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Prenatal Alcohol Exposure Disrupts γ-Secretase Activity and Impairs Learning and Memory in Wild-Type and 3xTg-AD Mice

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eLife Assessment

This **important** study shows that prenatal alcohol exposure produces lasting changes in amyloid precursor protein processing, including altered APP C-terminal fragments and Notch intracellular domain levels, that persist into adulthood and track with progressive spatial learning and memory deficits, in both wild-type and 3xTg-AD mice. The **convincing** study was carefully designed and combined biochemical, histological, and behavioral approaches across multiple ages. The work will interest researchers studying fetal alcohol spectrum disorders, Alzheimer's disease risk, and the developmental origins of neurodegeneration. Nonetheless, reviewers identified opportunities to strengthen the conclusions with additional samples and controls.

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Abstract

Although prenatal alcohol exposure (PAE) has been proposed as an early-life risk factor for Alzheimer's disease and related dementias (AD/ADRD), the mechanistic underpinnings are underexplored. Mutations in the *Presenilin* genes contribute to AD/ADRD, with Presenilin 1 acting as the catalytic subunit of the γ-secretase complex responsible for cleaving Notch and amyloid precursor protein (APP). We hypothesized that PAE disrupts γ-secretase activity during brain development, which persists and is associated with behavioral deficits later in life. Pregnant wild-type B6129 and 3xTg-AD mice were fed an ethanol-containing liquid diet during gestational days 13–15. From birth to adulthood, PAE increased APP C-terminal fragments and Notch intracellular domain (NICD) levels in cortical lysates. These changes were associated with impaired hippocampal-dependent learning and memory in wild-type mice at 3 and 6 months of age and exacerbated behavioral deficits in 4-month-old 3xTg-AD mice. Our findings provide the first mechanistic insight linking PAE to AD/ADRD vulnerability.

Introduction

There has been great interest in unraveling environmental risk factors that contribute to advancing our appreciation of the pathoetiology and treatment of Alzheimer's disease and Alzheimer's disease–related dementias (AD/ADRD) (Borenstein et al., 2006). To date, while such studies have largely focused on risk factors that are prevalent in adulthood, they also underscore that potential early-life risk factors have been unexplored (Yu et al., 2020).

Why consider early-life events as risk factors when the lore is that AD is an adult-onset neurodegenerative disease? Neurodevelopment and aging are stages in the continuum of life - neurodevelopment begins in the embryo, continues through childhood, adolescence, and tapers off in young adulthood. Disruption or impairment of neurodevelopmental processes at any point in this continuum can lead to indelible lifelong disabilities, many of which are reminiscent of

brain abnormalities or impaired cognition seen in neurodegenerative disorders. Along this line of thought, neurodevelopmental disorders such as autism spectrum have been reported to be associated with AD, a neurodegenerative disorder (Nadeem et al., 2021).

Early-life environmental insults can leave molecular and cellular imprints that persist long after development (Basha et al., 2005). The developing brain is particularly susceptible to environmental insults that enhance vulnerability to neurodegenerative diseases throughout the lifespan (Sarkar et al., 2019; Tartaglione et al., 2016). Prenatal alcohol exposure (PAE) may be one such insult, postulated in preclinical studies to be an early life risk factor that contributes to increased vulnerability to developing AD/ADRD later in life (Borenstein et al., 2006; Huang et al., 2023; Lahiri et al., 2008; Tousley et al., 2023). Indeed, PAE is recognized clinically as a potential early-life contributor to the development of neurobehavioral disorders (Coles et al., 2020; Hagan et al., 2016). Importantly, binge drinking is a common mode of PAE, particularly during the first trimester of gestation, before pregnancy is recognized, but just when neurogenesis and brain patterning peak (Dejong et al., 2019; Denny et al., 2019; Tough et al., 2006). Binge-like maternal alcohol consumption produces more severe neurodevelopmental outcomes than chronic low-level exposure, highlighting the importance of exposure pattern in determining long-term risk (Dejong et al., 2019; Maier & West, 2001; Muggli et al., 2024).

PAE adversely affects key neurodevelopmental processes, including neurogenesis, neuronal migration, and synaptogenesis (Coles et al., 2020). These disruptions alter the formation and maturation of neural circuits, leading to persistent structural and functional abnormalities. Clinically, such deficits manifest as long-term cognitive impairments, including deficits in learning, memory, and executive function, which are core features observed in individuals with Fetal Alcohol Spectrum Disorder (FASD) (Franklin et al., 2008; Mattson et al., 2019; Rasmussen, 2005). Although the behavioral and cognitive consequences of PAE are well documented (Chung et al., 2021), the molecular mechanisms linking early binge-like alcohol exposure to later-life neurodegenerative processes remain poorly understood. The present study is grounded in the perspective that indelible neurodevelopmental alterations may increase susceptibility to the development of neurodegenerative disorders, including AD/ADRD.

AD/ADRD are the most prevalent neurodegenerative disorders, characterized by progressive cognitive decline, synaptic dysfunction, and neuronal loss (Sheppard & Coleman, 2020). Mutations in the *Presenilin* genes (*PSEN1* and *PSEN2*) are among the most common causes of familial AD (Kelleher & Shen, 2017). Presenilin-1 (PS1) functions as the catalytic subunit of the γ-secretase complex, a protease that cleaves type I transmembrane proteins, including the amyloid precursor protein (APP) and Notch (Schroeter et al., 2003). For γ-secretase to become catalytically active, PS1 undergoes an endoproteolytic self-cleavage that generates stable N-terminal (NTF) and C-terminal (CTF) fragments (Gertsik et al., 2015; Schroeter et al., 2003; Wolfe, 2019). The γ-secretase complex, through its proteolytic activity, regulates key signaling pathways involved in neurogenesis, neuronal differentiation, and synaptic plasticity during brain development, and supports neuronal survival and synaptic function in adulthood (Jurisch-Yaksi et al., 2013; Wolfe, 2019). Disruptions in γ-secretase function during early development, driven by genetic mutations in *PSEN1*, result in loss-of-function alterations that impair Notch signaling and APP processing, leading to defective neurogenesis, altered neuronal differentiation, and long-lasting synaptic deficits (Handler et al., 2000; Zhou et al., 2011). PAE appears to converge on similar neurodevelopmental outcomes. PAE has been shown to interfere with key developmental signaling pathways, including those regulated by γ-secretase substrates, resulting in reduced neuronal proliferation, impaired neuronal migration and circuit formation, and persistent deficits in synaptic plasticity (Delatour et al., 2019; Guerri, 2002; Miller, 1993, 1996; Sarmah et al., 2016; Tong et al., 2013). These converging effects suggest that PAE may phenocopy aspects of γ-secretase dysfunction during brain development, thereby establishing a potential mechanistic framework through which early-life environmental exposures can increase vulnerability to AD/ADRD later in life.

In the context of APP processing, β-secretase (BACE1) cleavage first generates the membrane-bound APP C-terminal fragment (C99), which is subsequently processed by γ-secretase to produce amyloid-β (Aβ) peptides and the APP intracellular domain (Hur, 2022). Disruption of γ-secretase activity interferes with this process and leads to the intracellular accumulation of APP C-terminal fragments (APP CTFs), including C99. Elevated levels of APP CTFs are widely used as a biochemical indicator of reduced γ-secretase activity (Heilig et al., 2010; Montenegro et al., 2023; H. Yu et al., 2001). Importantly, the accumulation of C99 itself is increasingly recognized as a pathogenic contributor to AD, as it promotes endosomal dysfunction, synaptic deficits, and neurotoxicity independent of extracellular Aβ deposition (Bourgeois et al., 2018; Pulina et al., 2020). Supporting this, experimental studies in adult murine models have shown that ethanol exposure enhances amyloidogenic APP processing by increasing BACE1 activity, leading to elevated production of C99 and Aβ peptides (Huang et al., 2018). Ethanol-induced oxidative stress and membrane alterations have been proposed to shift APP processing toward the amyloidogenic pathway, thereby promoting the accumulation of neurotoxic APP-derived fragments (Huang et al., 2018). Consistent with these findings, studies have shown that excessive alcohol consumption disrupts the processing of APP, Aβ, and the expression of enzymes involved in these processes (Gong et al., 2021; Kim et al., 2011; Renau-Piqueras et al., 1997; Venkataraman et al., 2017). Moreover, multiple studies have linked PAE to AD-like pathology in mouse models (Alhowail, 2022; Canales et al., 2013; Gerlikhman et al., 2023; Tousley et al., 2023), suggesting that APP processing, hence γ-secretase activity, may be disrupted during neurodevelopment.

In this study, we hypothesize that PAE alters γ-secretase activity early during brain development, resulting in disrupted APP and Notch processing and increased vulnerability to learning and memory deficits in adulthood. To this end, we employed a binge-type PAE paradigm in both wild-type B6129 and 3xTg-AD mice, the latter harboring three human AD/ADRD-associated mutations *PSEN1* M146V, *Tau P301L,* and *APP K670_M671delinsNL* (Oddo et al., 2003). We conducted biochemical and histological analyses to assess the effects of PAE on γ-secretase function, as well as behavioral analysis to assess learning and memory. Our findings provide novel insights into how early-life alcohol exposure contributes to AD/ADRD pathogenesis, highlighting γ-secretase dysfunction as a potential mechanistic link between PAE and memory decline throughout life span.

Results

PAE impairs γ-secretase activity in neonatal wild-type B6129 and 3xTg-AD mice

Amyloid precursor protein C-terminal fragments (APP CTFs) and the Notch intracellular domain (NICD) are key readouts of γ-secretase activity, with APP representing the immediate substrate for γ-secretase cleavage and NICD a direct product of γ-secretase–mediated processing of the Notch receptor (Schroeter et al., 2003). In this light, we asked whether prenatal alcohol exposure (PAE) alters γ-secretase–dependent processing during cortical development by quantifying levels of APP CTFs and NICD in lysates harvested from the cortex towards the end stage of embryonic corticogenesis at postnatal day 0 (P0, birth).

