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RESEARCH ARTICLE

Interactions between menopause and high-fat diet on cognition and pathology in a mouse model of Alzheimer's disease

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Abstract

INTRODUCTION: Post-menopausal women constitute about two-thirds of those with Alzheimer's disease (AD). Menopause increases dementia risk by heightening the likelihood of metabolic disease, a well-known risk factor for dementia. We aimed to determine the effects of menopause and high-fat diet (HF) on cognitive and pathological outcomes in an AD mouse model.

METHODS: At 3 months old, App^{NL-F} mice received 4-vinylcyclohexene diepoxide (menopause model) or vehicle and were placed on a control (10% fat) or an HF diet (60% fat) until 10 months old.

RESULTS: An interaction between HF diet and menopause led to impaired recognition memory. No effects of menopause were observed on amyloid pathology. However, menopause induced alterations in microglial response, white matter, and hippocampal neurogenesis.

DISCUSSION: This work highlights the need to model endocrine aging in animal models of dementia and contributes to further understanding of the interaction between menopause and metabolic health in the context of AD.

KEYWORDS
Alzheimer's disease, amyloid, menopause, metabolic disease, microglia, neurogenesis, white matter

Highlights

- The combination of menopause and HF diet led to early onset of cognitive impairment.
- HF diet increased amyloid pathology in the hippocampus.

- Menopause led to an increase in microglia density and a decrease in myelin in the corpus callosum.
- Menopause altered hippocampal neurogenesis in a diet-dependent manner.

1 | BACKGROUND

Alzheimer's disease (AD) is the leading cause of dementia, affecting about 11% of people aged 65 years and older.[1] Two-thirds of those with Alzheimer's are women, the majority of whom are postmenopausal.[1] Menopause, characterized by the cessation of menstrual cycles and loss of ovarian estrogens, is a risk factor for AD and related dementias.[2–5] In fact, menopause leads to increased neuroinflammation, increased vulnerability to neural damage due to loss of circulating estrogens, and increased risk of metabolic disease.[6,7] Menopause, which usually occurs in mid-life, has been associated with increased weight gain and visceral fat accumulation, increased rates of type 2 diabetes, and altered brain glucose metabolism.[5,8,9] A younger age at menopause is associated with greater cognitive decline.[4,10,11] Further, the frequency of the vasomotor symptoms (hot flashes) experienced by women during the menopause transition (peri-menopause) is associated with more white matter damage and cognitive impairment, two common features of AD.[12–14] In addition, dynamic modifications to the immune and metabolic systems, during and after the menopause transition, are relevant to AD and related dementias and may provide a framework for prevention or precision therapeutic approaches.[15] It is therefore necessary to better understand and consider these interactions as treatments and their timing may be heavily affected by the endocrine status.

Metabolic disease is a modifiable risk factor for AD and related disorders.[16–19] Clinically, metabolic disease is characterized as having at least three of the following: obesity (excess abdominal weight or visceral fat), hyperlipidemia, low levels of HDL (high-density lipids), high blood pressure, and elevated blood sugar levels (pre-diabetes or type 2 diabetes). Metabolic disease in individuals older than 60 years is associated with an 11-fold increase in risk of AD compared to metabolically healthy individuals.[18] In mid-life, metabolic disease is considered a major risk factor for AD, as diabetes at that stage of life doubles dementia risk, and obesity increases AD risk by three-fold.[16–19] In line with this, about 80% of those with AD have prediabetes or type 2 diabetes.[20] Metabolic disease features are also observed in animal models of AD and seem to affect female rodents more than males. For example, female 3xTg-AD mice, on normal chow diet, display higher adiposity and body weight compared to male mice starting at 6 and 9 months of age, respectively.[21] Further, high-fat (HF) diet leads to worse AD pathology and cognitive deficits in animal models of dementia, with female rodents being more affected than males.[20,22–28] These observations prompt us to investigate the effects of menopause on AD pathology and cognitive decline and how they can be affected by comorbid metabolic disease.

In the following study, we used the knock-in mouse model of AD, $App^{NL\text{-}F}$ mice[29] to probe the effects of menopause on AD pathology and other dementia-related brain abnormalities in the presence of comorbid metabolic disease. We used a chemically induced accelerated ovarian failure model of menopause to model peri-menopause, an important transition period, and keep the ovaries intact. This model therefore has a clinically relevant advantage in modeling hormonal changes associated with natural menopause over gonadectomy, which leads to a sudden drop in estrogens and loss of ovarian androgen production. Metabolic disease was modeled by giving the mice an HF diet for the duration of the study as opposed to control mice that received a low-fat (LF) control diet. We hypothesized that menopause and metabolic disease may have negative effects on several outcomes, including cognitive decline, β-amyloid pathology, activation of microglia, white matter changes, and adult hippocampal neurogenesis (AHN).

2 | METHODS

2.1 | Animals and experimental design

All experiments were conducted in compliance with the ARRIVE guidelines and approved by the Albany Medical College Institutional Animal Care and Use Committee. $App^{NL\text{-}F}$ ($App^{tm2.1Tcs}$/$App^{tm2.1Tcs}$) mice were used to model AD, as they start accumulating plaques around 6 months of age.[29] $App^{NL\text{-}F}$ mice were acquired congenic on a C57BL/6J background from Dr. John Cirrito at Washington University following Material Transfer Agreement (MTA) from Riken, Japan. Mice, homozygous for the $App^{tm2.1Tcs}$ transgene were bred in-house and were weaned and fed a standard chow diet (Purina Lab Diet 5P76) until the start of this study. They were kept at three to five per cage, at ~21°C, 30%–70% humidity, with a 12-h light/dark cycle. At 3 months of age, cages of mice were randomized to treatment groups and placed on either an HF diet (60 kcal% fat, D12492, Research Diets, New Brunswick, New Jersey, USA) or an LF diet (10 kcal% fat, D12450J, Research Diets, New Brunswick, New Jersey, USA). At the same time of diet switch, mice were also randomized between control and post-menopausal cages. Menopause was modeled using accelerated ovarian failure as described previously.[30,31] Briefly, mice received daily intraperitoneal (i.p.) injections of 4-vinylcyclohexene diepoxide (VCD, 160 mg/kg) diluted in sesame oil for 20 consecutive days. Control mice were i.p. injected with the same volume vehicle (sesame oil). Vaginal cytology was used to monitor estrus cyclicity starting 2 months after the first

injection (mice ~ 5.5 months old). Mice that remained in diestrus for 10 consecutive days were declared acyclic/post-menopausal. Control (oil-injected) mice received vaginal cytology to check that they were still cycling and to control for any effects due to handling. A subset of mice received 3 consecutive daily i.p. injections of 5-ethynyl-2′-deoxyuridine (EdU at 10 μL/g body weight of a 10 mg/mL solution in saline) 40 days before the end of the study. At ~9 months old, mice received a battery of behavioral tests described below. At the end of study, vaginal cytology was performed, then under heavy isoflurane anesthesia, blood was collected by cardiac puncture. Mice (~10 months old) were then perfused with ice-cold heparinized saline. Ovaries were collected to confirm menopause (follicular atresia). Brains were collected and sagittally bisected, with one hemisphere randomly selected to undergo post-fixation for use with immunofluorescent labeling, and the other hemisphere regionally dissected and flash frozen on dry ice then stored at -80°C for molecular analysis.

2.2 | Open field testing

To evaluate locomotor activity and anxiety-like behavior, we used an open field test, as previously described.[28,30,32,33] Mice were placed in a square arena (45 × 45 cm) and allowed to explore freely for 10 min, then removed and placed in a "recovery cage" so as not to expose naïve cage mates to them. Videos were analyzed using ANY-maze software (Stoelting, Wood Dale, Illinois, USA). Total distance traveled (m) was measured as a proxy for locomotor activity. The percentage of time spent in the center of the arena was used as an inverse indicator of anxiety-like behavior.

