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# Mind the Gap: Estrogen receptor beta (ERβ) in astrocytes is a therapeutic target to prevent cognitive problems at menopause.

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# Abstract

Aging is a risk factor for cognitive decline and susceptibility to neurodegenerative diseases. Some aspects of aging, like the loss of sex hormones, may be preventable. Menopause is associated with cognitive deficits and brain atrophy. Since standard hormone replacement therapy (HRT) does not mitigate these brain aging outcomes, a gap in knowledge involves understanding brain region-specific, cell-specific, and receptor-specific mechanisms underlying this neurodegeneration. Here, cognitive testing and in vivo magnetic resonance imaging demonstrated that ovarian hormones in female mice at midlife protect against hippocampal-dependent cognitive impairment and dorsal hippocampal atrophy. Further, this neuroprotection in females at midlife is lost in mice with selective deletion of estrogen receptor beta (ERβ) in astrocytes, but not neurons. This preclinical evidence identifies ERβ in astrocytes as a novel therapeutic target to prevent hippocampal-dependent cognitive deficits and reduce posterior hippocampus atrophy in menopausal women, a major unmet need in half the population.


# Introduction

As advances in healthcare allow for longer lifespans, cognitive decline associated with brain aging has become as a major concern. Brain aging is associated with brain atrophy and neurodegeneration. It is also a risk factor for susceptibility and progression in many neurodegenerative diseases[1,2]. A better understanding of the effect of brain aging during health can provide insights into the effect of brain aging during disease.

While some factors of aging may not be reversible, others may be, such as the loss of endogenous sex hormones with aging. Menopause and andropause are each associated with cognitive decline in healthy people and are thought to worsen disabilities in Alzheimer's disease (AD) and multiple sclerosis (MS)[3–5]. Since testosterone is converted to estrogen in brain by aromatase, a decline in either sex hormone would result in less ligation of estrogen receptors in brain. This is relevant to neurodegeneration during aging since estrogens have neuroprotective properties when optimized for estrogen type and dose as well as timing of administration and age of recipients[6–9].

The study of sex differences is a way to capitalize on a known clinical observation, mechanistically disentangle it at the laboratory bench, and translate findings back to the clinic as a treatment trial[10]. The study of sex as a biologic variable has been recognized by the National Institutes of Health as a way to discover treatments optimally tailored for women and men[11,12]. Menopause in healthy women involves cognitive difficulties, termed "brain fog", that affect hippocampal-dependent verbal memory, attention, and working memory as quantified using objective cognitive testing[13,14]. Interestingly, healthy women have worse brain gray matter atrophy after 65 years[15,16], while men appear to have worse gray matter atrophy prior to 65 years of age[17–19]. AD is more common in women, which is not accounted for by greater longevity[20]. However, men may be at greater risk for Mild Cognitive Impairment (MCI) at younger ages[21]. The rate of progression from MCI to AD appears higher in women at older ages, but is higher in

men at younger ages[20]. In MS, older women have worsening of disabilities and gray matter atrophy after menopause[22–24], but gray matter atrophy and cognitive deficits are worse in MS men from young adulthood to midlife[25–27]. Overall, a pattern of sex differences in neurodegeneration seems to be emerging. Men appear to be more susceptible to neurodegeneration before midlife, while females start becoming more susceptible after midlife. We hypothesize that sex differences in the effect of brain aging during health and disease may reflect differential timing of menopause versus andropause. Andropause in men starts at age 30 years with very gradual decline of testosterone to age 75. In contrast, sex hormones are maintained longer in women, until menopause at around age 50 when there is an abrupt loss in estradiol and progesterone. By ages older than 75 years, there is near complete loss of neuroprotective sex hormones in both sexes. Since the decrease in testosterone with andropause is very gradual and the loss of estrogen at menopause is relatively abrupt, neurodegenerative mechanisms in menopause are highly amenable to study and target therapeutically. That said, merely repurposing standard hormone replacement therapy (HRT) for hot flashes to determine if this can mitigate menopause induced cognitive deficits and brain atrophy has yielded disappointing results[8,28]. A treatment specifically designed and optimized to target neurodegenerative mechanisms in the brain in menopausal women remains a major unmet need in half the population.

A new approach is needed to fill the gap in knowledge regarding mechanisms underlying cognitive deficits in otherwise healthy menopausal women. We propose that a brain region-specific, cell-specific, and receptor-specific approach is required to identify optimal therapeutic targets. A region-specific approach is warranted since in healthy brain, there are known differences in gene expression from one brain region to another within astrocytes[29,30], microglia[31], neurons[32], and oligodendrocytes[33]. A sex-specific approach is needed since sex differences in healthy brain structure and function are well established and exist across species[34,35], indicating a biologic effect of either sex hormones or sex chromosomes[36]. Finally, a receptor-specific approach is required since effects of estrogen receptors (ERα and ERβ) can be either synergistic or antagonistic depending on cell type[37,38].

Here, estrogen receptor beta (ERβ) in astrocytes, but not neurons, is shown to mediate protection from hippocampal-dependent cognitive decline and dorsal hippocampal atrophy by *in vivo* magnetic resonance imaging (MRI) in female mice at midlife. These preclinical data identify a candidate therapeutic target to prevent aging-related cognitive decline and its associated region-specific brain atrophy in menopausal women.

