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APOE4 genotype exacerbates the impact of menopause on cognition and synaptic plasticity in APOE-TR mice

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Abstract

The impact of sex and menopausal status in Alzheimer's disease remains understudied despite increasing evidence of greater female risk, particularly in *APOE4* carriers. Utilizing female *APOE*-TR mice maintained on a high-fat diet background we induced ovarian failure through repeated VCD injections, to mimic human menopause. At 12 months of age, recognition memory and spatial memory were assessed using object recognition, Y-maze spontaneous alternation, and Barnes maze. A VCD*genotype interaction reduced the recognition memory ($P < .05$), with *APOE4* VCD-treated animals unable to distinguish between novel and familiar objects. *APOE4* mice displayed an additional 37% and 12% reduction in Barnes ($P < .01$) and Y-maze ($P < .01$) performance, indicative of genotype-specific spatial memory impairment. Molecular analysis indicated both VCD and genotype-related deficits in synaptic plasticity with BDNF, Akt, mTOR, and ERK signaling compromised. Subsequent reductions in the transcription factors *Creb1* and *Atf4* were also evident. Furthermore, the VCD*genotype interaction specifically diminished *Ephb2* expression, while *Fos*, and *Cnr1* expression reduced as a consequence of *APOE4* genotype. Brain DHA levels were 13% lower in VCD-treated animals independent of genotype. Consistent with this, we detected alterations in the expression of the DHA transporters *Acsl6* and *Fatp4*. Our results indicate that the combination of ovarian failure and *APOE4* leads to an exacerbation of cognitive and neurological deficits.

KEYWORDS

Alzheimer's disease, docosahexaenoic acid, recognition memory, 4-vinylcyclohexene diepoxide

Abbreviations: AA, Arachidonic acid; AD, Alzheimer's disease; APOE, apolipoprotein E; APOE-TR, humanized-targeted replacement APOE; DHA, docosahexaenoic acid; FSH, follicle-stimulating hormone; HF, high fat; LOAD, late onset Alzheimer's disease; OVX, ovariectomized; PUFA, polyunsaturated fatty acid; VCD, 4-vinocyclohexene diepoxide.

David Vauzour and Anne-Marie Minihane are the authors share senior authorship.

1 | INTRODUCTION

The development of sporadic late-onset Alzheimer's disease (AD) is complex and multifactorial with more than 20 susceptibility loci including Apolipoprotein E (*APOE*).[1] *APOE4* is the strongest prevalent genetic risk factor with an allele frequency of 14% in the general population and is concentrated in AD (allele frequency ~40% in Caucasians).[2] The *APOE4* gene increases AD risk in a gene dose-dependent manner, rising from 2- to 3-fold for heterozygous *APOE4* carriers and 12-fold in homozygotes,[3,4] with age of onset considerably reduced. However, a large proportion of *APOE4* carriers do not develop AD indicating that the genotype is predictive rather than prognostic and may be modulated by other environmental and biological factors.[5] There is accumulating evidence that the penetrance of *APOE4* genotype is influenced by sex,[4,6] which may underlie the finding that almost two thirds of AD cases are females.[7] In a large meta-analysis Neu and colleagues reported increased MCI and AD risk in females compared to male *APOE4* carriers between 55-70 years and 65-75 years, respectively.[8] Despite this observation, little is known of the etiology underlying this susceptibility. However, given the age at which females are at increased risk[8] and the fact that earlier onset of menopause correlates with poorer cognition in later life,[9] it is plausible that menopause acts as a mediating factor.

Within the central nervous system APOE is the main lipid transporter.[10] In comparison to other organs, the brain is highly enriched with the n-3 polyunsaturated fatty acid (PUFA) docosahexaenoic acid (DHA) accounting for ~15% of total brain lipids.[11] We have recently reported lower DHA and specialized pro-resolving mediators in old *APOE4* female mice, indicating how sex, *APOE4,* and age contribute to the development of cognitive decline and AD pathology.[12] DHA is specifically concentrated within synaptosomal membranes,[13] influencing membrane dynamics, membrane protein function, secondary messenger systems, and neurotransmitter concentrations.[14] Thus it is unsurprising that reduced DHA status is consistently linked to poorer cognitive outcome and increased AD risk.[15] Synaptic loss and dysfunction which has been characterized in both menopause and *APOE4* individuals, is directly associated with cognitive decline and occurs in the initial stages of AD.[16]