2.3 | Object place and novel object recognition

The combined test was used for spatial recognition and object recognition memory testing. The open field arena was used, with a spatial cue (a horizontal piece of black tape) placed on the wall where objects were initially placed. The test was conducted as previously described[33] and consisted of three 10-min trials: a "training" trial, the object place recognition trial (OPRT) trial, and the novel object recognition trial (NORT) trial, with an inter-trial interval of one and a half hours.

2.4 | Barnes maze

Hippocampal-dependent spatial learning and memory were assessed using the Barnes maze (Stoelting, Catalog # 60170), as previously described.[30] Mice were trained to find an escape pod located under one of the 20 holes in the maze using spatial cues in the room. Mice were given two training trials a day (3 min each or until the mouse found the pod) and 3 h between trials during the same day for 3 days to test spatial learning. The search strategy was determined for each mouse on each trial according to the methods published by O'Leary et al.[34] Search strategy data were analyzed by comparing the percentage of mice adapting a random search strategy (the mouse failed to enter the escape hole or moved into the center of the maze and returning to areas already visited) within each group of a cohort (studies were conducted in four cohorts that were balanced so that each treatment group was equally represented).[34] Spatial memory was tested on the fifth day (1 day break) by allowing the mice to explore the maze for 2 min.

RESEARCH IN CONTEXT

1. **Systematic review**: Although two-thirds of those suffering from Alzheimer's disease (AD) are women who are most likely postmenopausal, preclinical research in the AD field has very few studies considering endocrine aging (menopause). Increased neuroinflammation, vulnerability to neural damage, and risk of metabolic disease make menopause an important sex-specific risk factor for dementia in women. Moreover, up to 80% of AD patients suffer from metabolic disease. In this study, we thought to determine the effects of menopause and high-fat (HF) diet-induced metabolic disease on cognitive and pathological outcomes in the $App^{NL\,F}$ mouse model of AD.
2. **Interpretation**: We used the accelerated ovarian failure model of menopause, which is more clinically relevant to natural menopause than ovariectomy. We found that menopause and HF diet have independent and synergistic effects on cognitive and pathological aspects of AD. An interaction between HF diet and menopause led to accelerated onset of impaired object recognition memory. When examining the underlying pathology, we did not detect any changes caused by menopause in amyloid pathology; however, we observed menopause induced changes in microglia response, myelin, and hippocampal neurogenesis. Potentially, further impairment might emerge with age/advanced pathology in this model.
3. **Future directions**: This work highlights the need to model endocrine aging in animal models of dementia and contributes to further understanding the interaction between menopause and metabolic health in the context of AD. Further studies are needed to elucidate different cellular and molecular mechanisms behind these interactions on diverse pathologies. Hormone therapies such as estrogen replacement can be beneficial in some cases. We are currently assessing the therapeutic potential of a brain-specific estrogen prodrug to protect against the detrimental effects of menopause on brain health. New therapeutic strategies are needed to alleviate the burden of AD, especially in post-menopausal women who represent the greatest percentage of AD patients.

2.5 | Immunofluorescent labeling

Half brains (right or left hemispheres) were randomly assigned to be used for immunofluorescent labeling. They were fixed overnight in a 4% paraformaldehyde (PFA) solution, cryoprotected in 30% sucrose, then frozen in optimal cutting temperature (O.C.T.) solution (23-730-571, Thermo Fisher Scientific), and stored at -80°C until further processing. Tissue sections (35 µm thickness) were obtained in eight alternating series of consecutive sections using a cryostat (Cm1950, Leica) and stored at 4°C in cryopreserve solution (1% polyvinylpyrrolidone, 30% ethylene glycol, 30% sucrose, and 0.01% sodium azide in phosphate buffered saline [PBS]).

Immunofluorescent labeling was performed for microglia and β-amyloid as previously described on four sections per animal (two containing the dorsal hippocampus between -1.34 and -2.06 mm from bregma and two containing the ventral hippocampus between -2.92 and -3.52 mm from bregma).[35] Briefly, slices were permeabilized and blocked for 1 h at room temperature using 0.3% Triton X-100 in PBS (TPBS) with 5% donkey serum solution. Primary antibodies: goat anti-Iba-1 (1:1000; PA5-18039, lot #TI2638761, Thermo Fisher Scientific), rat anti-CD68 (1:1000; MCA1957, lot #1708, Bio-Rad), and rabbit anti-β-amyloid (1:500; 71-5800, lot# SH257822, Thermo Fisher Scientific) were applied overnight at 4°C in blocking solution. Corresponding secondary antibodies were added in blocking buffer (1:500) for 1 h at room temperature. Slices were counter-stained with 4',6-diamidino-2-phenylindole (DAPI; 1:1000) and washed three times before being mounted.

Another set of sections (two per animal) containing the dorsal hippocampus (approximately −1.06 to approximately −2.06 mm from bregma) was labeled for neurogenesis as previously described.[36] EdU was labeled using the Click-iT EdU Alexa Fluor 647 Imaging Kit (C10340, Thermo Fisher Scientific). Sections were then permeabilized and blocked for 1 h at room temperature using 0.5% Triton X-100 in PBS (TPBS) with 10% donkey serum solution. Primary antibodies: rabbit anti-NeuN (neuronal-specific nuclear protein; 1:1000; ABN78, lot #3041797, Millipore) and guinea pig anti-doublecortin (DCX, 1:1000; AB2253, lot #3951142, Millipore) were applied overnight at 4°C in blocking solution. Corresponding secondary antibodies were added in blocking buffer (1:500) for 2 h at room temperature. Slices were counter-stained with DAPI (1:1000) and washed three times before being mounted.

A third set of sections (two per animal) containing the dorsal hippocampus (approximately −1.06 to approximately −2.06 mm from bregma) was used to label dividing cells using Ki67. After blocking and permeabilization for 1 h at room temperature using 0.3% Triton X-100 in PBS (TPBS) with 5% donkey serum solution. The rabbit anti-Ki67 primary antibody (neuronal-specific nuclear protein; 1:1000; ABN78, lot #3041797, Millipore) was applied overnight at 4°C in blocking solution. The corresponding secondary antibody was added in blocking buffer (1:1000) for 1 h at room temperature. Slices were counter-stained with DAPI (1:1000) and washed three times before being mounted.

Secondary antibodies used included Alexa Fluor 647 donkey anti-goat (705-605-147, Jackson ImmunoResearch), Alexa Fluor 488 Donkey anti-rabbit (711-545-152, Jackson ImmunoResearch), Rhodamine Red-X donkey anti-rat (712-295-150 Jackson ImmunoResearch), Alexa Fluor 488 Donkey anti-guinea pig (706-545-148, Jackson ImmunoResearch), and Alexa Fluor® 647 Donkey anti-rabbit (711-605-152, Jackson ImmunoResearch).

For white matter staining, two sections per animal containing the dorsal hippocampus were incubated for 1 h at room temperature in 0.3% TPBS with 5% donkey serum solution, then with FluoroMyelin green (1:500; F34651, lot#2079492, Thermo Fisher scientific) and DAPI (1:1000) in 0.2% TPBS for 25 min at room temperature before being washed three times in PBS.

All sections were mounted between slides and coverslips using ProLong Gold Antifade (P36930, Thermo Fisher scientific). Images of brain slices were obtained at 10x magnification using the Axio Observer Fluorescent Microscope (Carl Zeiss Microscopy, Oberkochen, Germany). The same exposure times for each stain/labeling were used across all animals.