# Results

### Sex differences occur in brain substructure atrophy during aging with females showing relative protection at midlife followed by abrupt volume loss thereafter

First, we assessed how aging affects spatial reference memory using Morris Water Maze (MWM) testing at young (3-4 months), midlife (12-14 months), and old (20-22 months) ages in females and male C57Bl/6 mice. There was no significant impairment of spatial memory in either females or males with

aging (Fig. 1a, b, c, d). Then, we determined if a biomarker of brain aging, namely substructure atrophy, might be more sensitive in detecting neurodegeneration. We collected *in vivo* MR images in both female and male mice at the same three ages: young, midlife, and old and used atlas-based morphometry to analyze substructure volumes (Fig. 1e). Both females and males showed significant atrophy at old age as compared to young in frontal cortex and striatum (Fig. 1f, g). However, at midlife there was a sex difference. Females had relative protection as compared to males. The trajectory in males was gradual atrophy from young to midlife to old ages. In contrast, females showed no significant atrophy from young to midlife, but thereafter had an abrupt drop in substructure volumes from midlife to old age. Furthermore, the dorsal hippocampus is primarily involved in cognition and memory and is analogous to the posterior hippocampus in humans which is known to atrophy with age[39,40]. In whole hippocampus, as well as dorsal hippocampus, female mice had no atrophy from young to midlife, but had significant atrophy at old age (Fig. 1h). Two-way ANOVA indicated a significant interaction between sex and age in dorsal hippocampus ($p = 0.0059$). This was region-specific in that neither females nor males showed atrophy in ventral hippocampus with age (not shown). Thus, females were relatively protected against aging associated region-specific atrophy at midlife, but thereafter the trajectory of atrophy was striking.

## Loss of endogenous ovarian hormones induces hippocampal-dependent cognitive impairment and dorsal hippocampus atrophy at midlife in females

We next determined the effect of loss of endogenous female and male hormones on cognitive behavioral testing and regional brain atrophy by *in vivo* MRI in females and males at midlife. Gonadectomy (GDX) versus sham surgery occurred at two months of age with cognitive testing in young and midlife mice. This revealed another sex difference. GDX females showed impairment on the MWM test of spatial reference memory at midlife, while GDX males performed well at midlife (Fig. 2a, b). An interaction between loss of ovarian hormones and aging in females was then discovered. GDX females at midlife had impairment, but GDX females that were young performed well (Fig. 2c, d). Thus, spatial memory impairment was due to both loss of ovarian hormones and aging.

To further investigate hippocampal-dependent cognitive impairment induced by loss of endogenous ovarian hormones in females at midlife, we performed Y-maze testing to assess working memory. There was a significant decrease in percent spontaneous alteration in GDX females at midlife compared to gonadally intact (sham) at midlife, and there was also a decrease in GDX females at midlife compared to GDX females that were young (Fig 2e). This result extended the observation of cognitive impairment in GDX females at midlife from spatial reference memory to working memory. Lastly, we addressed contextual fear conditioning. In concordance with the MWM and Y-maze tasks, GDX females at midlife showed a significant decrease in percent total freezing time 24 hours after conditioning compared to gonadally intact females at midlife (Fig 2f). These three hippocampal-dependent behavioral tasks demonstrated that loss of endogenous ovarian hormones in females induced cognitive impairment at midlife.

We next determined the effect of removal of endogenous ovarian hormones in females on region-specific brain atrophy at midlife, focusing on the hippocampus and its substructures. *In vivo* MR images from GDX and sham female mice were collected at midlife and old age. In gonadally intact female mice at old age, there was atrophy in dorsal hippocampus, with a trend in whole hippocampus, and no atrophy in ventral hippocampus (Fig 2g, h, i). At midlife, GDX females compared to gonadally intact demonstrated atrophy of dorsal hippocampus, with a trend in whole hippocampus, and no atrophy in ventral hippocampus (Fig. 2g, h, i). Together this revealed region-specific neuroprotective effects, whereby endogenous ovarian hormones in females conferred neuroprotection against atrophy of the dorsal hippocampus at midlife.

## Dorsal hippocampal atrophy as measured by *in vivo* MRI in aging mice is a biomarker for hippocampal-dependent cognitive impairment

To determine the relationship between hippocampal atrophy and spatial reference memory, we correlated hippocampal volumes with time spent in the target quadrant (TQ) on the MWM. We observed that there was a direct correlation between whole hippocampal volume and time spent in TQ ($r = 0.22$, $p = 0.049$). Smaller volumes (worse atrophy) in the hippocampus were associated with worse spatial reference memory performance. Correlations using hippocampal substructures demonstrated that this effect in whole hippocampus was driven by a direct correlation between dorsal hippocampus volume and time spent in TQ (Fig. 2j), with no correlation between ventral hippocampus volume and time spent in TQ (Fig. 2). This demonstrated that dorsal hippocampal atrophy as measured by *in vivo* MRI in aging mice is a sensitive biomarker for hippocampal-dependent cognitive impairment. This correlation with function underscored the importance of a region-specific approach in evaluating brain atrophy during aging.