With an etiological link accounting for the greater female *APOE4* susceptibility distinctly lacking,[17] we posit menopause as a contributing factor, given the neuroprotective properties of estrogen,[18] and greater cognitive decline associated with early menopause.[9] Here, in a transgenic mouse model we assessed the combined impact of *APOE* genotype and ovarian failure on cognitive performance hypothesizing that *APOE4* animals will be more susceptible to estrogen loss. Furthermore, we determine if changes in cognitive performance relate to alterations in brain fatty acid profiles and synaptic plasticity. Our model system combines the humanized-targeted replacement (*APOE*-TR) mouse model with 4-vinylcyclohexene diepoxide (VCD) treatment credited for its ability to establish an intermediary human-like "perimenopause" phase, while maintaining ovarian tissue integrity.[19]

2 | MATERIALS AND METHODS

2.1 | Study approval

All experimental procedures and protocols used in this study were reviewed and approved by the Animal Welfare and Ethical Review Body (AWERB) and were conducted within the provisions of the Home Office Animals (Scientific Procedures) Act 1986.

2.2 | Animal model and experimental design

Forty eight C57BL/6 background female humanized *APOE3* (B6.129P2-Apoe$^{tm2(APOE*3)Mae}$ N8) and *APOE4* (B6.129P2-Apoe$^{tm2(APOE*4)Mae}$ N8)-targeted replacement mice homozygous for the human *APOE3* or *APOE4* gene (Taconic, Germantown, NY, USA) were used in these experiments.[12,20,21] Mice were maintained in a controlled environment (21 ± 2°C; 12-h light-dark cycle; light from 07:00 hours) and fed ad libitum on a standard chow diet (RM3-P, Special Diet Services, Essex, UK) until the age of 4 months, ensuring normal development. Following this run-in period, mice were switched to a semi-purified high-fat diet (45 kCal% fat) (D17080301, Research diets, New Brunswick, NJ, USA) for the remaining duration of the experiment (See Table S1 for full dietary composition). A high-fat diet was utilized to mimic a human "western-style" high-fat diet and exacerbate AD age-related cognitive decline.[22]

At 8 months of age and to assess the impact of menopause, mice from each genotype received intra peritoneal (i.p.) injections of either VCD (160 mg/kg body weight) diluted in sesame oil, or sesame oil vehicle (sham) for a total of 14 injections over 3 weeks. The VCD model system is a well-established method for inducing a "menopause" like state in rodents[23] and is well-tolerated leading to a targeted degradation of ovarian follicles.[24] Eight months was selected as it is roughly midlife for the animals (when human menopause occurs) and before natural ovarian failure is known to occur in C57BL/6 mice.[25,26] Following completion of the final behavioral test, 12-month aged animals were sedated with isoflurane (1.5%) in a mixture of nitrous oxide (70%), and oxygen (30%) and transcardially perfused with an ice-cold PBS-containing protease (SIGMAFAST Protease inhibitor, Sigma-Aldrich, Devon, UK) and phosphatase (1 mM sodium pyrophosphate and 50 mM sodium fluoride, Sigma-Aldrich, Devon, UK) inhibitors. Sera were isolated via centrifugation at 2,000 ×*g* for 10 minutes. Brains were rapidly removed,