2.5.1 | Quantification of β-amyloid plaques

Image brightness was adjusted similarly for all animals in ImageJ (NIH) software. Plaques were manually counted by an experimenter masked to treatment conditions in (1) the area around the retrosplenial and motor cortex (Rsp Ctx), which plays a key role in memory and spatial learning, and is one of the first areas to show functional impairment in AD[37]; (2) the area around the entorhinal and piriform cortex (Ent Ctx) which is involved in processing spatial and non-spatial information and is often the earliest area to show histological alterations in AD[38]; (3) the hippocampus (Hipc), which is key for memory and spatial navigation, a location for adult neurogenesis, and is known to be severely affected in AD[39]; and (4) the corpus callosum (CC) a major white matter tract that is important for cognitive and executive functions and is shown to be atrophied in AD.[40]

2.5.2 | Quantification of microglia-related measures

Iba1 and CD68 images were thresholded using ImageJ (NIH) software. Regions of interest (ROIs) were drawn around the Rsp Ctx, Ent Ctx, Hipc, and CC to quantify the average area covered by cells positive for each of these antibodies, as well as their colocalization. Further, the hippocampus was divided into several areas of interest including the cornu ammonis region 1 and 3 areas of the hippocampus (CA1 and CA3, respectively), the dentate gyrus (DG), and in the ventral sections the subiculum (Sub). All measurements were performed by an experimenter masked to treatment conditions. Each value is an average of approximately two ROIs from the sections containing the dorsal or ventral hippocampus of each animal.

2.5.3 | Quantification of myelin-related measures

Image brightness was adjusted similarly for all animals in ImageJ (NIH) software and ROIs were drawn by a blinded experimenter in the central part of the CC (CC center), the CA1 area of the hippocampus (CA1), the area of the CC above CA1 (CC mid), the Rsp Ctx, and the Ent Ctx. The thickness of the CC was measured in the center of the section (in the area where the CC would cross to the contralateral hemisphere). Fluorescent staining intensity was then measured in all ROIs using the raw unmodified images. Intensity for each ROI was averaged across the sections of the same animals and used for statistical analysis.

2.5.4 | Quantification of neurogenesis-related measures

The numbers of Ki67+, EdU+, DCX+, EdU+/NeuN+, and EdU+/DCX+ cells were manually counted in the entire DG area in two sections throughout the dorsal hippocampus in ZEN software (blue edition, Carl Zeiss Microscopy) by two experimenters masked to the treatment conditions.

2.6 | Enzyme-linked immunosorbent assay

Frozen hippocampi and cortices were homogenized in 200 µL of T-Per buffer (ThermoScientific #78510) supplemented with protease and phosphatase inhibitor cocktail (HALT, ThermoScientific #1861284) and spun at 21,000 g for 20 min at 4°C. The supernatant containing soluble proteins was removed and stored at -80°C. The pellet containing insoluble proteins was resuspended in 100 µL of 70% formic acid and stored at -80°C. On the day of the enzyme-linked immunosorbent assay (ELISA), the samples were thawed, and 2 mL (20x) of neutralizing solution (1 M Tris base, 0.5 M Na2HPO4, 0.05% NaN_3) was added. The neutralized sample solution was diluted at 1:16 in sample diluent and processed for Aβ quantification using the Human Aβ40 ELISA (KHB3481, Thermo Fisher scientific) and Human Aβ42 ELISA Kits (KHB3441, Thermo Fisher scientific) according to the manufacturer's instructions. Aβ40 levels were below the detection level.

2.7 | Statistical analysis

Statistical analyses were completed using GraphPad Prism (GraphPad Software v10, San Diego, California, USA). Statistical outliers were removed following identification using Grubbs' test with alpha set at 0.01. For behavioral studies, mice were used as individual observations except in the analysis of search strategy over time in the training trial of Barnes maze, where each data point represents the percentage of mice/group that used a random search strategy within the cohort (four cohorts including each of the experimental groups equally represented). For histological studies, each data point represents the average value of sections analyzed per animal. Data were analyzed using two-way analysis of variance (ANOVA) followed by Tukey's post hoc test to adjust for multiple comparisons, except for measures tracked over time (Barnes maze) that were analyzed via three-way repeated measures ANOVA or mixed effects model analysis. A one-sample *t*-test was performed for measurements that were compared to chance (25% for time in the target quadrant of the Barnes maze and 0% for the object preference). Statistical significance was set at $p < 0.05$. Data are expressed as mean ± SEM.

3 | RESULTS

3.1 | Menopause and HF diet impair cognitive behavior

Metabolic disease is a known risk factor for AD. HF diet induces cognitive deficits in dementia mouse models, more so in female mice than in males.[28,32] Little is known about the interaction between menopause and HF diet. To gain further insight, we subjected control and post-menopausal female App^{NL-F} mice that were given either a LF or HF diet, to a battery of behavioral tests (Figure 1A). Using open field testing, we found that mice on an HF diet traveled less distance than those on an LF diet (Figure S1A, main effect of diet $p < 0.0001$); thus, we used measures for all subsequent analyses that were independent of activity levels. We used the Barnes maze to assess spatial learning and memory, respectively. We found a main effect of diet on impaired spatial learning, as evidenced by HF mice having a longer distance to escape the maze (Figure 1B, main effect of diet $p < 0.0001$), a higher number of errors (Figure 1C, main effect of diet $p < 0.0001$), and a higher percentage of a random search strategy throughout training trials (Figure 1D, main effect of diet $p = 0.0004$). Neither HF diet nor menopause seemed to impair spatial memory at early AD/pathology stages (APP^{NL-F} mice 10 mo), as no differences between groups were observed in the percentage of time mice spent in the target quadrant (Figure 1E), or the path efficiency to first find the target hole during the probe trial (Figure 1F). We used the novel object test to assess object recognition memory. We found that an HF diet worsened the performance of mice, as evidenced by a main effect of diet in lowering preference for the novel object (Figure 1G, main effect of diet $p = 0.0088$). Additionally, we found that post-menopausal mice on an HF diet had an impaired object recognition memory, as they were the only group that failed to show a significant preference (Figure 1G, *t*-test against chance, 0%). Further, post-menopausal mice on an HF diet were more impaired than their LF-fed controls (Figure 1G; Tukey's post-hoc test, $p = 0.0266$). To make sure low activity levels did not affect the novel object recognition test, we ran a correlation between the recognition preference and the distance traveled and found no association (Figure S1B, $r^2 = 0.0024$, $p = 0.7617$). These results show that HF diet leads to impaired spatial learning and impaired object recognition memory, and that a combination of HF diet and menopause causes the most severe impairment of object recognition memory.