## Dorsal hippocampal neuropathology

Given that atrophy of dorsal hippocampus was induced by GDX at midlife in females, we next assessed the effect of GDX on neuropathology within dorsal hippocampus. Reactive astrogliosis was determined using GFAP expression. There was an increase in $GFAP^+$ astrocytes in GDX females at midlife compared to gonadally intact (sham) at midlife. There was also an increase in GDX females at midlife compared to GDX females that were young (Fig. 3a, b). Next, microglia activation was assessed by colocalization of Iba1 and MHCII. There was a trend for an increase in microglia activation in GDX females at midlife compared to gonadally intact, and there was a significant increase in GDX females at midlife compared to GDX females that were young (Fig. 3c). Lastly, synaptic loss was assessed using the post-synaptic marker PSD95. GDX females at midlife showed significant synaptic loss as compared to GDX females that were young. Gonadally intact females at midlife as compared to gonadally intact females that were young also showed some synaptic loss, but to a milder degree (Fig. 3d).

We next examined the relationship between dorsal hippocampus neuropathologies. Astrogliosis and microglia activation showed a direct correlation ($r = 0.577$, $p = 0.0020$) with each other. There was an indirect correlation of $PSD95^+$ area fraction with $GFAP^+$ area fraction ($r = -0.4374$, $p = 0.0199$) and with microglia activation ($r = -0.7772$, $p < 0.0001$). Thus, synaptic loss correlated with increased astrogliosis and microglia activation. To address functional relevance, a cross-modality correlation analysis determined the relationship between dorsal hippocampus neuropathology and performance on the MWM test. Interestingly, there was a direct correlation between $PSD95^+$ area fraction and time spent in the TQ (Fig. 3e), and conversely an indirect correlation between $PSD95^+$ area fraction and time spent in the other three quadrants (Fig 3f). Finally, regarding the relationship between glial pathology and function, there was an inverse correlation between time spent in the TQ and astrogliosis ($r = -0.425$, $p = 0.024$) and microglia activation ($r = -0.4838$, $p = 0.0166$). Thus, glial activation and synaptic loss in dorsal hippocampus correlated with worse spatial reference memory function.

Together, this demonstrates that loss of endogenous ovarian hormones in females induces glia activation and synaptic loss in the dorsal hippocampus, atrophy of dorsal hippocampus by *in vivo* MRI, and hippocampal-dependent cognitive impairment at midlife, with significant relationships between each.

**Neuroprotection in females at midlife is mediated by ERb in astrocytes.**

Next, we investigated a region-specific, cell-specific, and receptor-specific mechanism through which endogenous estrogens could be mediating neuroprotection against hippocampal-dependent cognitive impairment and dorsal hippocampal atrophy at midlife. Hippocampal astrocytes play a role in memory formation and regulation of synaptic transmission, and ERβ is expressed in both neurons and astrocytes in hippocampus[29,30,41,42]. Focus on ERβ was supported by translational potential since a treatment targeting ERβ activation would not confer ERα mediated adverse effects on breast. Thus, we determined whether ERβ signaling in either neurons or astrocytes could mediate the effects of endogenous estrogens on hippocampal-dependent cognition and dorsal hippocampal atrophy. To test this, we created estrogen receptor b (ERb) conditional knock-outs in astrocytes (astrocyte ERb CKO) or neurons (neuron ERb CKO). At midlife, gonadally intact mice with selective deletion of either ERb in astrocytes or neurons, as well as wild type (WT) littermates, underwent cognitive behavioral testing and *in vivo* MRI. In the MWM task, mice with deletion of ERβ in astrocytes showed a significant reduction of percent time in the TQ, compared to WT littermates (Fig. 4a). In contrast, deletion of ERβ in neurons showed no impairment of spatial reference memory (Fig. 4a). Indeed, both WT and neuron ERb CKO females at midlife showed highly significant preference for the TQ, compared to other quadrants (Fig. 4b), while astrocyte ERβ CKO females did not show this preference for the TQ (Fig 4b). Regarding contextual fear conditioning, astrocyte ERβ CKO females at midlife showed a significant decrease in percent total freezing time at 24-hours, compared to WT littermates (Fig. 4c). In contrast, mice with deletion of ERβ in neurons showed no impairment of contextual fear conditioning as compared to WT littermates (Fig. 4c). Notably, females at

midlife with selective deletion of ERα in astrocytes had no impairment of contextual fear conditioning (Supplemental Fig. 1).

We next determined whether a protective effect of endogenous estrogens on hippocampal atrophy in females at midlife could be mediated through ERb in astrocytes or neurons. Interestingly, astrocyte ERb CKO mice showed atrophy in the hippocampus compared to WT littermates, while neuron ERb CKO mice did not (Fig. 4d). When we examined hippocampal substructure volumes, we found that atrophy in the dorsal hippocampus was primarily driving the effect in whole hippocampus. The dorsal hippocampus was significantly smaller in astrocyte ERb CKO compared to WT mice, while there was no difference between neuron ERb CKO and WT mice (Fig. 4e). In ventral hippocampus, astrocyte ERb CKO had a slightly smaller volume compared to WT, but there was no difference between astrocyte ERb CKO and neuron ERb CKO (Fig. 4f).

Since the Cre-system we utilized entailed selective deletion of ERβ in astrocytes or neurons throughout development and adulthood, we next addressed whether cognitive impairment or hippocampal atrophy had been induced during development. This was not the case. Young (age 3-4 month) female astrocyte ERβ CKO, as compared to WT littermates, had no deficits in spatial reference memory (Supplemental Fig. 2a, b) or 24-hour contextual fear memory (Supplemental Fig. 2c). Further, there was no hippocampal atrophy in young astrocyte ERβ CKO as compared to young WT littermates (Supplemental Fig. 2d, e, f).