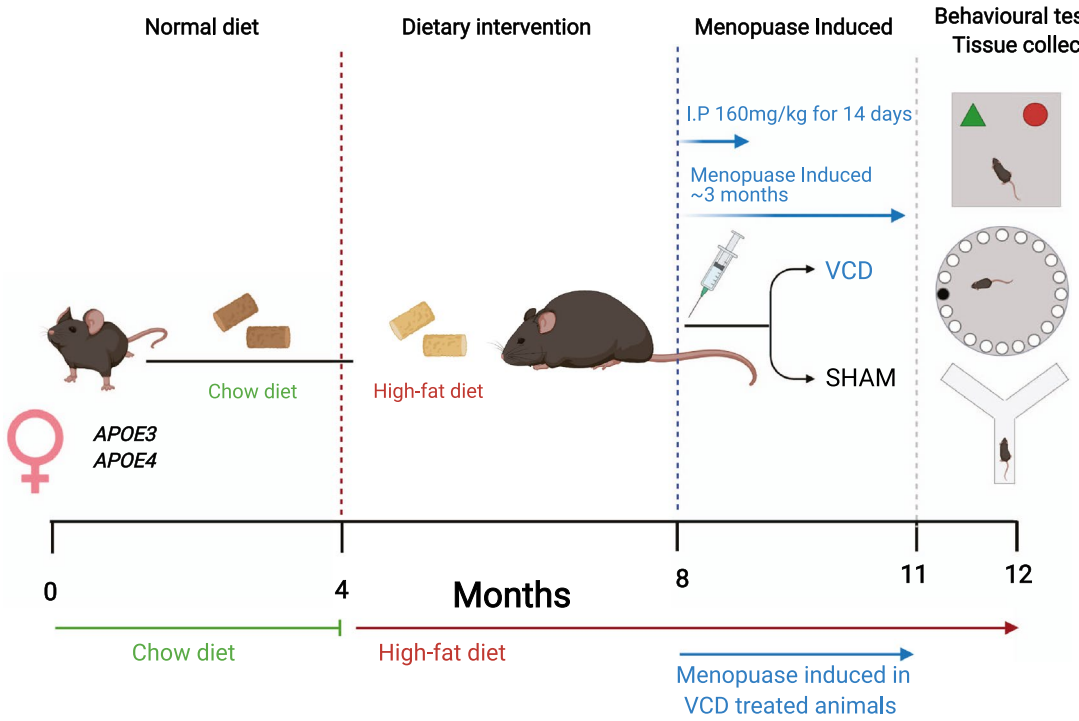

FIGURE 1 Experimental overview. At 4 months of age *APOE4* and *APOE3* TR mice were provided with a high-fat diet to model a western style diet. At 8 months of age mice received i.p. injections with 160 mg/kg VCD for 14 days in order to deplete ovarian follicles and mimic menopause by 12 months of age. At 12 months of age, mice were assessed cognitively through a battery of behavioral tests including Barnes maze, Y-maze, and object recognition. Upon completing the behavioral tests animals were immediately sacrificed. Created with BioRender.com

halved, snap frozen, and stored at −80°C until biochemical analysis. Ovaries were rapidly removed and processed for histology (see Section 2.4). Graphical representation of the experimental design is given in Figure 1.

2.3 | Behavioral assessment

All behavioral tests were performed when mice reached 12 months of age and immediately prior to sacrifice. A visual placing test was performed on each animal on the first day of testing, to ensure animals were not visually impaired.[27]

Spatial learning and memory were evaluated with the Barnes Maze as previously described.[28] Briefly, the maze consisted of a brightly illuminated (800 lux lighting) circular platform (92 cm diameter), with 20 evenly distributed holes located around the circumference and visual cues (4 simple shapes) placed at the periphery. The experiment was conducted over a 5-day period, with each mouse tested/trained on ability to locate the escape box four times per day during days 1-4. On day 5, a probe test was conducted, the maze was rotated 90°, the escape box was removed, and mice were placed in the center of the maze in which they were free to navigate for 1 minute. Percentage time in the correct quadrant was determined using the Smart 3.0 tracking software (Panlab, Kent, UK).

The novel object recognition (NOR), a measure of recognition memory, was performed as described previously,[29,30] with slight modifications. Briefly, on day 1 mice were habituated in gray 50 × 50 × 50 cm apparatus illuminated with low lux 100 lux lighting, mice were placed into the empty maze, and allowed to move freely for 10 minutes. On day 2, mice were conditioned to a single object for a 10-minute period. On day 3, mice were placed into the same experimental area in the presence of two identical objects for 15 minutes, after which they were returned to their respective cages and an inter-trial interval of 1 hour was observed. One familiar object was replaced with a novel object. Mice were placed back within the testing area for a final 10 minutes. Videos were analyzed for a 5-minute period, after which if total object exploration time failed to reach an accumulative 10 seconds, analysis continued until the 10 seconds was met. Animals not achieving 10 seconds were excluded from the analysis.[31] Similarly, animals not achieving a cumulative 10 seconds with the familiar object were excluded. Discrimination index was calculated as follows: DI = (TN − TF)/(TN + TF), where TN is the time spent exploring the novel object and TF is the time spent exploring the familiar object.