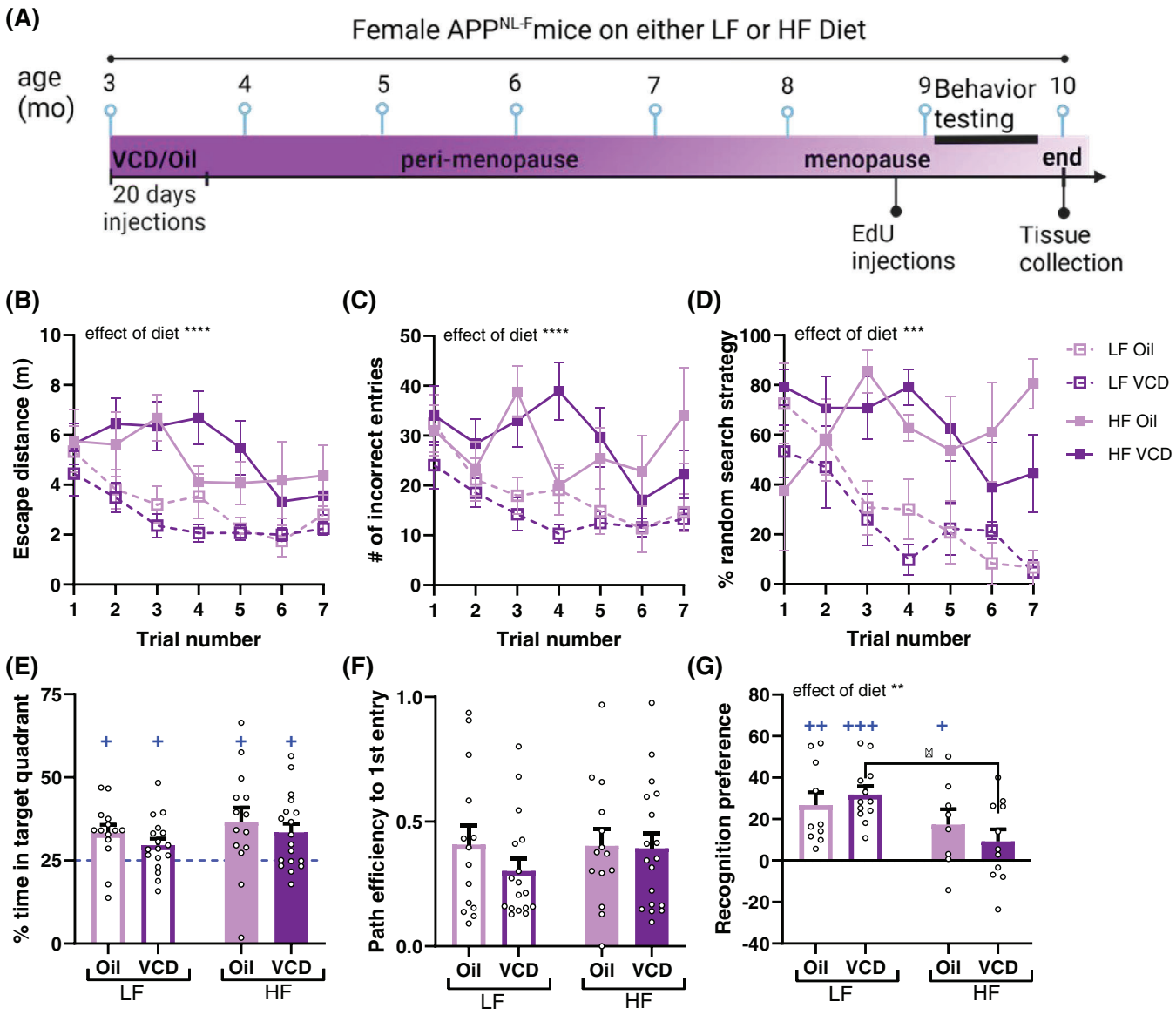

FIGURE 1 Metabolic effects of HF diet in WT versus APP^{NL-F} female mice. (A) An experimental timeline is shown. Spatial learning as assessed using escape distance (B) and number of errors/incorrect entries (C) during the training trials (learning phase) of the Barnes maze. **** $p < 0.0001$ main effect of diet three-way repeated-measure ANOVA; $n = 8–12$ mice/group. (D) The percentage of mice in each experimental group within the cohort using a random search strategy to escape the Barnes maze plotted over the seven learning trials. *** $p < 0.001$ main effect of diet three-way repeated-measure ANOVA; $n = 4$ cohorts/timepoint. Spatial memory was assessed using the % time spent in the target quadrant (E), and path efficiency for first entry (F) during the probe (testing) trial of Barnes maze. (E) Plus signs (+) are used for a *t*-test against chance (25%) indicating intact memory. + $p < 0.05$; $n = 14–18$ mice/group. Novel object recognition was used to test object recognition memory. (G) The preference difference between the old and new object at the same location. **$p < 0.01$ two-way ANOVA and Tukey's post-hoc test; $n = 8–12$ mice/group. Plus signs (+) are used for a *t*-test against chance (0) indicating intact memory. + $p < 0.05$, ++$p < 0.01$, +++$p < 0.001$. ANOVA, analysis of variance; HF, high-fat; LF, low-fat;VCD, 4-vinylcyclohexene diepoxide (menopause model).

3.2 | HF diet increases the number of β-amyloid plaques in the hippocampus

We sought to examine differences in β-amyloid plaques. We quantified the number of plaques in four different areas of the brain: the dorsal hippocampus, the area around the retrosplenial and motor cortex (Rsp Ctx), the area around the entorhinal and piriform cortex (Ent Ctx), and the CC (CC, Figure 2). We found an increase in the number of plaques in mice on an HF diet only in the hippocampus (Figure 2B, effect of diet $p = 0.0463$) and a trend in the entorhinal cortical area (Figure 2C, effect of diet $p = 0.0840$). Additionally, we evaluated the quantities of insoluble Aβ oligomers using ELISA in the cortex and hippocampus. We found no differences in Aβ42 levels in either region (Figure S2) and Aβ40 levels were below the detection level of the kit. These results indicate