**Selective deletion of ERβ in astrocytes, but not neurons, induced impairment in remote memory.**

Recent memory formation is hippocampal-dependent. Persistence of memory undergoes a transition from hippocampal-dependence for recent memories to hippocampal-independence for remote memories, through system memory consolidation. Medial prefrontal cortex supports remote memories, initially formed in hippocampus, by retrieving memory at remote time-points (28 days later)[43,44]. Thus, we next determined if loss of endogenous ovarian hormones affects remote memory. Remote contextual fear memory was evaluated in GDX versus gonadally intact (sham) females at midlife using the 1-month contextual fear memory test. GDX females at midlife showed a trend for reduction of percent total freezing time at 1-month compared to sham females (Supplemental Fig. 3a). Further, gonadally intact astrocyte ERβ CKO females showed a significant decrease in percent total freezing time at 1-month compared to WT littermate females (Supplemental Fig. 3b), while neuron ERβ CKO females did not. Together, these findings demonstrate an essential role for ERβ in astrocytes in females at midlife on recent and remote memory.

# Discussion

Here, we found a sex difference in the trajectory of regional brain atrophy by *in vivo* MRI in female versus male mice from young, to midlife, to old ages, even in the absence of cognitive deficits. Females had relative protection from substructure atrophy at midlife, followed by an abrupt decline, while males had

gradual atrophy across the lifespan. Gonadectomy also revealed a sex difference. While gonadectomy had no effect on cognitive behavioral testing in males at midlife, it induced hippocampal-dependent memory impairment in females. Loss of endogenous ovarian hormones in gonadectomized female mice also induced dorsal hippocampal atrophy at midlife, but not at young ages, revealing a hormone by age interaction. Even though previous research clearly demonstrated beneficial effects of estrogens on cognitive function and hippocampal synaptic pathology[45], which cell and receptor within hippocampus is essential for *in vivo* neuroprotection during midlife in otherwise healthy female mice has remained unclear. Here, we used gonadally intact female mice with selective deletion of ERβ in either astrocytes or neurons to determine if ERβ in either of these cells is essential for neuroprotection at midlife. Deletion of ERβ in astrocytes, but not neurons, induced hippocampal-dependent memory impairment and dorsal hippocampal atrophy at midlife, but not at young ages. This region-specific, cell-specific, and receptor-specific *in vivo* approach identifies ERβ in astrocytes as a candidate therapeutic target to prevent age related cognitive deficits and associated regional brain atrophy in females at midlife.

While selective deletion of ERβ in astrocytes was identified as a cell-specific and receptor-specific therapeutic target, additional targets are possible since finding one target is not mutually exclusive of another. For example, ERβ ligand treatment mediated neuroprotection in the MS model was shown to involve a critical role of ERβ in $CD11c^+$ cells of the microglia/macrophage lineage that was not mutually exclusive of an additional role of ERβ in $Olig1^+$ cells of oligodendrocyte lineage[46,47]. Two mechanistic pathways can be required for neuroprotection, and selective interruption of either may abrogate neuroprotection. Here, neuropathology of dorsal hippocampus during midlife in gonadally intact versus GDX females demonstrated that removal of endogenous estrogens induced astrogliosis, microglial activation, and synaptic loss, which was associated with cognitive impairment. Astrocytes can play a role in synaptic loss and function through either direct effects on the synapse or through indirect effects on complement mediated microglial activation and synaptic stripping[29,48–50]. Showing that interruption of a neuroprotective pathway in astrocytes is essential does not rule out an additional neuroprotective pathway in microglia. Thus, future studies using selective deletion of ERβ in microglia to determine the effect on hippocampal-dependent cognitive impairment and dorsal hippocampal atrophy at midlife in females should be pursued.

Thinking ahead about translation of this region-specific, cell-specific, and receptor-specific approach to future clinical trials, a look back at past clinical experience using a new lens, one focused on specificity, may provide insights. Cognitive problems during menopause have been known for decades. However, repurposing standard HRT using conjugated equine estrogens (such as Premarin), with or without progesterone, at doses to treat hot flashes and other menopausal symptoms has not provided neuroprotection[8,28]. The “timing hypothesis” proposed that estrogen treatment might be effective if started at early menopausal ages, but not if started after 65 years of age. Still, cognition was neither improved nor worsened by treatment with low dose oral estradiol (1 mg) compared to placebo in women within 6 to 10 years of menopause[8]. Thus, while appropriate timing is important, attention to only this is insufficient to achieve neuroprotection. Indeed, no specific estrogen/progesterone regimen has been FDA

approved to prevent cognitive deficits in women with menopause[28]. That said, can any positive signals be gleaned from past clinical trials? Perhaps, if one considers regional effects. While treatment with estradiol patch versus oral Premarin in early menopausal women aged 42–56 years showed no benefit of Premarin, estradiol treatment decreased regional gray matter atrophy in the dorsolateral prefrontal cortex and there was lower Pittsburgh compound B uptake in cortex during positron emission tomography (PET) imaging, albeit with no effect on whole brain volumes or global cognition[6,7]. An estrogen-specific and timing-specific influence on structure and function of prefrontal cortex has also been shown by a promising effect of treatment with ethinyl estradiol, but not Premarin, if administered during early, but not late, menopause[14,51]. Lastly, cognitive complaints in menopausal women age 50 to 60 years have been correlated with lower gray matter volume in the right medial temporal lobe[52]. Treatment of postmenopausal women of average age 58 years (range 51–75) with estradiol at 2mg, but not at 1mg or placebo, showed promise in reducing region-specific volume loss in posterior, but not whole, hippocampus[53], aligning with our preclinical findings.