Figure 1 illustrates Western blot data assessing γ-secretase–related processing of PS1 and APP expression in cortex lysates derived from P0 wild-type B6129 and 3xTg-AD mice with and without PAE. The summary graphs in Figure 1C and 1F indicate disrupted APP processing in wild-type B6129 and 3xTg-AD mice, respectively, exposed prenatally to ethanol (PAE-B6129 and PAE-3xTg-AD) relative to their isocaloric liquid diet controls. In wild-type B6129 mice, PAE significantly increased levels of APP CTFs up to 31.21% in the cortex relative to their non-PAE control cohorts ($p = 0.0026$, unpaired Student's t-test, Fig. 1A and 1C). NICD levels were also significantly elevated up to 34.14% in PAE-B6129 mice relative to their non-PAE controls ($p = 0.0315$, unpaired Student's t-test, Fig. 1B and 1C). In P0 3xTg-AD mice, cortical APP CTFs levels were significantly increased in both non-PAE (36.20%, $p = 0.0395$, one-way ANOVA with Tukey's post hoc

multiple comparisons) and PAE (64.35%, p = 0.0011, one-way ANOVA with Tukey's post hoc multiple comparisons) groups compared to non-PAE B6129 controls (Fig. 1D and 1F). Similarly, NICD levels were significantly elevated in PAE-3xTg-AD mouse cortex (53.96%, p=0.0087, one-way ANOVA with Tukey's post hoc multiple comparisons, Fig 1E and 1F). No significant increase in NICD levels, 34.76%, was detected in 3xTg-AD mice compared to B6129 controls. The increase in both cortical APP CTFs and NICD levels suggests that PAE affects the normal regulation of the γ-secretase proteolytic pathway at birth, resulting in increased intracellular accumulation. Importantly, the supranormal levels of APP CTFs and NICD observed in PAE-3xTg-AD mice suggest that PAE exacerbates the dysregulation of APP and Notch processing beyond that driven by AD-linked mutations.

We then asked whether the observed alterations in γ-secretase activity are associated with changes in the γ-secretase complex itself by examining the expression of the Presenilin 1 C-terminal fragment (PS1 CTF), a key functional component of the γ-secretase complex (Fig. 1A and 1D). As summarized in Figures 1C and 1F, PS1 CTF levels were unchanged in PAE-B6129 (p = 0.2269, unpaired Student's t-test), 3xTg-AD (p = 0.9923, one-way ANOVA with Tukey's post hoc multiple comparisons), and PAE-3xTg-AD (p = 0.8980, one-way ANOVA with Tukey's post hoc multiple comparisons) compared to wild-type controls, suggesting that PAE-induced alterations in γ-secretase activity are unlikely to result from changes in the abundance of its catalytic subunit.

To gain insight into how PAE influences the spatial distribution of the APP CTFs protein in brain regions that are vulnerable in patients with AD, we assessed immunohistochemically the intracellular accumulation of the APP CTFs protein in cortical and hippocampal sagittal sections derived from P0 wild-type B6129, PAE-B6129, 3xTg-AD, and PAE-3xTg-AD mice. APP CTFs were widely distributed throughout the cortical layers (Fig. 2, top panels). Quantification of mean fluorescence intensity revealed that the strongest immunoreactivity was observed across layers II–VI, with significant increases detected in the PAE-B6129 up to 32.54% (p = 0.0216, one-way ANOVA with Tukey's post hoc multiple comparisons) and PAE-3xTg-AD up to 34.01% (p = 0.0261, one-way ANOVA with Tukey's post hoc multiple comparisons) mice. In the hippocampus, APP CTFs were prominently localized within the CA regions, notably in the CA1 and CA2/3 subregions, compared to age-matched wild-type B6129 controls (Fig. 2, bottom panels; PAE-B6129, 28.50%, p = 0.0030; 3xTg-AD, 26.05%, p = 0.0088; and PAE-3xTg-AD, 26.46%, p = 0.0105. One-way ANOVA with Tukey's post hoc multiple comparisons.). Thus, brain regions essential for learning, memory, and the formation of cortical networks are already affected by altered APP processing by birth in the cortex and hippocampus. Together, these findings demonstrate that PAE is sufficient to induce early dysregulation of γ-secretase–related pathways in a wild-type B6129 background, as reflected by increases in APP CTFs and NICD at birth. In the presence of AD-associated mutations, this disruption is exacerbated, leading to greater accumulation of APP processing intermediaries. Thus, prenatal alcohol exposure may act as an early-life modifier of molecular pathways linked to Alzheimer's disease and related dementias, including impaired learning and memory behavior.

PAE impairs learning and memory and γ-secretase-related activity in adult wild-type B6129 mice

To determine if the disruption of γ-secretase–mediated processing observed at birth persists and is associated with learning and memory impairments in adulthood, hippocampal-dependent spatial learning was first assessed using the Morris water maze in wild-type B6129 mice at 3 and 6 months of age with or without PAE. The levels of APP CTFs and PS1 CTFs were analyzed using Western blot in cortical and hippocampal lysates derived from the same behaviorally tested mice.

Figure 3 illustrates the timeline of the Morris water maze regime (Fig. 3A) and behavioral data (Fig. 3B) derived from 3-month-old young adult B6129 mice with and without PAE. During hidden platform training in the Morris water maze, PAE-B6129 mice showed a significant longer escape latencies compared to control B6129 mice (p = 0.0036, two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means), indicating impaired spatial learning in the

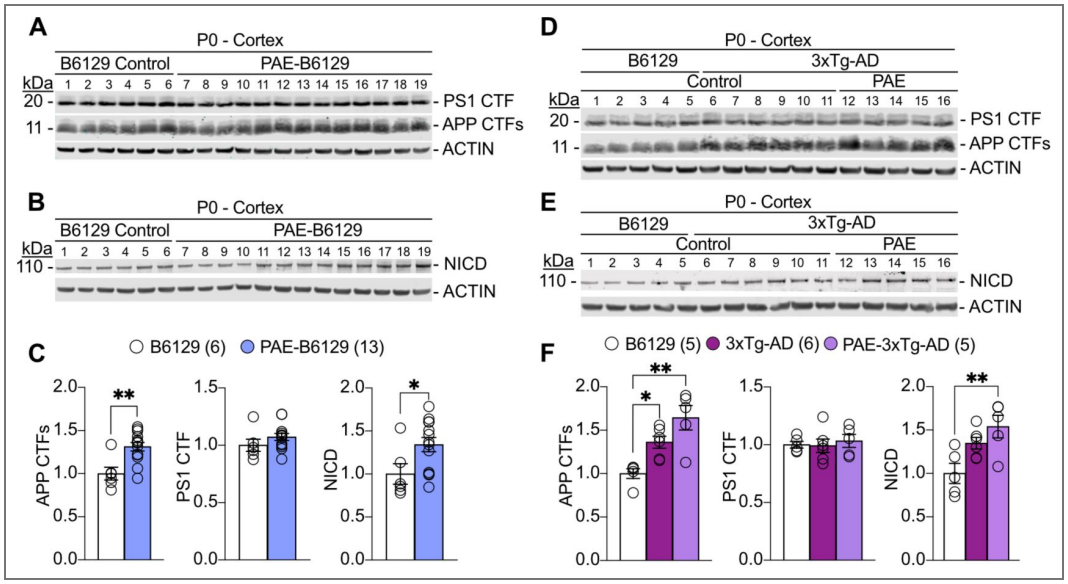

Figure 1. PAE reduces γ-secretase activity in B6129 and 3xTg-AD mice at birth.

(A) Western analysis of cortical lysates from B6129 controls (lanes 1-6) and PAE-B6129 (lanes 7-19) mice at postnatal day 0 (P0) shows no change in PS1 CTF levels between groups, and increased levels of APP CTFs in PAE-B6129 mice compared to B6129 controls. **(B)** Western blot analysis of cortical lysates from B6129 controls (lanes 1-6) and PAE-B6129 (lanes 7-19) mice at P0 shows increased levels of NICD in PAE-B6129 mice compared to controls. **(C)** Quantification of APP CTFs, PS1 CTF, and NICD shows that levels of APP CTFs ($p = 0.0026$) and NICD ($p = 0.0315$) are significantly increased compared to non-alcohol-exposed B6129. Quantification of PS1 CTF levels shows no significant differences between PAE-B6129 and B6129 controls ($p = 0.2269$). Unpaired Student's t-test. **(D)** Western analysis of cortical lysates from B6129 controls (lanes 1-5), 3xTg-AD controls (lanes 6-11), and PAE-3xTg-AD (lanes 12-16) mice at P0 shows no change in PS1 CTF levels between groups, and increased levels of APP CTFs in 3xTg-AD controls and PAE-3xTg-AD mice compared to B6129 controls. **(E)** Western analysis of cortical lysates from B6129 controls (lanes 1-5), 3xTg-AD controls (lanes 6-11), and PAE-3xTg-AD (lanes 12-16) mice at P0 shows increased levels of NICD in 3xTg-AD controls and PAE-3xTg-AD PAE-B6129 mice compared to B6129 controls. **(F)** Quantification of APP CTFs, PS1 CTF, and NICD shows that levels of APP CTFs in 3xTg-AD controls ($p = 0.0395$) and in PAE-3xTg-AD ($p = 0.0011$) were significantly higher compared to B6129 controls. Quantification of PS1 CTF levels shows no significant differences between 3xTg-AD controls ($p = 0.9923$), PAE-3xTg-AD ($p = 0.8980$), and B6129 controls. Levels of NICD in PAE-3xTg-AD ($p = 0.0087$) are significantly higher compared to B6129 controls. One-way ANOVA with Tukey's post hoc multiple comparisons. All data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. Sample size indicated in parentheses next to each experimental group label.

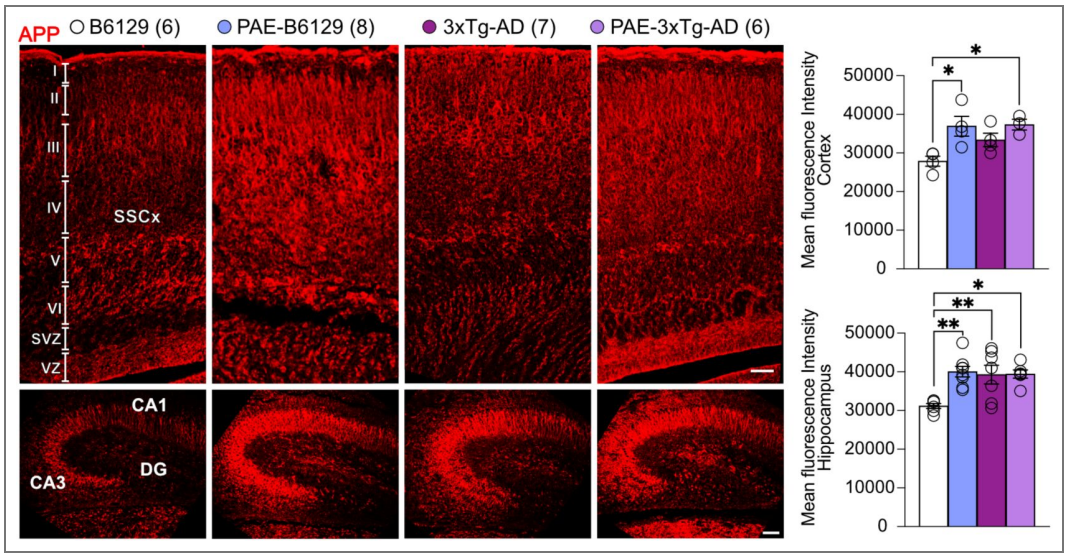

Figure 2. PAE-B6129 and PAE-3xTg-AD mice show high expression of APP CTFs in cortical regions at birth.