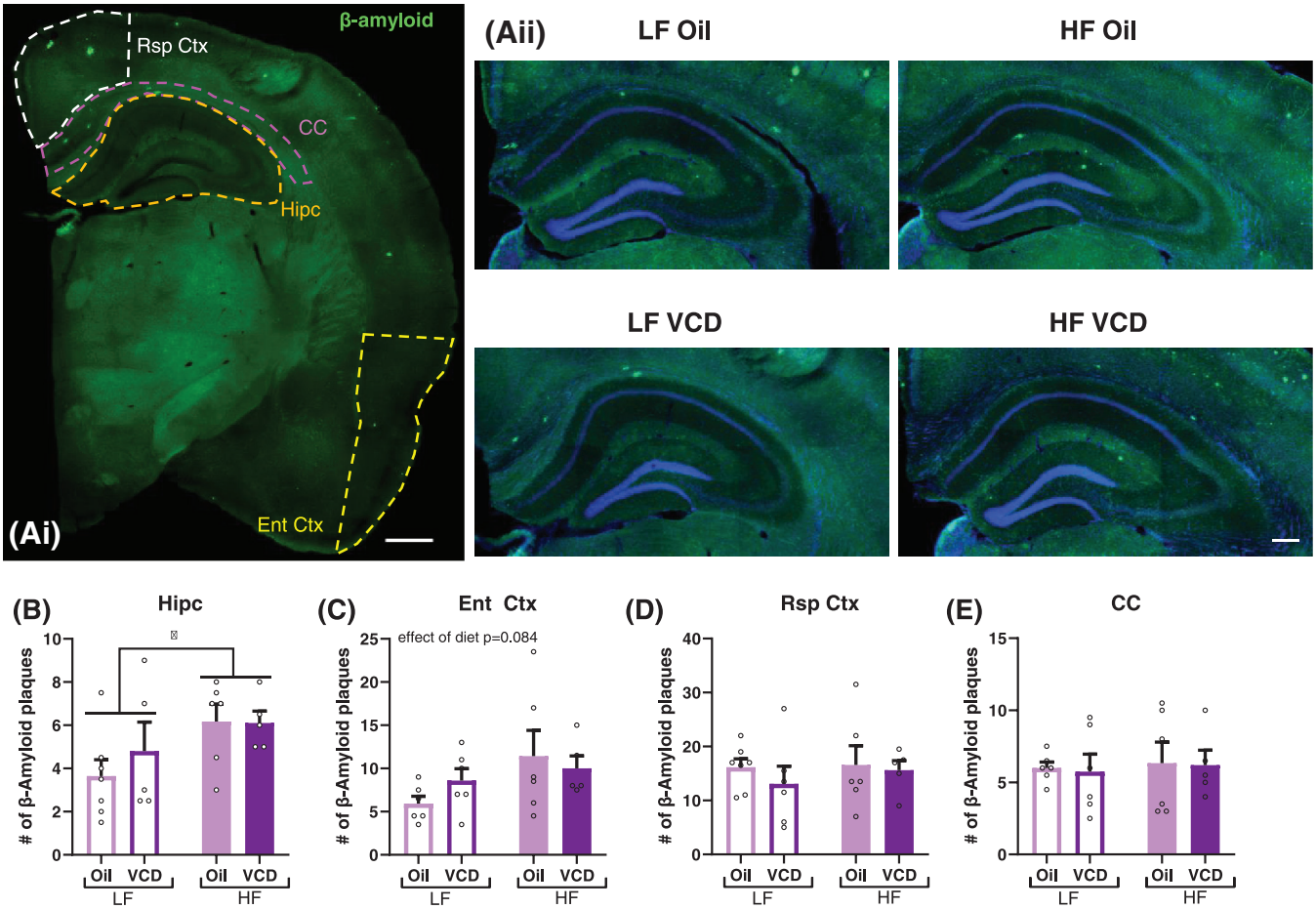

FIGURE 2 Effects of HF diet and menopause on amyloid pathology. Representative image of β-amyloid (green) immunofluorescent labeling in a full hemisphere (Ai). Dotted lines represent different regions of interest including the cortex areas around the retrosplenial and motor cortex (Rsp Ctx, white) and around the entorhinal and piriform cortex (Ent Ctx, yellow), the hippocampus (Hipc, orange), and the corpus callosum (CC, magenta). Zoomed in representative images of the hippocampus of each of the experimental groups are shown in Aii. Scale bar in Ai 500 μm and in Aii 200 μm. Quantification of the number of plaques in the hippocampus (B), the Ent Ctx (C), the Rsp Ctx (D), and the CC (E). A two-way ANOVA was used to assess the effect of diet and menopause; * $p < 0.05$ effect of diet; $n = 3$–7 mice/group. ANOVA, analysis of variance; HF, high-fat; LF, low-fat;VCD, 4-vinylcyclohexene diepoxide (menopause model).

that menopause, as opposed to HF diet, does not seem to exacerbate amyloid pathology in early AD/pathology stages.

3.3 | Menopause increases the area of microglia coverage in several brain areas

Both HF diet and menopause have been shown to increase neuroinflammation, notably microglia reactivity.[41–44] We assessed microglia reactivity in the cortex, hippocampus, and CC using immunolabeling for Iba1 and CD68 in the dorsal (Figure 3A), and ventral (Figure S3A) parts of the brain. In the sections containing the dorsal hippocampus, we did not observe any differences between groups for the area occupied by microglia labeling in the Hipc, Ent Ctx, or Rsp Ctx (Figures 3B, D, and F). In the CC, menopause led to an increase in Iba1+ area density (Figure 3H, main effect of menopause $p = 0.0115$). We further divided the hippocampus into several ROIs and found that menopause also led to an increase in Iba1+ area density in the dCA1 and dDG but not the CA3 (Figures 3J-N, main effect of menopause $p = 0.0244$ in CA1 and $p = 0.0448$ in the dDG). Additionally, we found that HF diet led to a decrease in the Iba1 area in the CA1 only (Figure 3J, main effect of diet $p = 0.0407$). No effect of diet nor menopause was observed on Iba1+CD68+ labeling (Figures 3C, E, G, I, K, M, and O). In the sections containing the ventral hippocampus (Figure S3), we only observe a diet by menopause interaction in the vDG (Figure S3G, $p = 0.0342$) in which the Iba1+CD68+ area was lower in menopausal mice on a LF diet but higher in menopausal mice on an HF diet. These results show that postmenopausal mice have a greater microglia density independent of diet in the white matter and subregions of the dorsal hippocampus.

3.4 | Menopause causes loss of myelin in a diet-dependent manner

White matter damage is a hallmark of AD and related dementias.[45] Menopause transition has been associated with greater white

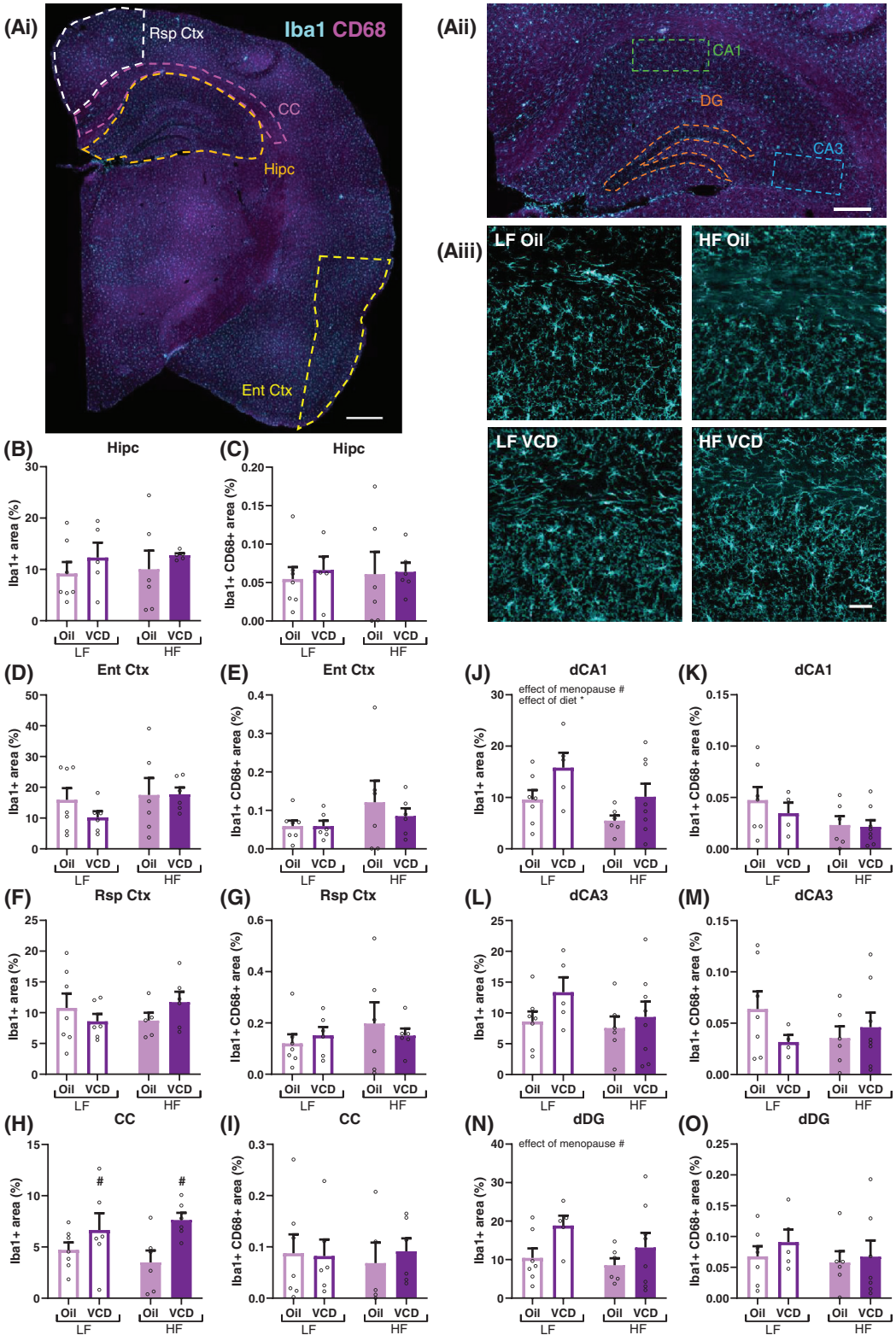

FIGURE 3 Effects of HF diet and menopause on microglia. Representative images of microglia labeling using anti-Iba1 (cyan, microglia marker) and CD68 (magenta, lysosomal marker) are shown in Ai and Aii for hippocampal specific ROIs. Zoomed in representative images of the corpus callosum (CC) and the CA1 of the hippocampus (Hipc) of each of the experimental groups are shown in Aiii. Scale bar in Ai 500 μm, in Aii 250 μm, and in Aiii 50 μm.Quantification of the area density (%) occupied by the Iba1 labeling in the Hipc (B), Ent Ctx (D), Rsp Ctx (F), CC (H), CA1 (J), CA3 (L), and DG (N). Quantification of the area density (%) occupied by the Iba1 and the CD68 labeling in the cortex the Hipc (C), Ent Ctx (E), Rsp Ctx (G), CC (I), CA1 (K), CA3 (M), and DG (O). A two-way ANOVA was used to assess the effect of diet and menopause; $^{\#}$ $p < 0.05$ effect of menopause; * $p < 0.05$ effect of diet; $n = 5–7$ mice/group. ANOVA, analysis of variance; CA, cornu ammonis; DG, dentate gyrus; HF, high-fat; LF, low-fat; ROI, region of interest; VCD, 4-vinylcyclohexene diepoxide (menopause model).