Are there any clinical insights from experience with estrogen treatment in other neurodegenerative diseases? A double-blinded, placebo-controlled, Phase 2 trial using treatment with the pregnancy hormone estriol 8 mg versus placebo, each in combination with MS standard-of-care glatiramer acetate, was done in women with MS ages 18–50 years[54]. Interestingly, estriol binds preferentially to ERβ over ERα[55,56]. An improvement in estriol treated compared to placebo treated subjects was observed for performance on the Paced Auditory Serial Addition Test (PASAT), a measure of processing speed including attention and working memory. Also, higher estriol blood levels correlated with more improvement in PASAT scores[54,57]. Further, there was a correlation between improvement in PASAT testing and sparing of cortical gray matter atrophy[54]. Voxel-based morphometry mapped estriol-mediated regional gray matter sparing to the medial frontal cortex, a region known to be involved in problem solving, attention, and arithmetic tasks, each with relevance to PASAT performance[58]. Another Phase 2 trial in MS women age 18–45 years repurposed oral contraceptives containing estradiol to determine effects on MS outcomes. Ethinyl estradiol at a dose of 40 mcg, 20 mcg, or placebo were each taken in combination with MS standard-of-care Interferon-beta[59]. The 40 mcg dose, but not the 20 mcg dose, resulted in fewer patients with cognitive impairment as measured by Rao's Brief Repeatable Battery. Since high doses of estradiol treatment carry significant risk when given for long durations in menopausal age women, treatments that bind predominantly ERβ, such as estriol or synthetic ERβ ligands, are needed to maximize dose and efficacy while minimizing toxicity conferred by ERα.

Future clinical trials targeting ERβ in astrocytes are warranted in early menopausal women using region-specific, cell-specific, and receptor-specific targeting. These would be neuroprotective treatment trials for a duration of one to two years, aiming to reduce regional brain atrophy and its neuropathology, not short term symptomatic treatment for weeks to months. Novel treatment development would require classic pharmacologic dose optimization strategies using ligands of ERβ or stimulation of downstream pathways activated by ERβ ligation in astrocytes, not repurposing of standard HRT regimens used for non-cognitive menopausal symptoms. A Phase 2 trial targeting ERβ in astrocytes should focus on early

menopausal women with cognitive complaints and have main clinical outcomes of improvement in objective tests of verbal memory, attention, and working memory (not global cognition) and biomarkers of preservation of posterior hippocampus and prefrontal cortex structure and function (not whole brain measures).

In conclusion, a future region-specific, cell-specific, and receptor-specific targeting strategy can build upon past insights regarding the importance of estrogen type, dose, and timing to begin to fill the gap in treatments for cognitive problems in menopausal women, an unmet need in half the population.

# Online Methods

See *Supplemental Materials*.

# Declarations


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### Data availability statement

The datasets generated during the current study are available from the corresponding author on reasonable request.

### Code availability statement

No custom codes or algorithms were used to generate results.

### Declaration of Conflicting Interests

The authors had full access to all of the data in this study and take complete responsibility for the integrity of the data and the accuracy of the data analysis. Dr. Rhonda Voskuhl is an inventor on UCLA patents pertaining to estriol and ERB ligand treatments.