(Top Panel) Representative images of cortical sagittal sections immunoassayed using antibodies specific for the C-terminal fragment of APP. Accumulation of APP CTFs is evident in the somatosensory cortex (SSCx), in layers II to VI, of PAE-B6129, 3xTg-AD, and PAE-3xTg-AD mice compared to B6129 controls. Quantification of fluorescence signal shows a significant increase in PAE-B6129 ($p = 0.0216$), and PAE-3xTg-AD ($p = 0.0261$) compared to B6129 controls. **(Bottom Panel)** Hippocampal sagittal representative images showing accumulation of APP CTFs mostly in CA1, CA2/3, and modest increased immunoreactivity in dentate gyrus (DG). Quantification of fluorescence signal shows a significant increase in PAE-B6129 ($p = 0.0030$), 3xTg-AD ($p = 0.0088$), and PAE-3xTg-AD ($p = 0.0105$) compared to B6129 controls. One-way ANOVA with Tukey's post hoc multiple comparisons. All data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. Sample size indicated in parentheses next to each experimental group label. Scale bars = 50 μm.

acquisition phase (Fig. 3A). These deficits in B6129 mice without PAE were detected at an age (3 months) when 3xTg-AD mice typically are presymptomatic for AD pathology, suggesting that PAE alone is sufficient to induce impaired hippocampal-dependent learning.

In Figure 4, Western blot analysis revealed persistent alterations in APP CTFs levels in cortical and hippocampal lysates obtained from the behaviorally tested 3-month-old mice PAE-B6129 mice compared with those from age-matched B6129 control cohorts. Specifically, PAE-B6129 mice exhibited significantly elevated levels of APP CTFs in both the cortex, up to 41.26% ($p = 0.0065$, unpaired Student's t-test, Figs. 4A and 4C), and hippocampus, up to 22.62% ($p = 0.0170$, unpaired Student's t-test, Figs. 4E and 4G). In contrast, the levels of PS1 CTF in PAE-B6129 showed a greater variability in the cortex (Coefficient of variation (CoV): B6129 = 5.147% vs. PAE-B6129 = 15.61%; $p = 0.0725$, unpaired Student's t-test, Figs. 4A and 4C) and hippocampus ($p = 0.4578$, unpaired Student's t-test, Figs. 4E and 4G) that did not differ significantly from the B6129 control cohort.

Cortical and hippocampal lysates from 3-month-old 3xTg-AD mice without PAE were also analyzed by Western blotting (Fig. 4). In the cortex, PS1 CTF levels from 3xTg-AD mice did not differ from those in B6129 controls ($p = 0.9235$, unpaired Student's t-test, Figs. 4B and 4D). However, in the hippocampus, PS1 CTF levels were significantly increased up to 33.12% ($p = 0.0165$, unpaired Student's t-test; Figs. 4F and H), likely reflecting overexpression of the human PS1 M146V transgene. By contrast, APP CTFs were significantly increased in 3xTg-AD mice in both the cortex, up to 56.22% ($p = 0.0097$, unpaired Student's t-test; Figs. 4B and 4D) and hippocampus, up to 86.41% ($p = 0.0096$, unpaired Student's t-test; Figs. 4F and 4H). When these increases were compared with APP CTF levels observed in PAE-B6129 mice, the data suggest that both prenatal alcohol exposure and the PS1 M146V mutation produce a comparable reduction in γ-secretase activity in young 3-month-old adult mice.

To determine whether the behavioral and γ-secretase alterations observed at 3 months of age persist or worsen with age, we next examined hippocampal-dependent learning using the same regime illustrated in Figure 3, and γ-secretase–related processing in PAE B6129 mice at 6 months of age (Figs. 5 and 6). Figure 5 illustrates that 6-month-old PAE-B6129 mice exhibited more severe deficits in hippocampal-dependent learning. During hidden platform training, PAE-exposed mice displayed highly significant longer escape latencies compared with age-matched non-PAE B6129 controls ($p < 0.0001$, two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means), and the differences in escape latencies between PAE-B6129 and 3xTg-AD controls mice are relatively modest ($p = 0.0220$, two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means). This indicates that spatial learning impairments not only persisted but also worsened into mid-adulthood, reaching a level comparable to that observed in the 3xTg-AD mouse model.

In cortical and hippocampal lysates from 6-month-old behaviorally tested mice, PAE-B6129 mice showed no significant changes in APP CTFs levels in the cortex compared with non-exposed B6129 controls ($p = 0.1219$, unpaired Student's t-test; Figs. 6A and 6C). In contrast, APP CTF levels in the hippocampus displayed a significant increase in PAE-B6129 mice up to 26.16% ($p = 0.094$, unpaired Student's t-test; Fig. 6E and 6G), which can be related to the learning and memory deficits observed in this group. Consistent with earlier observations, PS1 CTF levels did not differ significantly in cortex ($p = 0.4461$, unpaired Student's t-test) and hippocampus ($p = 0.7407$, unpaired Student's t-test) compared to the B6129 control group, indicating that the catalytic subunit of the γ-secretase complex remains unchanged (Figs. 6C and 6G). Analysis of APP CTF levels in the 3xTg-AD mouse model revealed a significant increase in the cortex up to 40.93% ($p = 0.0067$, unpaired Student's t-test; Figs. 6B and 6D), with an even greater increase in the hippocampus, up to 73.01% ($p = 0.0004$, unpaired Student's t-test; Figs. 6F and 6H), supporting a potential link between more pronounced γ-secretase deficits in hippocampal regions and learning and memory impairments. As observed in 3-month-old mice (Fig. 4), 3xTg-AD mice exhibited higher PS1 CTF protein levels in the cortex (24.39%, $p = 0.0162$, unpaired Student's t-test; Fig. 6D) and hippocampus (24.07%, $p = 0.0393$, unpaired Student's t-test; Fig. 6H) compared to B6129 controls, consistent with overexpression of the PS1 M145V transgene. Together, these

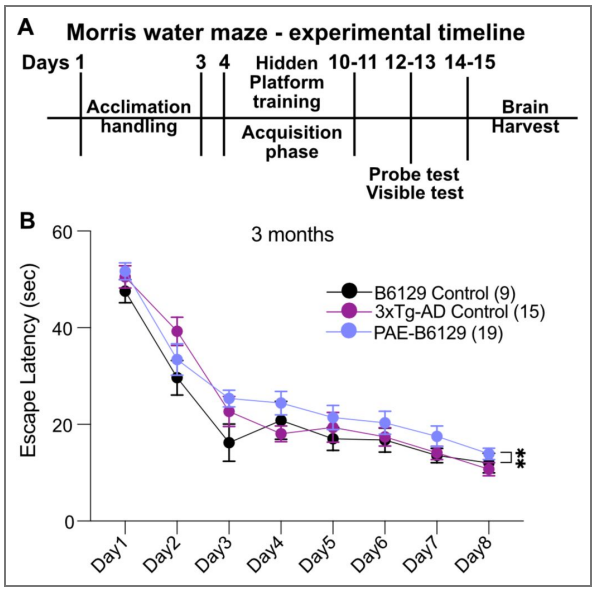

Figure 3. PAE impairs learning and memory in B6129 mice at 3 months of age.

(A) Morris water maze experimental timeline: Mice are individually acclimated to handling during days 1 to 3. The hidden platform training and acquisition phase is conducted from days 4 to 10-11, for a total of 7-8 days. Probe and visible platform tests are performed one day after completion of the acquisition phase, on days 12-13. Brains are harvested immediately following the behavioral study, on days 14-15. **(B)** During the hidden platform version of the Morris water maze test, PAE-B6129 mice at 3 months of age display significantly longer latencies compared to B6129 controls ($p = 0.0036$). At the pre-symptomatic stage (3 months of age), 3xTg-AD controls did not show significant differences in escape latencies compared to B6129 controls ($p = 0.2153$). Two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means. All data represent mean ± SEM **$p < 0.01$. Sample size indicated in parentheses next to each experimental group label.

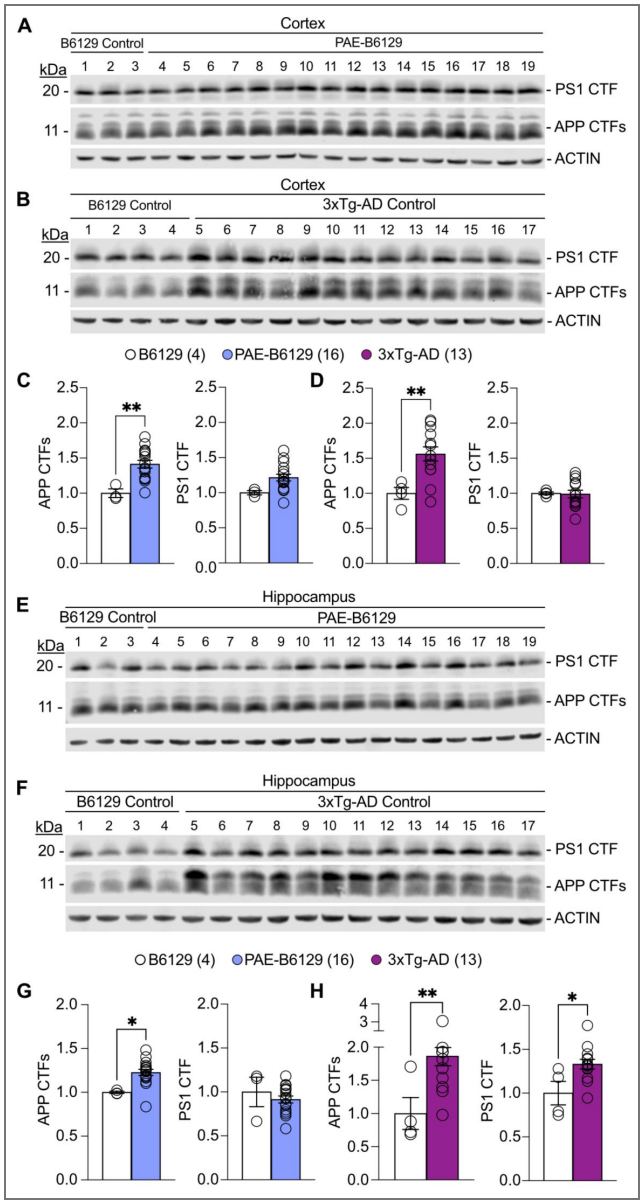

Figure 4. PAE reduces γ-secretase activity in B6129 mice at 3 months of age.