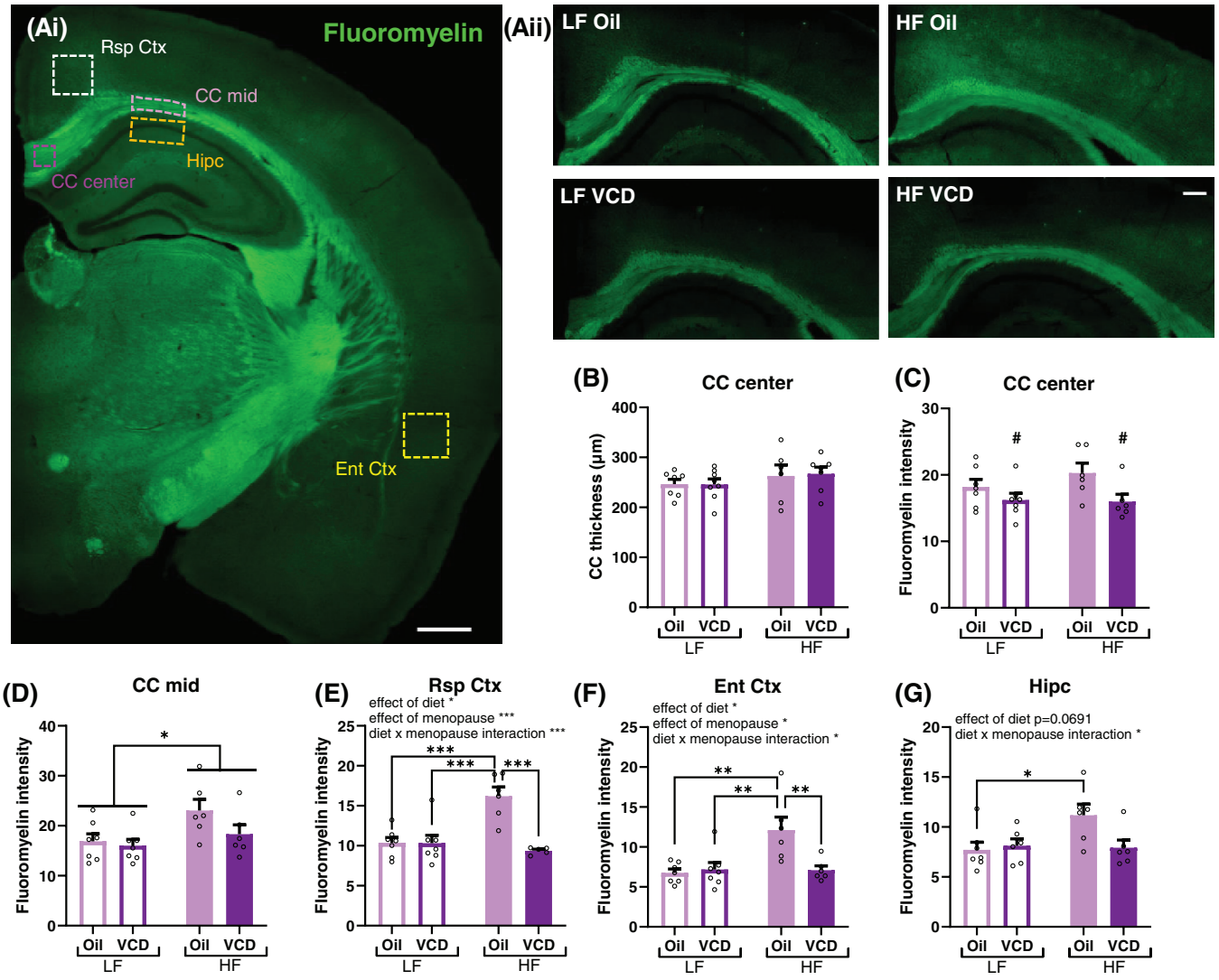

FIGURE 4 Effects of HF diet and menopause on myelin. Representative image of FluoroMyelin (green) staining in a full hemisphere (Ai). Dotted lines represent different regions of interest including the cortex areas around the retrosplenial and motor cortex (Rsp Ctx, white) and around the entorhinal and piriform cortex (Ent Ctx, yellow), the CA1 of the hippocampus (orange), the central part of the corpus callosum (CC center, magenta), and area of the CC above CA1 (CC mid, pink). Representative images of each experimental group are shown in Aii. Scale bar in Ai 500 µm and in Aii 200 µm. Measures of the center CC thickness in µm (B). Quantification of the fluorescent staining intensity in the CC center (C), the CC mid (D), the Rsp Ctx (E), the Ent Ctx (F), and the hippocampus (G). A two-way ANOVA was used to assess the effect of diet and menopause; $^{\#}p < 0.05$ effect of menopause, $^{*}p < 0.05$ effect of diet. Main effects are indicated above the graphs when there was a significant interaction or a trend. Tukey's post-hoc test was used for multiple comparisons; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $n = 5–7$ mice/group. ANOVA, analysis of variance; HF, high-fat; LF, low-fat; VCD, 4-vinylcyclohexene diepoxide (menopause model).

matter hyperintensities (WMHs), indicating greater damage.[12,46] To assess myelin changes, we measured the thickness of the CC at the interhemispheric connection and stained with FluoroMyelin (a highly specific myelin stain) and quantified staining intensity in different brain regions (Figure 4A). No changes were found in the CC thickness at the interhemispheric intersection (Figure 4B). Menopause led to a decrease in staining intensity in the central part of the CC (CC center) regardless of diet (Figure 4C, main effect of menopause $p = 0.0156$). In the area of the CC above the hippocampus (CC mid), we found a main effect of diet in increasing myelin (Figure 4D, main effect of diet $p = 0.0221$). Additionally, we measured staining intensity in gray matter areas such as the Rsp Ctx, the Ent Ctx, and the CA1 region of the hippocampus. In all three regions, we found a diet × menopause interaction wherein HF diet led to a higher staining intensity in oil injected (control) but not post-menopausal mice (Figure 4E, Rsp Ctx diet × menopause interaction $p = 0.0009$; Figure 4F, Ent Ctx diet × menopause interaction $p = 0.0100$; Figure 4G, CA1 diet × menopause interaction $p = 0.0444$). These results show that menopause affects myelin loss either independently or in a diet-dependent manner, depending on the brain region.

