowed to explore one familiar object from day two and one novel object. Exploration time for each object (familiar and novel) was recorded, and an object recognition index (ORI%) was calculated, such that $\text{ORI}\% = (t_n - t_f)/(t_n + t_f) \times 100$, where t_f and t_n represent times of exploring the familiar and novel objects, respectively.

Social interaction test

The social interaction test⁸⁴ was performed in an empty, neutral, and novel rectangular open arena (40 cm L × 35 cm W × 35 cm H). Two unfamiliar mice were placed in this arena and recorded for 5 minutes or until the first sign of aggression, whichever came first. Total time spent on social interaction was recorded by a blind scorer and divided by total time in arena to calculate percent of time spent on social interactions. Social interaction included sniffing (nose-to-nose or anogenital), crawling over or under each other, and allogrooming. Each mouse was tested twice, sequence randomized: once with a mouse from the same treatment group, and another with a mouse from the control group. The percentage of social interaction from both tests was averaged for each mouse. Pairing was randomized after controlling for sex and treatment group.

Brain preparation

Mice were euthanized and dissected the day following the last day of the 10-week experiments and behavioral testing. Hippocampus was dissected and homogenized with Branson Sonifier 150 in cold 1X PBS for Ca²⁺ assays and RIPA buffer (Thermo) plus 1% protease and 1% phosphatase inhibitors for Western blots. Homogenates were spun and supernatants collected for downstream assays.

Ca²⁺ colorimetric assay

Total calcium concentration in the hippocampus was determined by colorimetry (MAK022, Sigma) according to the manufacturer's instructions. 50 µL of hippocampus supernatant and standards were measured for absorbance of the chromogenic complex formed between Ca²⁺ and o-cresolphthalein at 575 nm. Concentration was calculated as mM (mol Ca²⁺ per L).

Western blot

Total protein concentration was quantified via BCA protein assay kit (Pierce) according to the manufacturer's instructions. 20 µg of lysates were run on a 10% pre-cast SDS-polyacrylamide gel (Bio-Rad) with Mini-PROTEAN (Bio-Rad) under constant voltage. Proteins were transferred onto a 0.2 µm PVDF Trans-Blot Turbo membrane (Bio-Rad) using Trans-Blot Turbo (Bio-Rad). Membranes were washed and blocked in 3% bovine serum albumin (BSA, Sigma) in 1X TBS + 0.1% (v/v) Tween 20 (TBST) blocking buffer for 1 h at room temperature, followed by overnight 4°C incubation in primary antibodies diluted 1:1000 in blocking buffer with gentle rocking. After washing, membranes were incubated in HRP-conjugated secondary antibodies, diluted 1:3000 in blocking buffer, at room temperature for 1 h with gentle rocking. Band signal intensities were detected using Pierce ECL Western Blot substrate and iBright FL1000 Imager (Thermo), quantified with ImageJ Gel Plugin, and normalized to intensity values of β-actin on the same blot. The following primary antibodies were used: rabbit anti-mouse pTau(Ser404) (Cell Signaling #35834), rabbit anti-mouse pTau(Thr181) (Cell Signaling, #12885), rabbit anti-mouse pTau(Thr217) (Cell Signaling, #51625), rabbit anti-mouse pTau(Thr231) (Cell Signaling #71429), and mouse anti-mouse β-actin (Cell Signaling #3700). The following secondary antibodies were used: goat anti-rabbit IgG (H+L)-HRP conjugate (Bio-Rad #1706515) and goat anti-mouse IgG (H+L)-HRP conjugate (Bio-Rad #1706516).

Immunohistochemistry fluorescent staining, imaging, and analysis

Mice were euthanized and dissected the day following the end day of the 10-week experiments and behavioral testing. Left brains were collected and fixed in 10% formaldehyde (FA, Sigma-Aldrich, USA) overnight at 4°C. Brain tissues were then rinsed with 1X TBS (Sigma) and transferred to 30% sucrose solution (Sigma) in a 15 mL conical tube at 4°C for 3 days until the tissues sank to the bottom. Brains were washed in cold TBS and flash frozen in isopentane chilled

with dry ice. Samples were immediately stored at -80°C. Prior to cryosectioning, frozen brains were embedded in a mold with O.C.T. compound (Sakura, USA) on dry ice. 20 µm sagittal brain slices were sectioned at -20°C with Microm HM525 Cryostat (Thermo) and transferred to SuperFrost microscope slides (VWR). Slides were stored in -80°C until staining.

Antibodies were diluted in 1X TBS-Tween20 (TBS-T) + 10% BSA (w/v). Slides were incubated in a humidifier in respective antibodies overnight at 4°C, with conjugated antibody incubations protected from light. Stained slides were mounted with DAPI mounting medium (Abcam ab104139), cover slipped, and stored in 4°C in dark until imaging. The following antibodies were used: rabbit anti-mouse IP3 receptor (Thermo #PA1-901), mouse anti-mouse GFAP Alexa Fluor 555 (Cell Signaling #3656), rabbit anti-mouse GLUT1 (Cell Signaling #73015), rabbit anti-mouse pTau(Ser404) (Cell Signaling #20194), goat anti-rabbit IgG (H+L) DyLight 550 (Thermo #84540), and goat anti-rabbit IgG (H+L) DyLight 488 (Thermo #A-11008).

Stained sections were scanned for high-resolution images with 20X objective lens using Cytation 5 cell imaging multi-mode reader (BioTek) or 63X objective lens in Airyscan super-resolution mode using LSM880 confocal microscope (Zeiss). For all analysis of stained sections, the region of interest was defined by the DAPI staining. Intensity was measured by calculating the mean gray value of the ROI, then subtracting mean gray value of background outside of the ROI. Astrocyte density was counted manually using the Cell Counter command on ImageJ. Density was normalized to the analyzed DG area and calculated as the number of astrocytes per mm². Astrocyte complexity was analyzed by Sholl analysis (FIJI plugin) using default settings. Three astrocytes were randomly selected and analyzed per sample. Image analysis was performed in a blinded manner.

485 *Quantification and statistical analysis*

486 All statistical analyses were performed using Prism v9.0.2. (GraphPad Software). Statistical details
487 of the experiments can be found in the results and figures. Significance was defined as $P \leq 0.05$.

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491 **Author Contributions**

492 S.W. designed and performed experiments, analyzed data, generated figures, and wrote the
493 manuscript.

494 J.N. performed experiments, analyzed data, and edited the manuscript.

495 J.C. performed experiments, analyzed data, and edited the manuscript.

496 A.G. performed experiments, analyzed data, and edited the manuscript.

497 X.M.S. performed statistical analysis and wrote the manuscript.

498 J.L. established the animal model, designed experiments, wrote the manuscript, obtained funding,
499 and supervised the project.

500 **Competing Interests**

501 The authors declare no financial interests or conflicts of interest.

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