A) Western analysis of cortical lysates from B6129 controls (lanes 1-3) and PAE-B6129 (lanes 4-19) mice at 3 months of age shows no change in PS1 CTF levels between groups and increased levels of APP CTFs in PAE-B6129 mice compared to B6129 controls. **(B)** Western blot analysis of cortical lysates from B6129 controls (lanes 1-4) and 3xTg-AD controls (lanes 5-17) mice at 3 months of age shows no change in levels of PS1 CTF and increased levels of APP CTFs in 3xTg-AD mice compared to B6129 controls. **(C-D)** Quantification of cortical levels of APP CTFs and PS1 CTF shows that **(C)** levels of APP CTFs are significantly increased in the cortex of PAE-B6129 ($p = 0.065$) compared to B6129 controls, and levels of PS1 CTF show no significant differences between PAE-B6129 ($p = 0.0725$) and B6129 controls. **(D)** Levels of APP CTFs ($p = 0.0097$) and PS1 CTF ($p = 0.0165$) in 3xTg-AD cortex are significantly increased compared to B6129 controls. Unpaired Student's t-test. **(E)** Western analysis of hippocampal lysates from B6129 controls (lanes 1-3) and PAE-B6129 (lanes 4-19) show no changes in PS1 CTF level, and increased levels of APP CTFs in PAE-B6129 compared to B6129 controls. **(F)** Western analysis of hippocampal lysates from B6129 controls (lanes 1-4) and 3xTg-AD controls (lanes 5-17) shows increased levels of PS1 CTF and APP CTFs in 3xTg-AD mice compared to B6129 controls. **(G-H)** Quantification of hippocampal levels of APP CTFs and PS1 CTF shows that **(G)** levels of APP CTFs in PAE-B6129 ($p = 0.0170$) are significantly increased compared to B6129 controls. Levels of PS1 CTF were not significantly different between groups ($p = 0.4578$). **(H)** Levels of APP CTF in 3xTg-AD controls ($p = 0.0096$) were significantly higher compared to B6129 controls. Levels of PS1 CTF in 3xTg-AD ($p = 0.0165$) are significantly higher compared to B6129 controls. Unpaired Student's t-test. All data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$. Sample size indicated in parentheses next to each experimental group label.

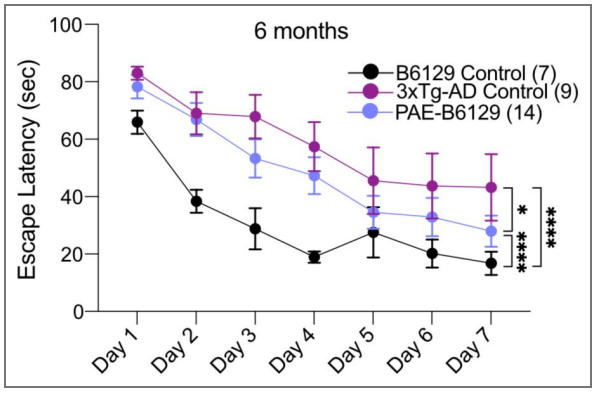

Figure 5. PAE impairs learning and memory and γ-secretase activity in B6129 mice at 6 months of age.

During the hidden platform version of the Morris water maze test, PAE-B6129 mice at 6 months of age display significantly longer escape latencies compared to B6129 controls ($p < 0.0001$). Similarly, 3xTg-AD controls ($p < 0.0001$) show significant differences in escape latencies compared to B6129 controls. The difference in escape latency between PAE-B6129 and 3xTg-AD mice reached modest statistical significance ($p = 0.0220$). Two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means. All data represent mean ± SEM *$p < 0.05$ ****$p < 0.0001$. Sample size indicated in parentheses next to each experimental group label.

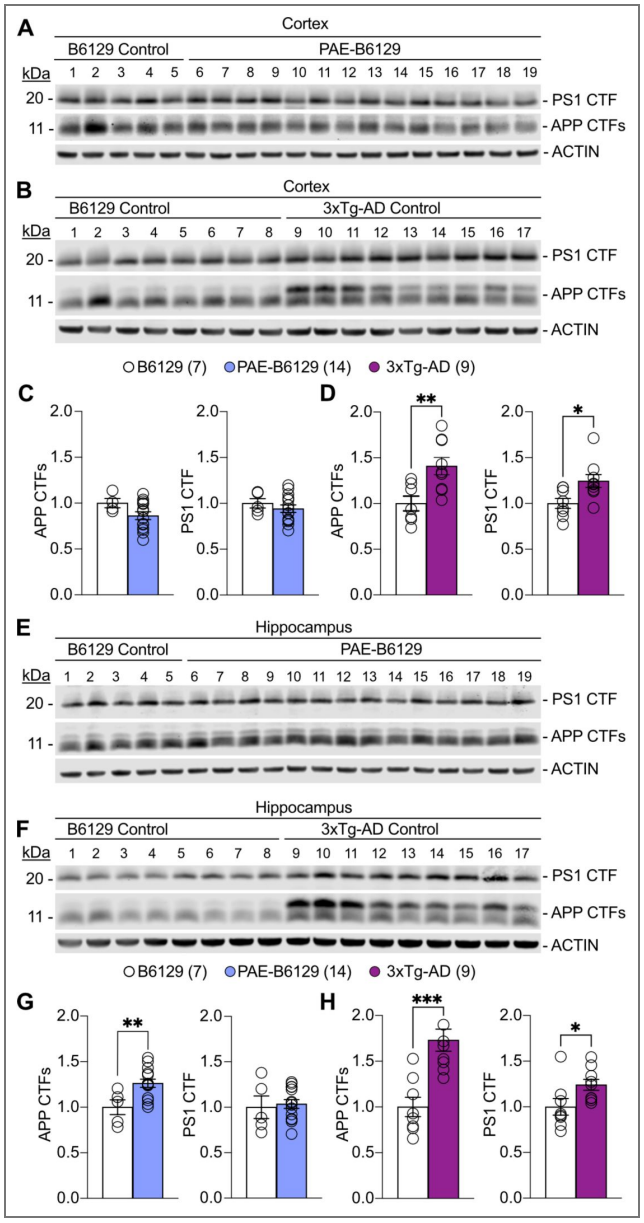

Figure 6. PAE reduces γ-secretase activity in the hippocampus of B6129 mice at 6 months of age.

(A) Western analysis of cortical lysates from B6129 controls (lanes 1-5) and PAE-B6129 (lanes 6-19) mice at 6 months of age shows no change in PS1 CTF and APP CTFs levels between groups **(B)** Western blot analysis of cortical lysates from B6129 controls (lanes 1-4) and 3xTg-AD controls (lanes 5-17) mice at 6 months of age shows no change in levels of PS1 CTF and increased levels of APP CTFs in 3xTg-AD mice compared to B6129. **(C-D)** Quantification of cortical levels of APP CTFs and PS1 CTF shows that **(C)** levels of APP CTFs ($p = 0.1219$) and PS1 CTF ($p = 0.4461$) are not significantly different in the cortex of PAE-B6129 compared to non-alcohol-exposed B6129 **(D)** Levels of APP CTFs ($p = 0.0067$) and PS1 CTF ($p = 0.0165$) in 3xTg-AD cortex are significantly increased compared to B6129. Unpaired Student's t-test. **(E)** Western analysis of hippocampal lysates from B6129 controls (lanes 1-3) and PAE-B6129 (lanes 4-19) shows no changes in PS1 CTF level, and increased levels of APP CTFs in PAE-B6129 compared to B6129 controls. **(F)** Western analysis of hippocampal lysates from B6129 controls (lanes 1-4) and 3xTg-AD controls (lanes 5-17) shows increased levels of PS1 CTF and APP CTFs in 3xTg-AD mice compared to B6129 controls. **(G-H)** Quantification of hippocampal levels of APP CTFs and PS1 CTF shows that **(G)** levels of APP CTFs in PAE-B6129 ($p = 0.0094$) are significantly increased compared to B6129 controls. Levels of PS1 CTF were not significantly different between groups ($p = 0.7407$). **(H)** Levels of APP CTF in 3xTg-AD controls ($p = 0.0004$) are significantly higher compared to B6129 controls. Levels of PS1 CTF in 3xTg-AD ($p = 0.0393$) are significantly higher compared to B6129 controls. Unpaired Student's t-test. All data represent mean ± SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Sample size indicated in parentheses next to each experimental group label.

findings suggest that early PAE-induced disruption of γ-secretase–mediated APP processing produces long-lasting brain region-selective alterations. In particular, the pronounced accumulation of APP CTFs observed in the cerebral cortex at birth and in the hippocampus at adulthood of PAE-B6129 mice strongly suggests a mechanistic link to the persistent hippocampal-dependent learning deficits observed in 3 and 6-month-old mice. Given the central role of the hippocampus in spatial learning and memory, these findings support the possibility that PAE-induced disruption of APP processing correlates with long-lasting behavioral dysfunction.

PAE alters learning and memory in 3xTg-AD mice

To investigate whether PAE contributes to pathological outcomes in mice carrying AD–linked mutations, we asked whether PAE exacerbates learning and memory deficits in 3xTg-AD mice at a presymptomatic stage (4-month-old) and, if so, whether it is associated with alterations in γ-secretase–dependent APP processing. Hippocampal-dependent learning and memory were assessed in PAE and non-exposed 3xTg-AD mice (Fig. 7A). As expected, non-exposed 3xTg-AD mice already displayed early impairments relative to B6129 controls by 4 months of age ($p = 0.0299$, two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means), consistent with baseline deficits driven by PS1, APP, and Tau mutations. PAE-3xTg-AD mice exhibited further delays in escape latency in the Morris water maze compared to non-exposed 3xTg-AD mice, with escape latency significantly greater than B6129 controls ($p = 0.0005$, two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means), indicating that early-life alcohol exposure amplifies pre-existing spatial learning deficits. These results suggest that PAE interacts with the underlying AD pathology to exacerbate hippocampal-dependent learning and memory impairments.

Western blot analyses of γ-secretase activity in cortical (Fig. 7B, C) and hippocampal (Fig. 7D, E) lysates showed increased levels of APP CTFs, with a more pronounced increase in APP C99, in both PAE and non-exposed 3xTg-AD mice compared with B6129 controls, consistent with the effects observed in PS1 mutant models. Significant overexpression of the PS1 M146V transgene was detected in cortical tissue from non-exposed 3xTg-AD (38.51%, $p = 0.019$, one-way ANOVA with Tukey's post hoc multiple comparisons) and PAE-3xTg-AD mice (34.18%, $p = 0.0022$, one-way ANOVA with Tukey's post hoc multiple comparisons) relative to the B6129 control group (Fig. 7C). In the hippocampus, PS1 CTF levels were elevated but did not reach statistical significance (3xTg-AD, 28.70%, $p = 0.1584$, and PAE-3xTg-AD, 21.87%, $p = 0.2186$, one-way ANOVA with Tukey's post hoc multiple comparisons), likely reflecting greater inter-animal variability in transgene expression and/or differential regional susceptibility to PAE (Fig. 7E).