3.5 | Menopause alters hippocampal neurogenesis in a diet-dependent manner

Metabolic disease and HF diet have been shown to dysregulate neurogenesis.[36,47–52] Estrogens are shown to modulate AHN; however, their effects are dependent on the type of estrogen and the duration of exposure.[53] Little is known about how menopause affects this process with comorbid metabolic disease. We first probed cell proliferation within the DG of the dorsal hippocampus using immunolabeling for Ki67 (a nuclear protein expressed in actively dividing cells, Figure 5A) and injections of EdU (a thymidine analog that incorporates into the cell's DNA during mitosis, Figure 5C). There was no effect of either diet or menopause on the number of Ki67+ cells (Figure 5B, Figure S4A). However, we observed a diet × menopause interaction wherein the HF diet or menopause on their own led to a decrease in the number of EdU+ cells in the DG (Figure 5D, diet × menopause interaction $p = 0.0489$, Figure S4B). We then counted the number of doublecortin positive cells (DCX, neuroblasts/immature neurons). DCX is specific to cells committed to neuronal fate and is expressed by new neurons from ~4 days until ~4 weeks after cell birth in mice.[54,55] There was no change in the number of DCX+ cells (Figure 5E, F, Figure S4B). Additionally, we quantified the % of DCX+ cells among the EdU+ ones (arrowhead in Figure 5) and found a diet × menopause interaction wherein post-menopausal mice on HF diet had a greater % of cells compared to those on a LF diet (Figure 5G, diet × menopause interaction $p = 0.0464$). As EdU injections were done ~40 days before the mice were euthanized, these cells are likely to be ones that began differentiating into neurons but remained in an immature phase. We therefore quantified the % of NeuN+ cells (mature neuron marker) among the EdU+ ones (Figure 5H, I) and found a main effect of diet ($p = 0.0094$) and a main effect of menopause ($p = 0.0316$) in decreasing the percentage of newly formed mature neurons (arrow in Figure 5). This effect was strengthened by HF VCD mice having the lowest percentage (Tukey's post hoc test Oil LF vs. VCD HF $p = 0.0044$). These results suggest that menopause effects on AHN are dependent on the HF diet.

4 | DISCUSSION

Menopause is understudied in AD preclinical models. The loss of estrogens' protective effects, and the vasomotor symptoms (hot flashes) that occur during peri-menopause, have been associated with an increased risk of metabolic disease, worse cognitive decline, and increased pathology such as WMHs.[2,4–6,10–13,56] Our previous study using these mice confirmed that menopause led to metabolic impairment (weight gain and glucose intolerance) and exacerbated metabolic disease caused by HF diet.[35] Here, we sought to understand how menopause affects AD in the presence of metabolic disease. To model menopause, we used the accelerated ovarian failure model by VCD injections. This is a follicle-depleted, ovary-intact model where mice go through peri-menopause then post/menopause where the ovaries cease to produce estrogens but continue to produce other hormones (similar to post-menopausal women).[31,57] Using the App^{NL-F} AD mouse model, we show that the combination of menopause and HF diet led to accelerated cognitive decline. Mice showed impaired object recognition memory at 10 months old, which equates to early disease stage; further impairment might emerge with age. Additionally, we show that menopause led to an increase in microglia density and a decrease in myelin. Further, menopause altered AHN in a diet-dependent manner. Conversely, we observed that HF diet impaired spatial learning and caused increased amyloid pathology in the hippocampus. Our findings highlight the impact of menopause and emphasize the need for more inclusivity of sex-specific factors and comorbidities in preclinical dementia research. This also suggests the need for considering metabolic health as a covariable in clinical studies assessing sex differences in AD or other sex-specific factors such as menopause or use of hormone therapy.

Cognitive decline increases after menopause.[2,4,6,12,13,56] Metabolic disease is associated with increased cognitive impairment in dementia patients and preclinical models.[20,22–27,58,59] Here, we show that, at early disease stages in the App^{NL-F} mice, menopause on its own did not affect cognitive function. However, the combination of menopause and HF diet led to impairment of object recognition memory. This is consistent with our previous study where menopause, in wild-type mice, did not alter cognition but, in combination with cerebral hypoperfusion, led to increased deficits in object recognition memory and activities of daily living.[30] These results may indicate that menopause increases the vulnerability to negative effects of other comorbidities such as metabolic and cerebrovascular disease which can be due to the loss of neuroprotective effects of estrogen.[60,61]

In women, earlier age at natural or surgical menopause and late start of hormone replacement therapy (HRT) are associated with increased AD pathology and cognitive decline.[4,14,62–64] In ovariectomized nonhuman primates, estradiol HRT reduces the number of amyloid-β plaques even in monkeys on a Western-style diet.[65] Several mechanisms of protection have been described in vitro, as estrogens promote Aβ degradation[66] and decrease Aβ generation by primary cultures of rat, mouse, and human embryonic neurons.[67] However, we did not observe any effects of menopause induced estrogen loss on the number of plaques. This may be because we are examining a timepoint which equates to early disease stage in this model. Ovariectomy in APP(SWE) mice has been shown to increase the number of amyloid-β plaques.[68] Future studies are needed to examine menopause effects at later ages and disease stages. Indeed, we have recently demonstrated that, in mice, menopause effects on metabolic changes are influenced by chronological age.[57] These studies suggest an increased vulnerability to dementia in those who go through menopause at a younger age and may require a special clinical follow up plan compared to normally cycling women.

Metabolic disease has been shown to be associated with increased Aβ accumulation.[28,69,70] To date, one other study has examined the effects of an HF diet in App^{NL-F} mice and found that, in male App^{NL-F}, HF diet for 12 months led to an increase of Aβ in the hippocampus.[71] Here, we show that in female App^{NL-F} mice, an HF diet for 7 months had similar effects. In APP/PSEN1 mice, insoluble Aβ42 levels in the cortex were increased after 12 months of dietary intervention.[70]