Y-maze spontaneous alternation test, a measure of spatial working memory was performed on the final day of behavioral testing as previously described.[32] Briefly, the Y-maze apparatus comprised of white Plexiglas in the following

dimensions (38.5 × 8 × 13 cm, spaced 120° apart) was illuminated with low lux (100 lux) lighting. Mice were placed into the maze and allowed to explore freely for 7 minutes while tracking software recorded zone transitioning and locomotor activity (Smart 3.0 tracking software, Panlab, Kent, UK). Spontaneous alternation was calculated using the following formula: Spontaneous Alternation = (Number of alternations/Total Arm entries − 2) × 100.

2.4 | Histological analysis

Ovaries were trimmed of fat and fixed in 10% formalin for 24 hours before being paraffin-embedded, and processed for hematoxylin and eosin (H&E) staining as described previously.[33]

2.5 | Fatty acid profile in the brain

Total lipids were extracted from sub-cortical brain tissues (n = 5/6 per group) using the Folch extraction method.[34] Fatty acid methyl esters (FAME) were obtained using acid-catalyzed transesterification.[35] FAME were evaporated under oxygen-free nitrogen, and re-suspended in 500 μL of isohexane, SPE silica cartridges (Clean-up 203 Cusil 156, UCT) were used for purification purposes. The purified FAMEs were separated by gas chromatography using a Trace 1300 series Gas Chromatograph (Thermo Fisher Scientific, Stafford House, Boundary Way, Hemel Hempstead. HP2 7GE, U.K.) equipped with a PTV injector modified for on-column injection, and a flame ionization detector. A fused silica capillary column (TraceGOLD TG-5MS Guard GC Column with SafeGuard column, 30 m × 0.32 mm × 0.25 μm; Thermo Fisher Scientific, Hemel, Hempstead, UK) was employed, and hydrogen was used as carrier gas. The temperature gradient was from 50 to 150°C at 40°C/min and then, to 200°C at 2°C/min followed by 214°C at 1°C/min and finally to 220°C at 40°C/min, where it was held for 5 min. Individual methyl esters were identified by comparison to known standards (Marine oil FAME mix RESTEK #35066). Data were collected and processed using the Chromeleon software package (version 7.2).

2.6 | Immunoblotting and ELISA

Cortices were homogenized in lysis buffer (CelLytic MT, Sigma-Aldrich, UK) containing protease (cOmplete, Mini, EDTA-free Protease Inhibitor Cocktail, Roche, UK) and phosphatase (PhosSTOP, Roche, UK) inhibitors. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, UK). Protein electrophoresis was conducted under denaturing conditions as previously described.[36] The following antibodies were used: anti-APOE (1:1000; Cell Signaling, UK), anti-phospho-mTOR (Ser2448) (1:1000; Cell Signaling, UK), anti-mTOR, (1:1000; Cell Signaling, UK), anti-phospho-ERK1/2 (Thr202/Tyr204) (1:1000; Cell Signaling, UK), anti-ERK1/2, (1:1000; Cell Signaling, UK) anti-GAPDH (1:2500, Cell Signaling, UK), anti-beta-actin (1:2500, Cell Signaling, UK), and anti-Rabbit IgG (H + L) DyLight 680 Conjugate (1:10,000, Cell Signaling, UK). Bands were revealed by fluorescence using an Odyssey 9120 Infrared Imaging system (LI-COR Biosciences, Ltd, UK). Relative band intensities were quantified using Image studio software Version 5.2.5 (LI-COR Biosciences, Ltd, UK).

Follicle-stimulating hormone (FSH) concentrations were determined by ELISA (Abnova, Taipei, Taiwan; ref KA2330) in sera samples as per the manufacturer's instructions and are displayed as ng/mL. Brain-derived neurotrophic factor (BDNF) concentrations were determined using DuoSet ELISA kit (R&D systems Minneapolis, MN, USA; ref DY248) from brain homogenates, as per the manufacturer's instructions, and the concentration (pg/mL) was normalized to total protein content (pg/mg total protein).