In both cortex and hippocampus, protein analysis revealed a significant increase in APP C99 levels in the cortex, 6 times higher (Figs. 7B and 7C, 3xTg-AD controls, $p < 0.0001$; PAE-3xTg-AD, $p < 0.0001$, one-way ANOVA with Tukey's post hoc multiple comparisons) and hippocampus, 20 times higher (Figs. 7D and 7E, 3xTg-AD controls, $p = 0.0084$; PAE-3xTg-AD, $p = 0.0009$, one-way ANOVA with Tukey's post hoc multiple comparisons) compared with the B6129 control group. APP C99 levels did not differ significantly between 3xTg-AD and PAE-3xTg-AD mice; however, greater variability was observed within the PAE group (CoV: cortex 3xTg-AD vs. PAE-3xTg-AD = 35.11% vs. 42.04%; hippocampus 3xTg-AD vs. PAE-3xTg-AD = 58.41% vs. 73.81%), which may reflect differences in individual vulnerability to prenatal alcohol exposure, variability in transgene expression, or an interaction between both factors.

Discussion

Preclinical evidence suggests that PAE may be a potential early-life risk factor that predisposes the brain to neurodegenerative vulnerability later in life, including susceptibility to AD/ADRD (Alhowail, 2022; Gerlikhman et al., 2023; Tousley et al., 2023; Walter et al., 2023). However, there are currently no human epidemiological datasets to substantiate this preclinical concept. One reason may be that FASD was not formally identified as a neurodevelopmental condition until the late 1960s (Lemoine, 1968; Jones et al., 1973; Jones & Smith, 1973). As a result, the earliest individuals who were identified under the FASD framework are only now

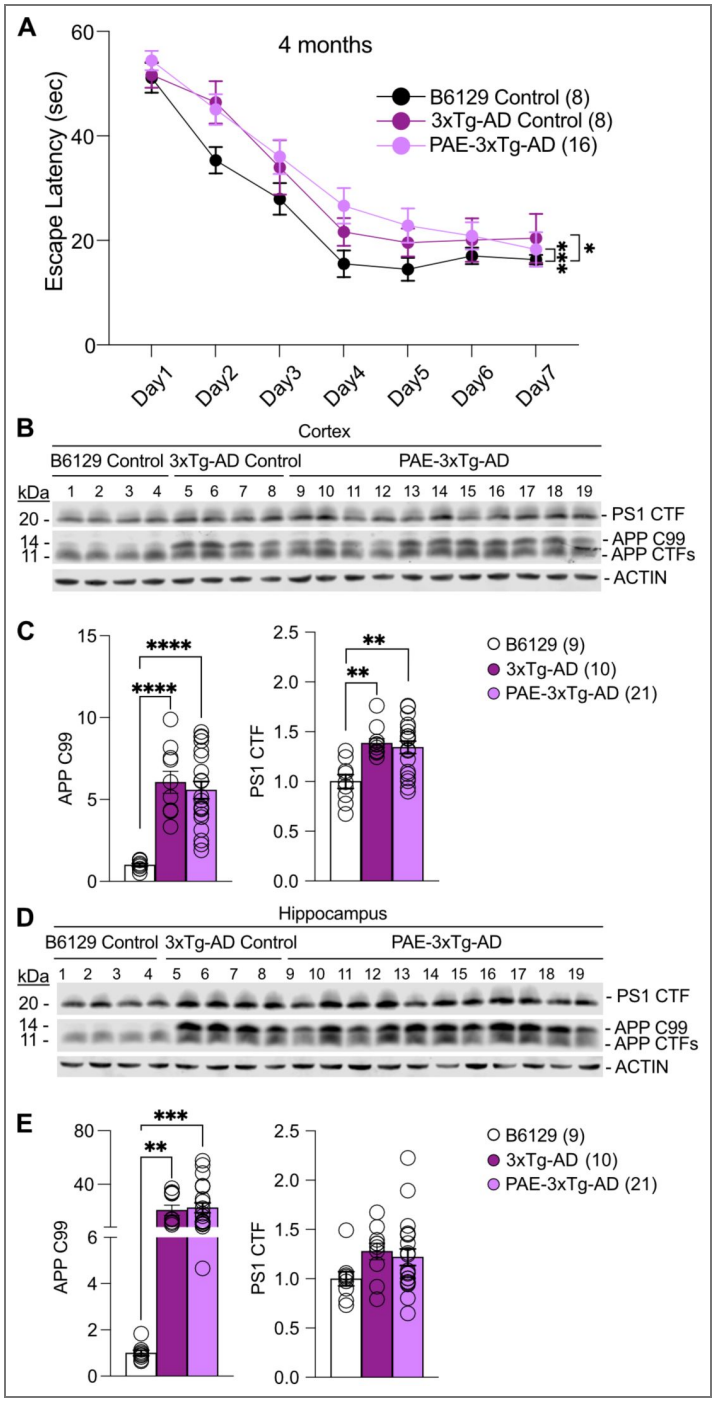

Figure 7. PAE alters learning and memory in 3xTg-AD mice in the pre-symptomatic stage.

(A) During the hidden platform version of the Morris water maze test, PAE-3xTg AD mice at 4 months of age (pre-symptomatic stage) display significantly longer escape latencies compared to B6129 controls ($p = 0.0005$). 3xTg-AD controls ($p = 0.0299$) show less significant differences in escape latencies compared to B6129 controls. Two-way ANOVA with Tukey's post hoc multiple comparisons of the main columns means. All data represent mean ± SEM *$p < 0.05$ ***$p < 0.001$. Sample size indicated in parentheses next to each experimental group label.

approaching the age at which AD/ADRD pathology and clinical symptoms typically begin to emerge. Consequently, there are no records of clinically diagnosed FASD individuals who have been systematically evaluated for Alzheimer's disease pathology or dementia, creating an opportunity for clinical and epidemiological studies to determine whether early-life alcohol exposure confers long-term risk for age-related neurodegeneration. In this context, preclinical studies are essential for establishing the biological basis for PAE as a developmental contributor to AD/ADRD risk.

The present study provides evidence that PAE disrupts γ-secretase–related processing during brain development, and that these alterations are associated with persistent deficits in hippocampal-dependent learning and memory later in life, reminiscent of AD/ADRD. Using a binge-like gestational exposure paradigm, we demonstrate that PAE induces early accumulation of APP CTFs and alters Notch-related processing in the short term that persist into adulthood and are accompanied by behavioral impairments, consistent with the hypothesis that PAE establishes long-lasting neurobiological changes that increase vulnerability to learning and memory decline.

It is noteworthy that the selection of ages for this study captures both pre-symptomatic and early symptomatic stages of pathology. In the 3xTg-AD mouse model, subtle synaptic dysfunction emerges as early as 3–4 months of age, preceding cognitive decline (Oddo et al., 2003). This pre-symptomatic window provides an opportunity to interrogate early alterations, particularly those related to γ-secretase activity and memory loss, before the onset of confounding widespread neurodegeneration. Incorporating age-matched wild-type cohorts allows for the detection of subtle, early memory impairments. The presence of early deficits in wild-type mice is especially relevant in the context of PAE, where neurodevelopmental insults may shift the baseline trajectory of cognitive function independent of AD transgenes. Assessing animals at 3, 4, and 6 months of age captures the progression from pre-symptomatic to more advanced stages, enabling differentiation between early vulnerability and later-stage pathology.

A key finding of this study is that a brief maternal binge-like ethanol exposure during mice gestational days 13–15, a window that approximates the late first trimester to early second trimester of human gestation (Peavey & Dotters-Katz, 2021), and is characterized by robust neurogenesis, neuronal migration, and early cortical and hippocampal circuit formation (Hill, 2023; Otis & Brent, 1954; Parnell et al., 2014), is sufficient to disrupt γ-secretase–dependent APP processing in the neonatal brain. The accumulation of APP CTFs in both cortex and hippocampus suggests reduced γ-secretase activity, as impaired cleavage of APP C99 is a recognized biochemical indicator of γ-secretase dysfunction (Bourgeois et al., 2018; Pulina et al., 2020). These effects were observed not only in 3xTg-AD mice carrying AD-linked mutations but also in wild-type mice, indicating that alcohol exposure alone can induce a molecular phenotype resembling presenilin-related dysfunction. This finding provides a potential mechanistic framework linking developmental alcohol exposure to pathways implicated in neurodegenerative disease.

The increased accumulation of APP CTF in cortical layers II–VI and hippocampal CA subregions is particularly relevant given the known vulnerability of these regions to alcohol-induced developmental insult (Cuzon et al., 2008; Delatour et al., 2019, 2020; Skorput & Yeh, 2016). Previous work has shown that PAE disrupts neuronal migration, dendritic maturation, and synaptic development in the cortex and hippocampus (Berman & Hannigan, 2000; Delatour et al., 2019, 2020; Guerri, 2002; Livy et al., 2003; Miller, 1993; Subbanna & Basavarajappa, 2022). Our findings suggest that impaired γ-secretase signaling may contribute to these abnormalities by altering developmental pathways regulated by APP and Notch processing.

The PAE effect on γ-secretase–related processing of APP persisted into adulthood. At both 3 and 6 months of age, PAE wild-type mice exhibited impaired performance in the Morris water maze, accompanied by persistent accumulation of APP CTFs in the hippocampus. These data suggest that developmental alcohol exposure produces long-lasting alterations in protein processing pathways

that may contribute to sustained hippocampal dysfunction. The progressive worsening of escape latency by 6 months further supports the notion that early molecular injury induced by alcohol exposure has enduring functional consequences.

Morris Water Maze performance reflects multiple cognitive and behavioral processes and should not be interpreted solely as a generalized measure of "memory impairment." In the present study, hidden platform acquisition trials primarily assessed hippocampal-dependent spatial learning. PAE mice did not exhibit deficits during visible platform testing, suggesting that the observed impairments were unlikely to result from visual or motor dysfunction. These findings support the interpretation that PAE correlates with a disruption of hippocampal-dependent spatial learning and memory processes in adulthood.

The findings in 3xTg-AD mice extend this interpretation and suggest that PAE acts as a disease modifier in the context of pre-existing genetic vulnerability. PAE exacerbated learning deficits in 4-month-old transgenic mice, an age at which early cognitive abnormalities begin to emerge in this model (Bourgeois et al., 2018; Javonillo et al., 2022). Although APP C99 accumulation was already elevated due to AD-associated mutations, the greater behavioral impairment observed following PAE suggests an interaction between prenatal alcohol exposure and AD-linked pathogenic mechanisms.