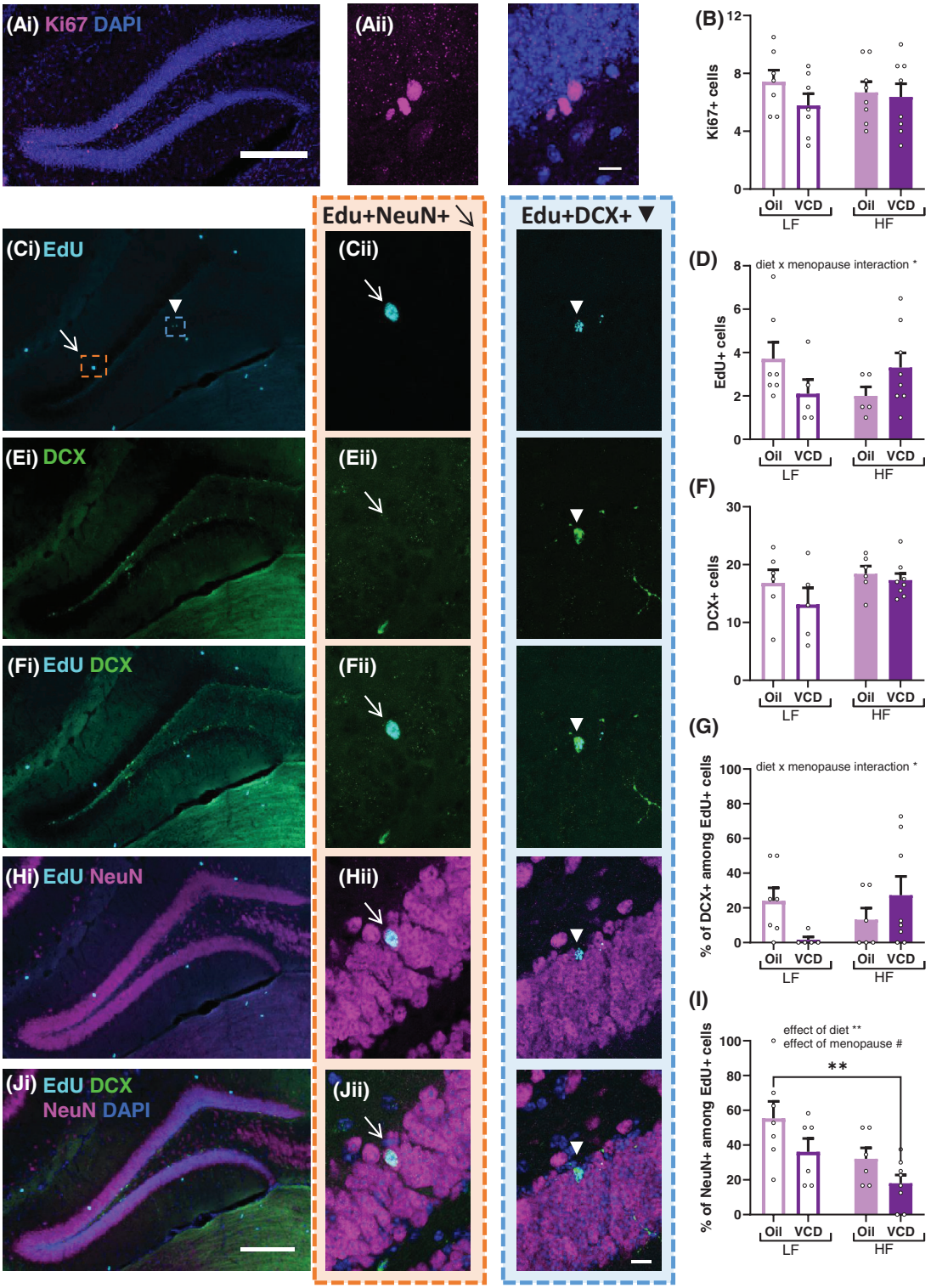

FIGURE 5 Effects of HF diet and menopause on neurogenesis. The number of proliferating cells in the DG was assessed using Ki67 immunolabeling (Magenta, Ai, Aii). Representative images of EdU staining (Cyan, cells born ~40days before endpoint), DCX (green, immature neurons), and NeuN (magenta, mature neurons) are shown in panels C-J with i panels showing the entire Hipc and ii panels zoomed in on specific cells (orange shaded boxes and arrows show EdU+NeuN+ cells, blue shaded boxes and arrowheads show EdU+DCX+ cells). DAPI (cell nuclei) is in blue in panels A and J. Scale bar in i 200 μm and in ii 10 μm. Quantification, in the dentate gyrus, of the number of Ki67+ cells (B), EdU+ cells (D), DCX+ cells (F), the percentage of DCX+ cells among the EdU+ cells (G), and the percentage of NeuN+ cells among the EdU+ cells (I). A 2-way ANOVA was used to assess the effect of diet and menopause. Main effects are indicated above the graphs when there was a significant interaction. Tukey's post-hoc test was used for multiple comparisons; $^{*}p < 0.05$; $n = 5–8$ mice/group. ANOVA, analysis of variance; DCX, doublecortin; EdU, 5-ethynyl-2′-deoxyuridine; HF, high-fat; LF, low-fat; VCD, 4 vinylcyclohexene diepoxide (menopause model).

These data indicate that HF diet leads to exacerbation of amyloid pathology.

Microgliosis is observed in AD and metabolic disease.[41–43,72] During menopause, microglia are thought to transition to an activated state[15,46,73] potentially due to loss of estrogen (modulator of microgliosis in rodents).[74,75] We did not detect any effects of HF diet or menopause on Iba1+ or Iba1+CD68+ areas in most brain regions except the CC, where menopause led to an increase in microglia area density. This is in line with our previous study where we did not find any effects of menopause (accelerated follicular atresia) on cortical expression of microglial genes (Iba1 and CD68) in wild-type mice even after cerebrovascular injury.[30] The increase in microglia only in the white matter region (CC) is in line with previous findings in rats, where endocrine aging leads to selective microglial reactivity targeted to the white matter.[74] The lack of effects of HF diet on microglia in most brain areas and the decrease in the hippocampus CA1 are consistent with previous studies showing that HF diet led to no change or decrease in microglia density and activation in female mice compared to males.[36,76,77] The controversial nature of these results can simply be because most studies are conducted exclusively in males. Further studies are needed to investigate microglia and neuroinflammation in female brains and how it is modulated by other factors.

WMHs are an indicator of underlying damage, including myelin loss, and have been reported in up to 90% of AD patients.[78] During perimenopause, hot flashes are associated with greater WMH burden.[12] Our study shows that menopause leads to a decrease in myelin in the CC center. We further observe an increase of intensity with HF diet in other brain regions that is specific to non-menopausal females. A possible explanation could be the increased amount of energy provided by the diet preventing myelin catabolism in response to decreased brain glucose metabolism induced by menopause.[79] Further studies are needed to test this hypothesis and elucidate if and how menopause might hinder this phenomenon.

Deficits in AHN may contribute to cognitive decline and increase the risk of dementia. Estrogens are known modulators of AHN and neuronal plasticity, and their effects vary depending on the type and other events such as pregnancy/parity.[80–84] Estradiol has been shown to increase proliferation of progenitors and survival of new neurons in the hippocampus of young adult female mice.[53,84–86] When assessing proliferating cells using Ki67, we did not observe any effects of menopause or diet. However, we did find differences in proliferating cells that survived (EdU+, 40-day survival). The discrepancy between the Ki67 and EdU results could indicate a problem in newborn cell survival rather than proliferation. Further examination of these surviving cells revealed that menopause and HF diet each caused a decrease in the number of those that became mature neurons (Edu+NeuN+), this decrease was most severe in the HF post-menopausal mice. Another possibility is that there could be delayed maturation of the granule neurons. In line with this, we found that menopausal mice on a HF diet had a greater percentage of surviving cells of immature neuronal fate (Edu+DCX+). An in-depth investigation is needed to elucidate the exact mechanism. Animal studies show that AHN is suppressed by HF diet and metabolic disease.[36,87] We have previously shown that HF diet did not affect neurogenesis in male mice but reduced the number of proliferating cells (Ki67+) and neuroblasts/immature neurons (DCX+) in females.[36] Here, we found that the combination of menopause and HF diet did not lead to a significant decrease in proliferating cells. However, it resulted in an increased number of EdU+ cells that maintained DCX labeling 40 days post-EdU injection, indicating a potential dysregulation in new neuron maturation. These results indicate that menopause alters AHN through a diet-dependent manner. Further studies are needed to elucidate the underlying mechanisms.

In conclusion, our data demonstrate that menopause affects multiple aspects of AD pathology. These effects can be dependent on or modulated by HF diet highlighting the interaction between endocrine aging and metabolic disease as risk factors for AD. Further studies are needed that consider these comorbidities and other sex-specific factors as single sex studies, especially in males, lead to incomplete knowledge, hindering the advancement of understanding and curing diseases. On the clinical side, closer attention is needed for those who go through menopause at a younger age. Moreover, it is important to consider the metabolic health, and the hormonal status, of women as some of the menopause effects observed in this study were exacerbated by or dependent on HF diet. Menopause-induced loss of estrogens had multiple negative effects on brain pathology hinting to potential benefits for brain-specific estrogen therapies. Even though HRT is not currently recommended after menopause as a dementia therapeutic, recent human studies and meta-analyses show that HRT could reduce dementia risk by ~ 33%.[62,88,89] This highlights the benefits of a more personalized HRT approach, which considers dose, timing, type of hormones, comorbidities, and genetic risk.[90] For example, our studies and those of others, show promising results to enhance cognitive function in rodent models using a brain-specific estrogen prodrug which helps avoid unwanted peripheral effects of classical HRT.[33,91,92] Our lab is currently assessing the effects of this prodrug in several different dementia models. The results will pave the way to potentially promising therapeutic strategies that would alleviate the burden of AD in post-menopausal women who represent the greatest percentage of AD patients.

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CONFLICT OF INTEREST STATEMENT

The authors have no conflicts to report. Author disclosures are available in the Supporting information.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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