2.7 | RNA isolation and qRT-PCR

RNA isolation, cDNA synthesis, and qRT-PCR were carried out as previously described.[37] Briefly, total RNA was isolated from the brain samples using the Qiazol reagent (Qiagen, UK). One μg of total RNA was treated with DNase I (Invitrogen, UK) and used for cDNA synthesis using Invitrogen Oligo (dT) primers and M-MMLV reverse transcriptase. Quantitative real-time PCR (qRT-PCR) reactions were performed using SYBR green detection technology on the Roche light cycler 480 (Roche Life Science, UK). Results are expressed as relative quantity scaled to the average across all samples per target gene and normalized to the reference gene glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), which was identified as the optimal housekeeping selection using the software RefFinder.[38] Primer sequences are given in Supplementary Table S2.

2.8 | Statistical analysis

All data are presented as mean ± S.E.M. Data analysis was performed in GraphPad Prism version 8 (GraphPad Software, CA, USA). After identifying outliers using the Grubbs method, data were checked for normality/equal variances, performing log or box-cox transformation if necessary. Comparisons among groups were performed on normally distributed data

using two-way ANOVA, followed by post hoc Tukey's test when two-way ANOVA resulted in a significant interaction effect. Pearson's correlation was used in behavioral analysis to assess the association of travel distance and movement speed on corresponding behavioral test performance. *P* values of less than .05 were considered statistically significant.

3 | RESULTS

3.1 | Repeated injections of VCD result in ovarian failure independently of APOE genotype

Repeated injections of VCD in *APOE3-TR* and *APOE4-TR* mice resulted in a considerable loss of ovarian follicles (Figures 2A and S1) and a fourfold increase in serum FSH levels (VCD effect: $F(1, 21) = 27.64$ $P < .0001$ Figure 2B), irrespective of *APOE* genotype. Neither VCD treatment nor genotype had any impact on body weight gain ($P > .05$ Figure 2C) nor food intake ($P > .05$; Supplementary Figure S2).

3.2 | While APOE4 impairs working spatial memory, VCD causes additional deficits to recognition memory

Y-maze, Barnes maze, and NOR were employed to establish the impact of genotype and VCD treatment on cognitive performance (Figure 3). VCD had no effect on spatial learning and memory tasks, as assessed by the Y-maze and the Barnes maze (Figure 3A,B). However, a genotype-dependent effect was observed, with *APOE4* animals displaying ~12% lower cognitive performance than their *APOE3* counterparts as assessed by the Y-maze (genotype effect: $F(1, 38) = 7.75$ $P < 0.01$ Figure 3A). This was consistent with the Barnes maze, where *APOE4* animals spent ~40% less time in the correct quadrant, (genotype effect: $F(1, 37) = 8.09$ $P < .01$ Figure 3B). A genotype effect was also apparent during the learning phase (Supplementary Figure S3). Pearson's correlation revealed that these effects were not influenced by movement speed nor travel distance ruling out these as potential confounding factors (Figure 3C-F) Representative trajectory maps for Barnes probe test are shown in Figure 3G.