The absence of PAE-induced changes in PS1 CTF levels suggests that prenatal alcohol exposure does not directly affect the abundance of the presenilin protein. Rather, these findings support the presence of a regulatory mechanism acting at the level of γ-secretase function. Such regulation may involve altered catalytic efficiency, impaired complex maturation, changes in subcellular localization, or modifications in the interaction between γ-secretase and its substrates. Together, this suggests that PAE may influence APP processing through functional impairment of the proteolytic machinery rather than through altered expression of its core components.

The findings of the present study bear important implications, given that binge drinking during early pregnancy is clinically relevant, particularly during periods when pregnancy may not yet have been recognized - even a brief exposure during a relatively narrow developmental window may produce persistent molecular alterations in pathways critical for brain maturation and cognitive function later in life. This supports the concept that PAE may not only contribute to neurodevelopmental disorders such as FASD but may also increase vulnerability to age-related neurodegenerative processes.

Several limitations should be acknowledged. While the accumulation of APP CTFs strongly supports impaired γ-secretase activity, this interpretation is based on indirect biochemical readouts. Direct quantification of γ-secretase cleavage products, including Aβ40, Aβ42, and AICD, will be important in future studies to more precisely define how PAE alters enzyme activity, substrate processing, and peptide production. Assessing Aβ42/Aβ40 ratios will determine whether PAE shifts γ-secretase cleavage toward more aggregation-prone species, a key feature of AD/ADRD pathology.

In addition, the present study focuses on early and mid-adulthood time points and therefore does not address whether PAE accelerates the progression of AD-like neuropathology in older mice. Extended longitudinal studies will be necessary to associate early-life disruption of γ-secretase function with enhanced amyloid deposition, tau pathology, or progressive neuronal loss later in life. Future work should incorporate comprehensive neuropathological analyses, including assessment of amyloid plaque burden, tau hyperphosphorylation, synaptic function, and region-specific neurodegeneration in vulnerable brain areas such as the hippocampus and cortex.

While our behavioral and biochemical findings suggest persistent hippocampal dysfunction, direct evaluation of neurodegenerative processes, such as neuronal loss, dendritic spine alterations, and neuroinflammatory responses, was not performed. Given that accumulation of APP CTFs, particularly C99, has been implicated in synaptic toxicity and endosomal dysfunction (Bourgeois et al., 2018), it will be important to determine whether PAE-induced alterations in APP processing contribute to progressive synaptic degeneration and circuit-level dysfunction.

Assessing markers of gliosis and neuroinflammation will also be essential to establish whether PAE primes the brain for heightened inflammatory responses that may exacerbate neurodegenerative processes over time.

The variability observed in PAE cohorts may reflect differences in transgene expression, differential susceptibility to PAE, or interactions between genetic background and environmental exposure (Chen et al., 2011; Everson & Eberhart, 2022). These findings open avenues for future research aimed at elucidating the molecular mechanisms underlying individual variability in vulnerability to PAE-induced effects.

Future investigations should systematically assess how the timing, duration, and developmental stage of prenatal alcohol exposure (PAE) influence γ-secretase regulation, APP processing, and, commensurately, learning and memory. Direct comparisons with acute exposure paradigms, such as the 3-day binge drinking model, will be needed to determine whether the observed molecular alterations reflect cumulative developmental reprogramming or transient ethanol-induced perturbations. In addition, PAE at different gestational time points should be evaluated, as the embryonic and fetal brain undergoes stage-specific wave of neurogenesis, synaptic formation, and circuit refinement that may differentially determine vulnerability to long-term Alzheimer's disease–related molecular and neurobehavioral changes.

In summary, our findings identify disrupted γ-secretase–related processing as a novel molecular consequence of PAE and support a mechanistic link between early-life binge alcohol exposure and persistent hippocampal-dependent learning and memory dysfunction. These data provide the first experimental evidence that developmental alcohol exposure impacts core proteolytic pathways central to AD/ADRD pathogenesis.

Materials and Methods

Wild-type and Transgenic Mice

Genotype-verified breeder pairs were obtained directly from The Jackson Laboratory. Wild-type B6129SF1/J (B6129) Strain # 101043 (*https://www.jax.org/strain/101043*) and B6;129-Tg (APPSwe, tauP301L)1Lfa *Psen1*^{tm1Mpm}/Mmjax (3xTg-AD) MMRRC Strain # 034830-JAX (*https://www.jax.org/strain/004807*) were bred and maintained following guidelines and procedures approved by the Institutional Animal Care and Use Committee (IACUC) at Dartmouth College. Animals were housed in a 12 h light/dark cycle with light periods occurring between 7:00 AM and 7:00 PM.

Prenatal Alcohol Exposure (PAE)

As previously described, in the binge-type alcohol exposure paradigm (Delatour et al., 2019, 2020; Skorput & Yeh, 2016; Tousley et al., 2023), breeding trios of one male and two female mice were housed overnight for breeding, with the following day designated as embryonic day 0.5 (E0.5) and after which dams were singly housed and acclimated to an *ad libitum* control liquid diet from E10 to E12. From days E13 to E15. Feeding bottles were replaced daily with either 5% (w/w) ethanol liquid diet or an isocaloric control liquid diet (Table 1), with water available *ad libitum*. Dams were weighted daily throughout the PAE period. Wild-type B6129 dams reached a mean blood ethanol concentration (BEC) of 0.20 g/dL ± 0.006 g/dL, whereas 3xTg-AD dams reached a mean BEC of 0.11 g/dL ± 0.005 g/dL, as measured at 11:00 PM on E15.5 using an Analox AM1 series III analyzer (Analox Instruments). The lower BEC observed in 3xTg-AD dams versus the B6129 dams may reflect differences in ethanol preference, sensitivity, or metabolic processing (Lim et al., 2012; Rhodes et al., 2005). Importantly, both groups achieved BECs exceeding 0.08 g/dL, thereby meeting the NIAAA criteria for binge-level alcohol exposure (NIAAA, 2025).

Age-matched mice fed with the control liquid diet were used as non-ethanol-exposed control groups for all experimental comparisons. Non-ethanol-exposed B6129 mice served as genotype-matched controls for the PAE-B6129 cohort. Similarly, non-exposed 3xTg-AD mice served as baseline transgenic controls, providing the reference for AD-like pathological profile. This cohort

Diet ID	EtOH (mL)	Premix[1] (g)	Olive Oil (g)	Maltose[2] (g)	ddH2O (mL)
Control	0	109	28.4	87.5	775.1
5% EtOH	65	109	28.4	0	812.6

1. Premix L10251A (Research Diets)
2. Maltose Dextrin 42 I20005 (Research Diets)

Table 1. **Isocaloric liquid diet formulas for 1 liter preparation (w/w)**

served not only as the direct control for the PAE-3xTg-AD group, but also as an AD-relevant benchmark for comparison with the PAE-B6129 group, facilitating evaluation of whether PAE alone induces molecular changes that approach those observed in the 3xTg-AD model.

Western Blot Analysis

After birth, brains from P0 mice were harvested, whereas the remaining experimental mice were maintained for subsequent behavioral and biochemical analyses at 3, 4, and 6 months of age. The entire right hemisphere was reserved for immunohistochemical analysis. From the left hemisphere, the whole cortex was dissected from P0 mice, while the cerebral cortex and hippocampus were separately dissected at 3, 4, and 6 months of age. All tissues were fast frozen and stored for subsequent protein lysate preparation. Cortical and hippocampal tissues were manually homogenized using pestle homogenizers followed by passage through 1ml syringes fitted with 25G needles in radioimmunoprecipitation assay buffer, Pierce RIPA buffer (ThermoFisher Scientific, Cat No. PI89900) supplemented with phenylmethylsulfonyl fluoride (PMSF) protease inhibitor (Sigma, Cat No. 10837091001) and Halt protease inhibitor cocktail, EDTA-free (ThermoFisher Scientific, Cat No. 78425). Protein concentrations were quantified using the Pierce BCA protein assay kit (ThermoFisher Scientific, Cat No. 23225). Protein lysates were normalized to 3 ug/ul in NuPAGE LDS sample buffer (ThermoFisher, Cat No. NP0007) and NuPAGE sample reducing agent (ThermoFisher, Cat No. NP0009). Protein samples (20 - 40 µg total protein) were resolved on NuPAGE 4-12% Bis-Tris midi SDS-PAGE gels (ThermoFisher Scientific, Cat No. WG1402BOX) and transferred to a 0.45 µm nitrocellulose membrane (ThermoFisher Scientific, Cat. No. 88018) using wet transfer conditions. Membranes were blocked for 1 h in Intercept (TBS) blocking buffer (LICORbio, Cat. No. 927-60001) and incubated overnight at 4°C with primary antibodies specific for PS1 CTF, APP CTFs, and NICD (Table 2). Detection was performed using infrared-conjugated secondary antibodies (Table 2) and the Odyssey CLx imaging system (LICORbio). Protein bands were quantified with Fiji image processing software (ImageJ), with target protein levels normalized to actin as a loading control.

Behavioral Analysis

Mice were individually handled daily for 3 days, 5 min each, before testing. Three cohorts of naïve mice: Cohort 1: ~2.5 months of age; wild-type B6129 control (N=9), 3xTg-AD control (N=15), PAE-B6129 (N=19), Cohort 2: ~5.5 months of age; B6129 control (N=7), 3xTg-AD control (N=9), PAE-B6129 (N=14), Cohort 3: ~3.5 months of age; B6129 control (N=8), 3xTg-AD control (N=8), PAE-3xTg-AD (N=16). Each mouse was trained in the hidden platform version of the task for a total of 7-8 days. The Morris water maze is a circular pool of 120 cm in diameter. The release point for each training trial was varied among all four quadrants in a pseudorandom manner. Four distal visual cues were placed on the walls during training and full-cue probe tests. The escape platform (10 cm in diameter) was submerged 1 cm beneath the water surface, which was covered with plastic beads to prevent visual localization of the platform and maintained in a constant position. Mice received four training trials daily with a maximum duration of 60-90 s and an inter-trial interval of at least 15 min. Mice that did not locate the platform during a training trial were guided to the platform and allowed to remain on it for 15 s. The location of mice in the pool was continuously monitored using an automated video tracking system (Ethovision XT, Noldus). Probe trial tests were conducted on the morning of the last day (days 8-9) by removing the hidden platform and releasing mice from the opposite quadrant, allowing them to search for the platform location for 60s. To verify visual and swimming ability, the visual-cue version of the task was performed on days 8 or 9 with all distal cues removed. Four trials were administered, with the platform position marked by a visible object.