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# Figures

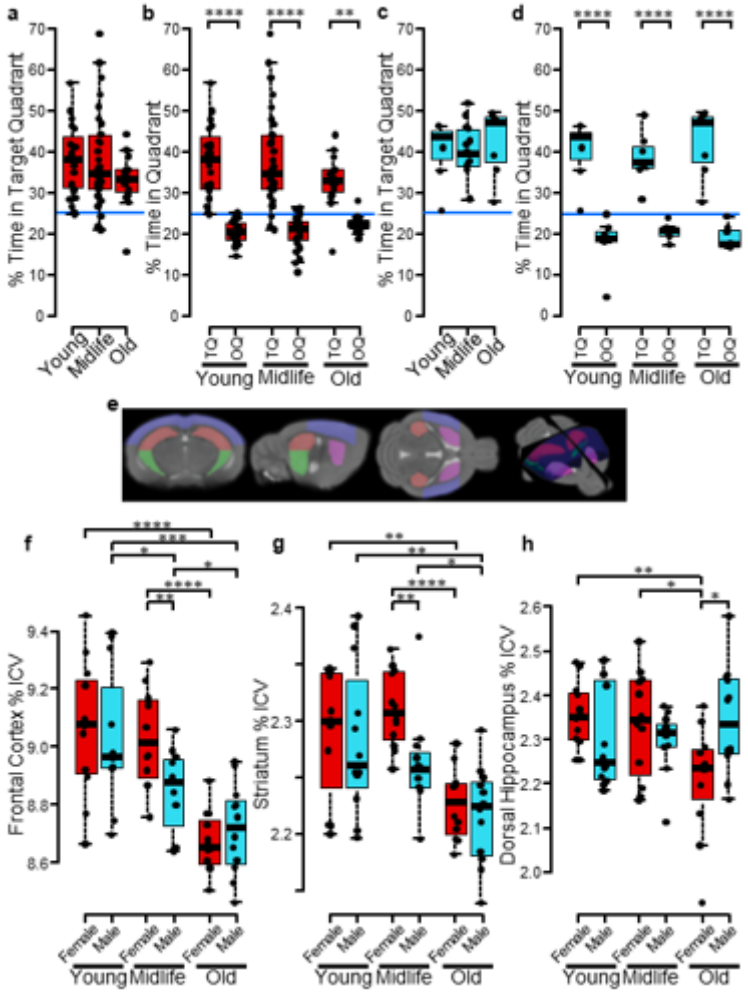
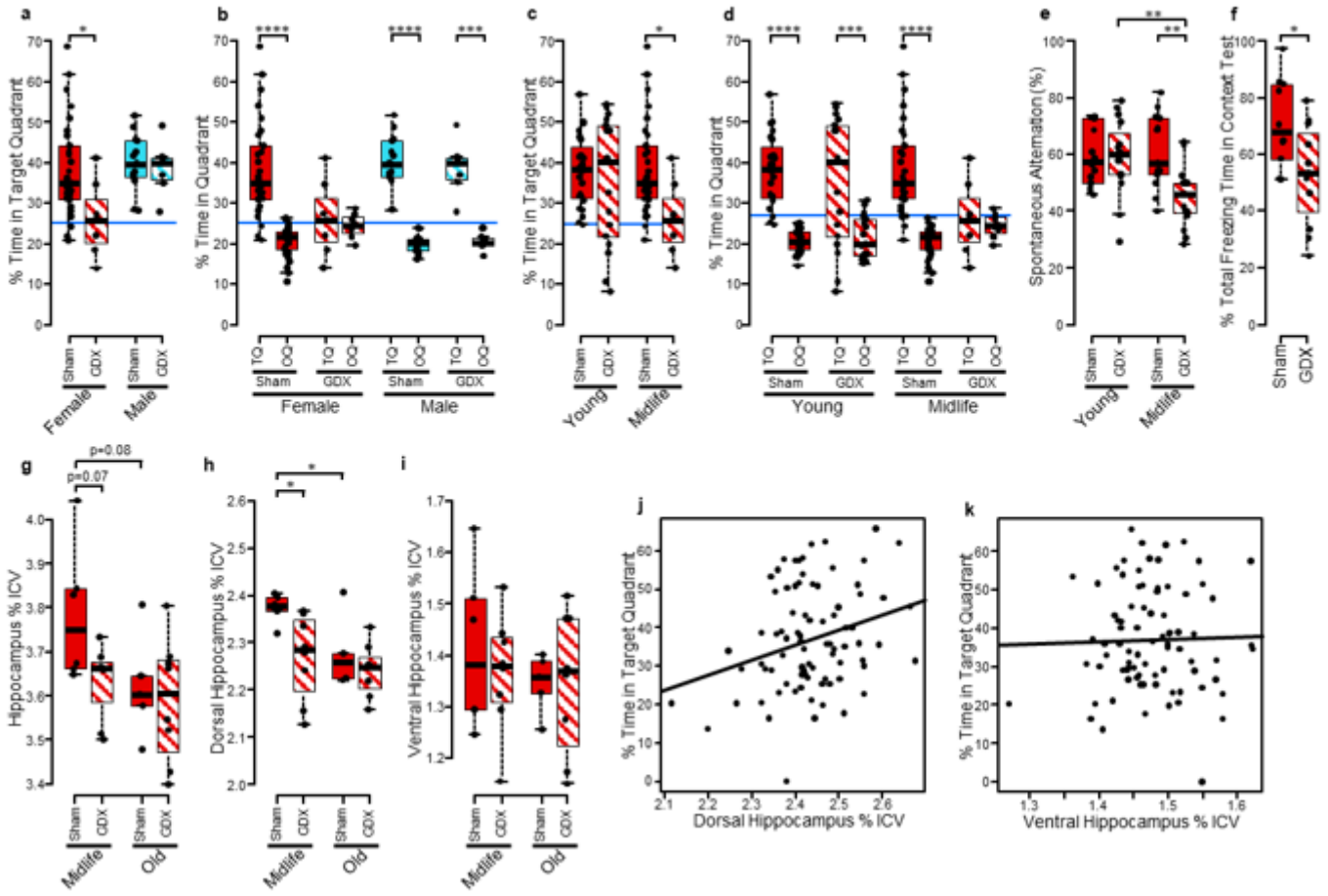