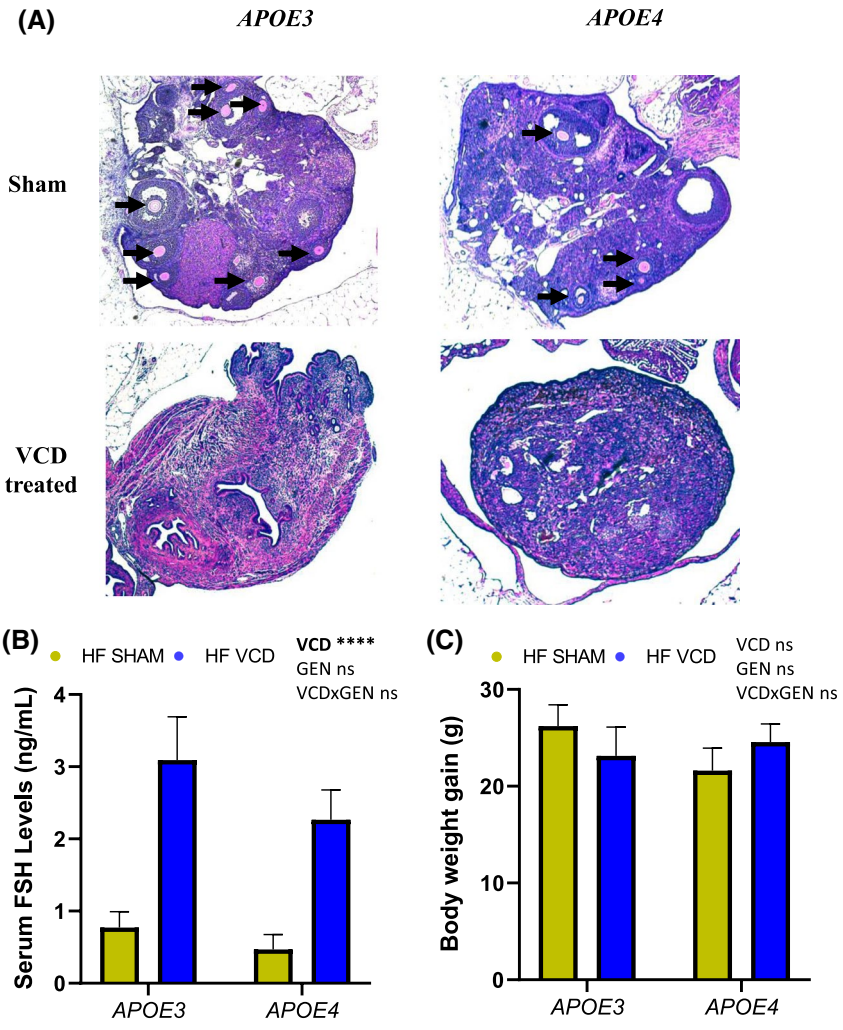

FIGURE 2 VCD treatment leads to ovarian failure independently of *APOE* genotype in *APOE3-TR and APOE4-TR* mice. A, Representative images of *APOE3-TR* and *APOE4-TR* female mice ovaries stained with hematoxylin and eosin show important loss of ovarian follicles following repeated i.p. injections of VCD (arrows indicate follicles); B, VCD-injected groups display elevated serum follicle-stimulating hormone (FSH) levels (n ≥ 5); C, Body weight was unaffected by VCD treatment or genotype (n ≥ 8); Data are presented as mean ± S.E.M **P* < .05, ***P* < .01, ****P* < .001, *****P* < .0001