Histological Analysis

Upon completion of behavioral testing, mice were asphyxiated with CO2 and transcardially perfused with phosphate-buffered saline solution (PBS, pH 7.4) containing 0.25 g/L heparin (Sigma, Cat. No. H3393). Brains were hemisected, and the right hemispheres were post-fixed in 4%

Antibody Name	Vendor	Cat. No.	RRID	Titer
Rabbit anti-APP-Y188	Abcam	ab32136	AB_2289606	WB: 1:5000 IF: 1:200
Rabbit anti-PS1-CTF	Cell Signaling	5643S	AB_10706356	WB: 1:1000
Rabbit anti-cleaved Notch1	Cell Signaling Technology	4147S	AB_2153348	WB: 1:1000
Mouse anti-β-actin	Sigma Aldrich	A5441	AB_476744,	WB: 1:5000
Goat anti-mouse IRdye680	LI-COR Biosciences	926-68020	AB_2651128	WB: 1:10000
Goat anti-rabbit IRdye800	LI-COR Biosciences	926-32211	AB_2651127	WB: 1:10000
Alexa Fluor 555 goat anti-rabbit IgG	ThermoFisher Scientific	A-21429	AB_2535850	IF: 1:250

Table 2. **List of antibodies used in this study**

paraformaldehyde (Sigma, Cat. No. 158127) in PBS at 4°C with gentle shaking overnight. Subsequently, the brains were embedded in paraffin following standard procedures (Kang et al., 2021). Sagittal paraffin sections (10 μm) were obtained using a Epredia HM 355 S rotary microtome. For immunofluorescence staining, paraffin sagittal sections were first deparaffinized in Histo-Clear (Mercedes Scientific, Cat. No. NAT HS202), alcohol-dehydrated in sequential baths, and then incubated in antigen retrieval buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) for 30 min at 95°C. After cooling down, the sections were subjected to permeabilization with Tris-buffered saline containing 0.5% Triton X-100 for 30 min on a horizontal rocker. Sections were thoroughly washed with 1X PBS solution, transferred to a humid chamber, and blocked with 10% normal goat serum (MP Biomedical, Cat. No. 219135680) for 1.5 h, at RT. Sections were incubated with primary antibody against APP CTF (Table S2) overnight at 4 °C, followed by 1.5 h RT incubation with fluorophore-conjugated Alexa 555 secondary antibody (Table S2). All sections were counterstained with DAPI (Sigma, Cat.No. D9542). Immunofluorescent images were taken and analyzed using Zeiss LSM 510 Meta laser confocal microscope (Zeiss).

Data Quantification and Statistical Analysis

Data acquisition and quantification were performed blinded to genotype and treatment, with the exception of the Western blot analyses. All statistical analyses were performed using Prism (Version 11; GraphPad) and Excel (Microsoft). All data are presented as the mean ± SEM. The exact sample size (e.g. the number of mice) of each experiment is indicated in the figure. Statistical analysis was conducted using unpaired Student's t test for two groups comparison (Figs 1C – 4C, D, G, H – 6C, D, G, H), one-way ANOVA followed up by Tukey's multiple comparisons for three or more groups comparison (Figs. 1F – 2 – 7C, E), and two-way ANOVA followed up by Tukey's multiple comparisons (Figs. 3B – 5 – 7A). All statistical comparisons were performed on the data from ≥ 3 independent samples. Significance is shown as *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Data availability

All biochemical, immunichemical, and behavioral data have been deposited at *https://dataverse.harvard.edu/dataverse/PAE-PS1* and are publicly available.

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Additional information

Author Contributions

PM, HY designed the research; PM, RK, MZ, MC performed the research and analyzed the data; PY provided the transgenic mice; PM, HY wrote the paper with input from PY.

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Peer reviews

Reviewer #1 (Public review):

Summary:

The manuscript by Montenegro and colleagues reports a uniquely significant set of compelling results from a carefully designed study. The findings are fundamental and should substantially advance our understanding of whether prenatal exposure to high levels of alcohol produces neural changes that are precursors to the development of Alzheimer-like pathology in an animal model of Fetal Alcohol Spectrum Disorder (FASD). The quest was to test whether prenatal exposure to high-dose alcohol in the mouse would result in selective damage that would result in Alzheimer disease-like cellular disorder and mnemonic impairment. Both outcomes emerged and were exacerbated in relevant transgenic mice. The untoward effect on memory endured and even worsened with age.

Strengths:

The authors noted the importance of using a validated animal model to test their hypotheses related to AD-like outcomes because human postmortem data are unavailable and even in vivo data are limited to younger FASD cohorts. The authors further noted limitations, which also anticipate experiments that could chart the temporal course of the effect and windows of prenatal alcohol exposure that result in damage or resilience. In short, as the authors state on lines 435-7, "These data provide the first experimental evidence that developmental alcohol exposure impacts core proteolytic pathways central to AD/ADRD pathogenesis."

Weaknesses:

Addressing the following would clarify several points in an already well-written paper:

(1) It would be useful to have a timeline of the study, much like the one provided for the water maze protocol. On that timeline, please include the sample sizes examined, ages at exposure, and other pertinent procedures, indicating which animals remained alive for testing, etc.

(2) What were the attrition rates for each study group?

(3) What are the human age equivalents of the maternal mice?

(4) It would be helpful to see a graph of the BECs of each animal relative to the doses given. That would clarify how the alcohol exposure amount and timing are the same and where they are different for all exposed mice, given that the alcohol levels were somewhat different by group, as noted in the Methods. Were these BEC differences at all related to group differences in outcome measures or memory performance?

https://doi.org/10.7554/eLife.111776.1.sa3

Reviewer #2 (Public review):

Summary:

In this study, the impact of prenatal alcohol (PAE) on amyloid precursor protein (APP) C-terminal fragments and notch intracellular domain (NICD) levels in adulthood is measured in 3xTg-AD mice.

Prenatal alcohol alters gamma secretase activity with development and aging. This could have implications for Alzheimer's disease risk in populations without inherited Alzheimer's risk genetics.

Strengths:

Strengths include the model, the use of orthogonal approaches, and the rigorous, high-quality data.

Weaknesses:

Some figures lack prenatal alcohol treatment in the 3xTg-AD mice.

Some overstatements should be tempered. For instance, one cannot conclude that the changes in CTFs are driving the changes in learning and memory (as suggested in the last line of the abstract) without a direct intervention testing this. For instance, though PAE caused a more robust learning deficit at 6 mo in WT, the impact on CTFs was less than it was at 3 mo. PAE did not significantly change CTFs or learning/memory in 3xTg-AD mice at 4 months,

suggesting the genotype effect takes over at this point. The text should be adjusted to reflect this.

Conclusion:

In summary, this is a rigorous assessment of the long-term impacts of PAE on CTFs and learning/memory in adult WT and 3xTg-AD mice.

https://doi.org/10.7554/eLife.111776.1.sa2

Reviewer #3 (Public review):

Summary:

The goal of this study was to test the hypothesis that prenatal alcohol exposure (PAE) can affect Alzheimer's disease (AD) pathogenesis using biochemical proxies, histology, and a behavioral paradigm sensitive to AD-related memory decline. Major strengths include the breadth of techniques used, consideration of different AD-related molecular markers, and the use of different ages as well as appropriate controls. The authors largely achieved their aims to show that PAE does affect amyloid precursor fragments (CTFs) and notch signaling very early on as well as long-term effects in adulthood, which may uncover a previously underappreciated mechanism that may contribute to AD-related neuropathology and behavioral outcomes during lifespan.

Strengths:

(1) Several techniques are used to address molecular, behavioral, and histological PAE-related changes.

(2) There is use of appropriate controls and an AD-relevant mouse model.

(3) Different ages are used to address age-related and long-term effects in AD and control mice.

(4) The novelty of results shows early changes in amyloid-related processes, affected by PAE.

Weaknesses:

(1) It is unclear as to whether there are sex differences, particularly in the adult cohort.

(2) More clarity is needed on sample size per cohort and whether mice that were used for anatomy and biochemical analyses were previously used for behavior. Including a table and noting any overlap would be useful.

(3) In many instances, two-way ANOVAs with treatment (PAE vs vehicle) and genotype as factors will be useful to report (e.g., Figure 1).

(4) In Figure 5 and line 253, it is stated that older mice have more severe deficits, but there are no direct statistical comparisons with younger AD mice.

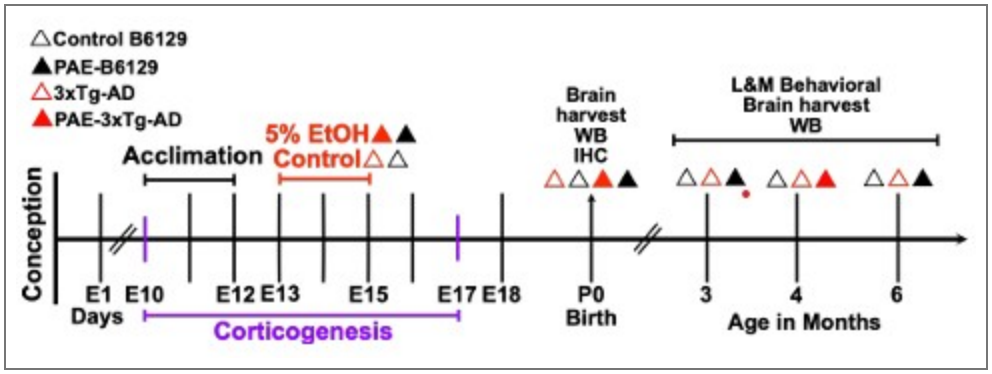
(5 Lines 270-271 refer to mice as "presymptomatic", but these mice do have behavioral symptoms. Do the authors mean no neuropathology yet? Any data showing lack of robust neuropathology would be useful.

https://doi.org/10.7554/eLife.111776.1.sa1

Author response:

> ***Public Reviews:***
>
> ***Reviewer #1 (Public review):***
>
> *(1) It would be useful to have a timeline of the study, much like the one provided for the water maze protocol. On that timeline, please include the sample sizes examined, ages at exposure, and other pertinent procedures, indicating which animals remained alive for testing, etc.*

We agree with the reviewer that a timeline would be helpful for clarifying the PAE exposure paradigm and the subsequent sample collection and processing. Sample sizes vary across the different analyses; therefore, we have indicated the sample size for each experiment in the corresponding figure. To further improve clarity, we will add Author response image 1, which will include a schematic of the overall experimental timeline, including the PAE regimen, collection time points, and sample processing, as shown below.



Author response image 1.