**Figure 1**

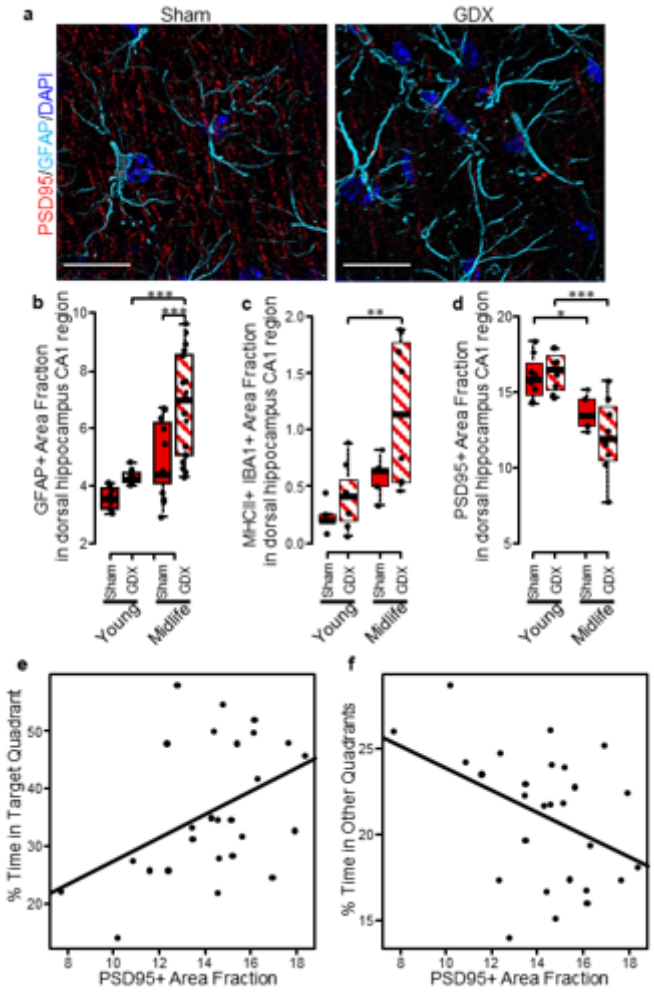
Females have substructure volume preservation from young age to midlife followed by an abrupt loss from midlife to old age. a) Morris water maze (MWM) testing with comparison of % time in target quadrant (TQ) among females (red) at young, midlife, and old age. No significant difference was observed between groups. The blue line indicates the null hypothesis (25% in TQ). b) Significant preference for the TQ compared to the other quadrants (OQ) for all ages was observed in females, indicating intact reference memory in each group with no between group differences. (all female: young n = 24, midlife n = 30, old n = 16, in a, b). c) Comparison of % time in target quadrant (TQ) among males (cyan) at young, midlife, and old age. No significant difference was observed between groups. d) Significant preference for the TQ compared to the other quadrants (OQ) for all ages was observed in males indicating intact reference memory in each group with no between group differences. (all male: young n = 7, midlife n = 14, old n = 7, in c, d). e) In vivo MRIs were collected from young, midlife, and old females and males. Structure volumes visualized on the mean template (cortex = blue, striatum = magenta, dorsal hippocampus = red, ventral hippocampus = green). Female (red) and male (cyan) volumes expressed as a percentage of intercranial volume (ICV) for f) frontal cortex, g) striatum, and h) dorsal hippocampus, at each of the three ages (young, midlife, and old). Males had gradual atrophy in frontal cortex and striatum from young to midlife to old age . Females had preservation of frontal cortex, striatum, and dorsal hippocampus volumes from young to midlife, with an abrupt drop in volumes between midlife and old age (females: frontal cortex young to midlife p = 0.692, midlife to old p = p= .000004685; striatum young to midlife p = 0.2691 , midlife to old p = p= .000002197; dorsal hippocampus young to midlife p = 0.6536 , midlife to old p = .0197). All groups n = 12 in f, g, h). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.




## Figure 2

Gonadectomy worsens hippocampal-dependent cognition and hippocampus atrophy at midlife in females. a) MWM testing with % time in target quadrant (TQ) among females (red) and males (cyan) at midlife that underwent gonadectomy (GDX, stripe) or sham (solid) surgery. GDX females performed significantly worse than sham females at midlife ($p = 0.0203$), while males showed no difference between GDX and sham ($p = 0.9936$). Blue line indicates the null hypothesis (25% in TQ). b) Significant preference for the TQ compared to the other quadrants (OQ) was observed in all groups at midlife except for GDX females. (all midlife: female sham n = 30, female GDX n = 8, male sham n = 14, male GDX n = 7, in a,b). c) % time in TQ among young and midlife females that underwent GDX or sham surgery. Significant cognitive impairment occurred at midlife in GDX females as compared to sham ($p = 0.0464$), but not in young females that were GDX versus sham. d) Significant preference for TQ compared to OQ for all groups except GDX females at midlife. (all female: young sham n = 24, young GDX n = 17, midlife sham n = 29, midlife GDX n = 8, in c, d). e) Significant working memory impairment, assessed by Y maze, was observed in GDX females at midlife ($p = 0.0038$ vs midlife sham and $p = 0.0085$ vs GDX young). (all female: young sham n = 14, young GDX n = 16, midlife sham n = 13, midlife GDX n = 16, in e). f) GDX females at midlife had impaired recent contextual fear memory, compared to sham females at midlife ($p = 0.0239$). (sham n= 10, GDX n=11, in f). Neither % time of freezing or locomotor activity difference at