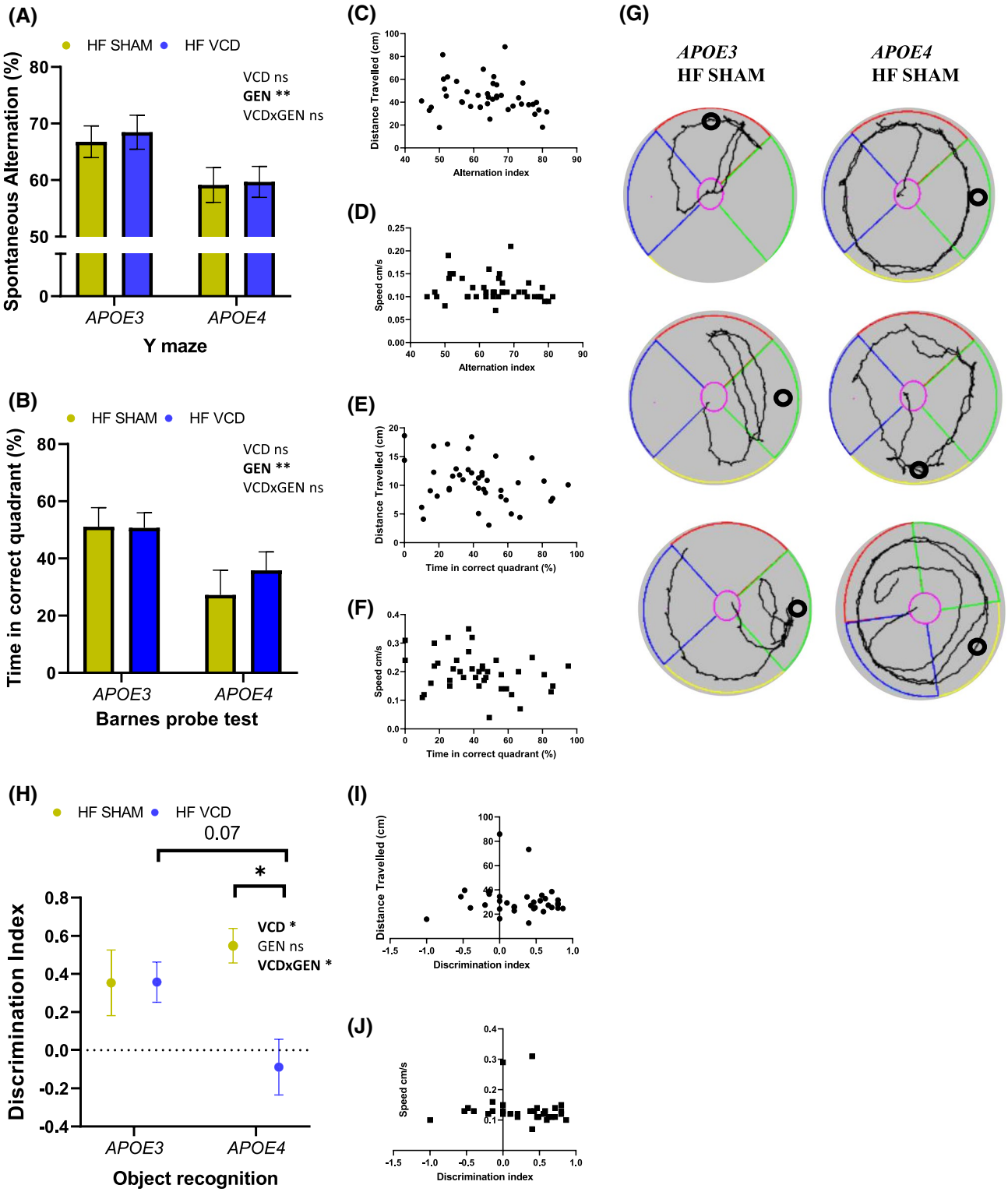

FIGURE 3 *APOE4* and VCD treatment influence cognition. A, Y-maze spontaneous alternation task (n ≥ 8) and B, Barnes probe test (n ≥ 8) identify deficits in spatial memory as a result of *APOE4* genotype, VCD treatment had no impact; C-F, Pearson's Correlation: Analysis of travel distance and movement speed do not correlate with Y-maze nor Barnes maze performance $P > .05$; G,Representative trajectory's from *APOE3* Sham (left) and *APOE4* sham (right) during the probe test, circle denotes former location of the escape box; H, Performance on Novel Object Recognition task (n ≥ 7) was severely compromised in the *APOE4* VCD group; I and J, Object recognition test score did not correlate with travel distance nor movement speed; Data are presented as mean ± S.E.M $^{*}P < .05$, $^{**}P < .01$

Unlike spatial learning and memory, recognition memory as assessed by the NOR test was influenced by the menopausal status, and in a genotype-dependent manner, with VCD-injected *APOE4* animals losing the ability to distinguish between novel and familiar objects (VCD effect: $F(1, 31) = 5.40$ $P < .05$, Interaction effect: $F(1, 31) = 5.52$ $P < .05$ Figure 3H). Again, these effects were not influenced by movement speed nor travel distance (Figure 3I,J).

3.3 | VCD injections reduce brain DHA levels independently of APOE genotype

A significant 13% genotype independent lower brain DHA concentration was observed in VCD-injected animals independent of their *APOE* genotype (VCD effect: $F(1, 18) = 17.94$ $P < .001$ Figure 4A). Higher total mono-unsaturated fatty acids (MUFAs) were observed in *APOE3* carriers