> *(2) What were the attrition rates for each study group?*

We thank the reviewer for raising this important point. There was no attrition of animals within the experimental groups; the number of animals included at the beginning and end of the study remained the same. However, we did observe a reduction in litter size following prenatal alcohol exposure (PAE). In our 3xTg-AD colony, litters typically consisted of approximately 8 pups under control conditions, whereas PAE litters occasionally contained 4–6 pups. Thus, the reduction in animal numbers reflects decreased litter size associated with PAE rather than attrition during the study. We would also like to clarify that the primary scope of this study was not to provide a terminal/end-point analysis of disease progression, but rather to investigate the emergence of Alzheimer's disease (AD)-related phenotypes during early adulthood following PAE. Accordingly, our longitudinal experimental design focused on identifying the earliest molecular, synaptic, behavioral, and neuropathological alterations that emerge during this period. This approach allowed us to examine whether PAE accelerates or precipitates the onset of AD-related symptomatology in the 3xTg-AD model, rather than following the animals through advanced disease stages. We will clarify this rationale in the revised manuscript.

> *(3) What are the human age equivalents of the maternal mice?*

We appreciate the reviewer's question regarding the age of the maternal mice. The dams used in our study were young adult females (2 to 3 months of age) at the time of breeding. Because chronological age does not translate linearly between mice and humans, particularly during development and reproductive maturation, we have avoided assigning a precise human-age equivalent. Based on established comparative developmental frameworks and

calculations, these animals represent a 20 years old young-adult in the reproductive stage, rather than an advanced maternal-age condition [1]. We will clarify this point in the revised manuscript.

> *(4) It would be helpful to see a graph of the BECs of each animal relative to the doses given. That would clarify how the alcohol exposure amount and timing are the same and where they are different for all exposed mice, given that the alcohol levels were somewhat different by group, as noted in the Methods. Were these BEC differences at all related to group differences in outcome measures or memory performance?*

We appreciate the reviewer's suggestion to provide a more detailed representation of the BEC data. We agree that displaying the BECs for individual animals would provide additional clarity regarding the consistency of alcohol exposure across groups. We have therefore included the individual BEC values in the new Figure 1. We observed some variability in BECs between the 3xTg-AD and B6129 groups, as noted in the Methods. Importantly, however, the average alcohol consumption was comparable between the two genotypes, indicating that the difference in BECs was not due to differences in the amount of alcohol consumed. All dams in the PAE groups reached BECs above 0.08 g/dL, the commonly used legal blood alcohol concentration limit in the United States, supporting the use of our paradigm as a binge-like alcohol exposure model. We further examined whether the variability in BECs was associated with the differences observed in outcome measures, including memory performance. We did not find evidence that the differences in BECs accounted for the group differences in behavioral or molecular outcomes. Thus, although some intergroup variability in BECs was present, the overall alcohol exposure was comparable, and the observed phenotypic differences were not attributable to differences in alcohol consumption.

> ***Reviewer #2 (Public review):***
>
> *(1) Some figures lack prenatal alcohol treatment in the 3xTg-AD mice.*

We appreciate the reviewer's observation and agree that the rationale for the different experimental groups across the figures should be clarified. The primary focus of this study is to characterize the effects of prenatal alcohol exposure (PAE) in wild-type B6129 mice, with the 3xTg-AD mice serving primarily as a disease-model reference to determine whether the effects observed following PAE in wild-type animals overlap with or resemble features of AD pathology. Accordingly, the initial figures focus on the effects of PAE in B6129 mice and include the non-exposed 3xTg-AD group as a reference for the AD phenotype. The last two figures specifically address the effects of PAE in the 3xTgAD model, with the 3xTg-AD mice becoming the experimental subject of interest rather than serving solely as a disease reference. For this reason, the PAE-3xTg-AD group is not included in the earlier figures, whereas it is included in the final two figures where the effect of PAE on the AD model is directly evaluated. We will clarify this experimental rationale in the revised manuscript and figure legends.

> *(2) Some overstatements should be tempered. For instance, one cannot conclude that the changes in CTFs are driving the changes in learning and memory (as suggested in the last line of the abstract) without a direct intervention testing this. For instance, though PAE caused a more robust learning deficit at 6 mo in WT, the impact on CTFs was less than it was at 3 mo. PAE did not significantly change CTFs or learning/memory in 3xTg-AD mice at 4 months, suggesting the genotype effect takes over at this point. The text should be adjusted to reflect this.*

We appreciate the reviewer's careful consideration of this point. We agree that the relationship between APP CTF accumulation and learning and memory deficits should not be interpreted as causal in the absence of a direct intervention experiment. We were careful in choosing the wording throughout the manuscript to describe these findings as associated

changes rather than evidence of a causal interaction. Our data demonstrate the presence of APP CTF accumulation and learning and memory deficits following PAE, but they do not establish that CTF accumulation directly drives the behavioral phenotype. We therefore will temper the language in the Abstract and throughout the manuscript to avoid overstatement. We also acknowledge that the relationship between these phenotypes is not necessarily linear across age: although PAE produced a more pronounced learning deficit at 6 months in B6129 mice, the magnitude of APP CTF accumulation was greater at the earlier time point. Importantly, we consider the possibility that the greater APP CTF accumulation observed at earlier ages may represent an early molecular insult whose functional consequences become evident later in life. In this context, the temporal dissociation between the molecular and behavioral phenotypes could be consistent with a "two-hit" model, in which an early-life insult induced by PAE creates or primes a pathological vulnerability that subsequently manifests as cognitive dysfunction with ageing [2]. We recognize, however, that this interpretation remains a hypothesis and would require longitudinal mechanistic studies to establish. This temporal relationship may also contribute to the broader concept of early-life origins of AD/ADRD, suggesting that prenatal environmental exposures may initiate molecular alterations during neurodevelopment that remain detectable or predispose the brain to later-life dysfunction. Similarly, the absence of significant changes in APP CTFs or learning and memory in 4-month-old 3xTg-AD mice suggests that the effects of the AD genotype may become dominant at this stage. Consistent with this interpretation, we state in the Discussion that future studies are needed to identify and experimentally test the direct molecular pathways affected by PAE that ultimately contribute to learning and memory impairment. We will revise the text accordingly to make this distinction clear while highlighting the potential significance of an early molecular insult preceding the later emergence of behavioral phenotypes.

> ***Reviewer #3 (Public review):***
>
> *(1) It is unclear as to whether there are sex differences, particularly in the adult cohort.*

We appreciate the reviewer's comment regarding potential sex differences. We agree that considering sex as a biological variable is important, particularly for the adult cohorts. To address this point, we will include identifying marks for male and female animals separately in our plots where sample size permits. This will allow the reader to better evaluate potential sex-dependent effects of PAE and to determine whether the observed phenotypes are consistent across sexes. We will also clarify this approach in the revised manuscript.

> *(2) More clarity is needed on sample size per cohort and whether mice that were used for anatomy and biochemical analyses were previously used for behavior. Including a table and noting any overlap would be useful.*

We appreciate the reviewer's suggestion and agree that greater clarity regarding the sample sizes and use of animals across analyses is important. The sample size for each cohort and experimental group is indicated in the corresponding figures, and we will make this information more explicit in each figure legend to facilitate interpretation. Animals that underwent behavioral testing were subsequently used for biochemical analyses, allowing us to examine molecular changes in the same animals in which behavioral phenotypes were characterized. In contrast, for the neonatal cohort, we performed both anatomical and biochemical analyses, to assess the distribution and extent of APP CTF accumulation across the brain during this early developmental period. This approach was selected to provide a broader assessment of the spatial distribution of APP CTF accumulation at birth. We will clarify these experimental details in the revised Methods and figure legends.

> *(3) In many instances, two-way ANOVAs with treatment (PAE vs vehicle) and genotype as factors will be useful to report (e.g., Figure 1).*

We appreciate the reviewer's suggestion regarding the use of two-way ANOVA with treatment and genotype as factors. However, we respectfully disagree that this approach is appropriate for all of the analyses presented in this manuscript. Our experimental design and the specific biological questions addressed in each experiment were not uniform across cohorts. In particular, the primary objective of the study was to characterize the effects of PAE in B6129 mice, with the 3xTg-AD mice serving primarily as a disease-model reference, while the effects of PAE in the 3xTg-AD model were specifically examined in the later experiments. Therefore, combining genotype and treatment as factors across all datasets would not always reflect the experimental questions or the structure of the cohorts. In addition, some experiments did not include all four groups, making a two-way ANOVA inappropriate for those analyses. Nevertheless, we agree that a two-way ANOVA may be informative for experiments in which both genotype and treatment are fully represented and the experimental design supports this analysis. We will therefore consider and apply two-way ANOVA, where appropriate, to those datasets, including evaluation of the main effects of genotype and treatment and their interaction. We will clarify the statistical approach and its rationale in the revised Methods and figure legends.

> *(4) In Figure 5 and line 253, it is stated that older mice have more severe deficits, but there are no direct statistical comparisons with younger AD mice.*

We appreciate the reviewer's observation. We agree that, in the absence of a direct statistical comparison between age groups, the statement that older mice have "more severe deficits" may be too strong. Our intention was to describe the apparent progression of the phenotype across age rather than to imply that we had statistically demonstrated an age-dependent increase in severity. We have therefore revised the text in Figure 5 and at line 253 to use more cautious language, describing the greater magnitude of the observed deficits in older mice without implying a direct statistical comparison between age groups. We agree that a formal conclusion regarding age-dependent progression would require a statistical analysis, which we will include in the revised manuscript.

> *(5) Lines 270-271 refer to mice as "presymptomatic", but these mice do have behavioral symptoms. Do the authors mean no neuropathology yet? Any data showing lack of robust neuropathology would be useful.*

We appreciate the reviewer's careful observation. We agree that the term "presymptomatic" was not sufficiently precise, particularly because the mice already exhibit measurable behavioral alterations at this age. Our intention was not to suggest that these animals were free of phenotypic abnormalities, but rather that they were at an early stage of disease progression, before the emergence of robust neuropathological features. We have therefore revised the terminology to avoid referring to these mice as "presymptomatic." Instead, we describe them as being in an early stage of disease development, characterized by emerging behavioral and molecular alterations but without the extensive cardinal neuropathology typically associated with later stages of the 3xTg-AD phenotype. We agree that the distinction between behavioral symptoms and neuropathological progression is important. In this study, our focus was on the emergence of early AD-related phenotypes during young adulthood, rather than on establishing the absence of neuropathology. We have therefore avoided making a definitive claim regarding the lack of neuropathology and have revised the text to more accurately reflect the scope of our data.

Additional References

(1) Dutta, S. & Sengupta, P. Men and mice: Relating their ages. Life Sci. 152, 244–248 (2016).

(2) Gunn, J. S. et al. Exploring the 'Multiple-Hit Hypothesis' of Neurodegenerative Disease: Bacterial Infection Comes Up to Bat'. Frontiers in Cellular and Infection Microbiology | *www.frontiersin.org* 1, 138 (2019).

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