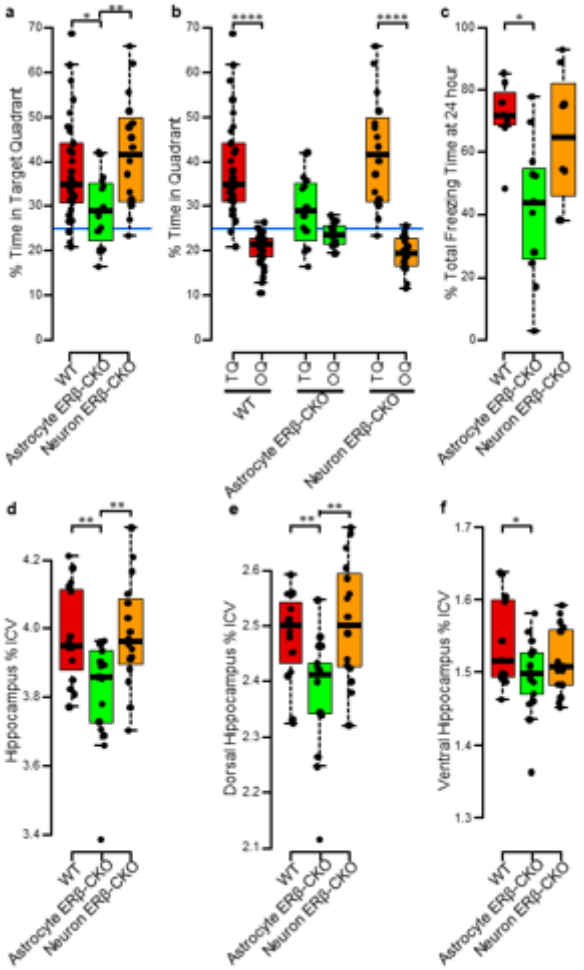
baseline was observed during acquisition. Female sham and GDX hippocampal volumes were assessed by MRI and expressed as a percentage of intercranial volume (ICV) for g) whole hippocampus, h) dorsal hippocampus, and i) ventral hippocampus. At midlife, GDX females had smaller dorsal hippocampus volumes than sham females ($p = 0.0137$). There was also smaller dorsal hippocampus volumes in old sham compared to midlife shame ($p = 0.04378$) (all female: midlife sham n = 6, midlife GDX n= 8, old sham n= 5, old GDX n= 8, in g, h, i). j) MWM % time in TQ correlated with dorsal hippocampus volume ($r = 0.28$; $p = 0.011$), k) MWM % time in TQ did not correlate with ventral hippocampus volume ($r = 0.027$, p = not significant). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.




## Figure 3

The effect of gonadectomy on glial activation and synaptic loss in dorsal hippocampus at midlife in females. a) Representative 40X images of GFAP (cyan), PSD95 (red), and DAPI (blue) in dorsal hippocampal CA1 region for sham (left) and GDX (right) females at midlife. Scale bar = 20um. Quantitative analysis of b) GFAP+ area fraction, c) MHCII+Iba1+ area fraction, and d) PSD95+ area fraction in gonadally intact, sham (solid) and GDX (stripe) females at young and midlife. b) GFAP+ area fraction was significantly increased in GDX females at midlife as compared to GDX females that were young ($p = 0.0002$) and as compared to sham females at midlife ($p = 0.0006$), (young sham and GDX each n = 8, midlife sham n = 15, midlife GDX n = 18, in b). c) MHCII+Iba1+ area fraction was significantly

increased in GDX females at midlife as compared to GDX females that were young (p = 0.0090), but not as compared to sham females at midife (p = 0.0962), (young sham n = 6, young GDX n = 7, midlife sham n= 5, midlife GDX n = 8, in c) d) PSD95+ area fraction was significantly decreased in GDX females at midlife as compared to GDX females that were young (p = 0.0003). (young sham n = 8, young GDX n = 8, midlife sham n= 6, midlife GDX n = 8, in d). e) MWM % time in TQ correlated directly with PSD95+ area fraction (r = 0.428, p = 0.029) and f) MWM % time in OQ correlated indirectly with PSD95+ area fraction (r=-0.417, p=0.027). * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.




## Figure 4

Selective deletion of ERβ in astrocytes worsens hippocampal-dependent cognition and hippocampus atrophy at midlife in females. a) Gonadally intact wild type (WT, red) females along with conditional knock-outs of ERβ in astrocytes (astrocyte ERβ CKO, green) or ERβ in neurons (neuron ERβ CKO, orange) were assessed at midlife for spatial reference memory by MWM. WT mice and neuron ERβ CKO each showed intact reference memory, while astrocyte ERβ CKO had significant impairment (p = 0.0425 vs WT; p = 0.0065 vs neuron ERβ CKO). Blue line indicates the null hypothesis (25% in TQ). b) WT and neuron ERβ CKO showed preference for the TQ over the OQ (p < 0.0001), while astrocyte ERβ CKO did not. (WT n = 30, astrocyte ERβ CKO n = 15, neuron ERβ CKO n = 16, in a, b). c) Recent contextual fear memory testing showed a significant decrease of % total freezing time in astrocyte ERβ CKO compared to littermate WT

(p = 0.0164) but not in neuron ERβ CKO (p = 0.7779). (WT n = 7, astrocyte ERβ CKO n = 11, neuron ERβ CKO n = 8, in c). Neither % time of freezing or locomotor activity difference at baseline was observed during acquisition. Structure volumes, assessed by MRI, expressed as a percentage of intercranial volume (ICV) for d) whole hippocampus, e) dorsal hippocampus, and f) ventral hippocampus. d) Astrocyte ERβ CKO females had worse atrophy than both WT and neuron ERβ CKO at midlife in whole hippocampus (p = 0.0048 astrocyte ERβ CKO vs WT; p = 0.0050 astrocyte ERβ CKO vs neuron ERβ CKO) and e) dorsal hippocampus (p = 0.0055 astrocyte ERβ CKO vs WT; p = 0.0016 astrocyte ERβ CKO vs neuron ERβ CKO), f) with less difference in ventral hippocampus (p = 0.0275 astrocyte ERβ CKO vs WT, not significant astrocyte ERβ CKO vs neuron ERβ CKO). (WT n = 15, astrocyte ERβ CKO n = 17, neuron ERβ CKO n = 18, in d, e, f). * p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001.

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- RVSupplementalMaterials91321.pdf