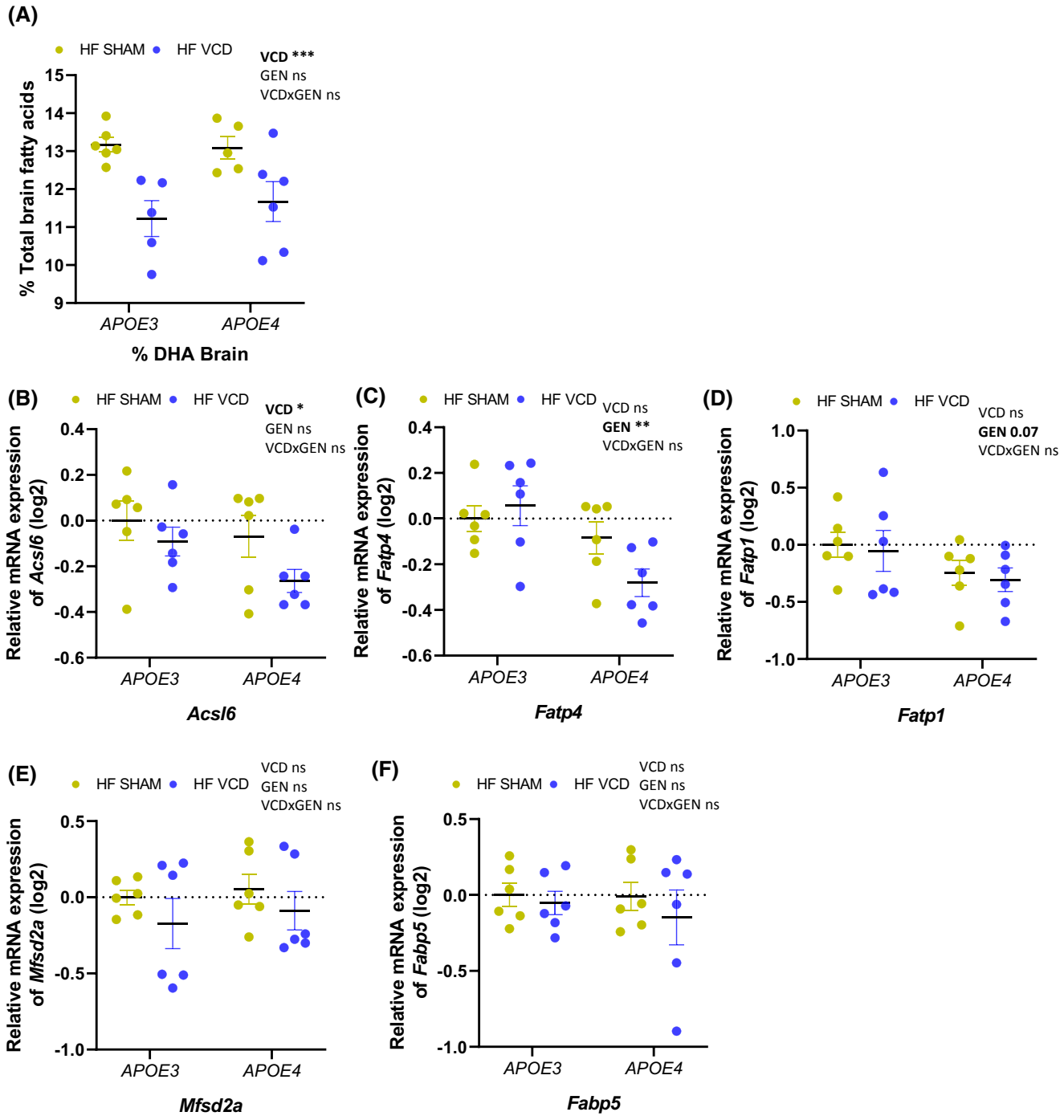

FIGURE 4 HF VCD treatment reduces brain DHA levels. A, GC-FID analysis revealed lower brain DHA levels in VCD-treated groups (n = 5/6); B-F, Expression of the key transporter *Aclsl6, Fatp4, Fatp1, Mfsd2a,* and *Fabp5* involved in DHA transport and uptake at the blood-brain barrier level. Data are presented as the mean ± S.E.M $*P < .05$, $**P < .01$, $***P < .001$

following VCD injections (VCD effect: $F(1, 18) = 6.44$ $P < .05$; Interaction effect: $F(1, 18) = 7.66$ $P < .05$; Table 1). These changes were mirrored by a nominal decrease in total saturated fatty acids (SFAs) in *APOE3* (VCD effect: $F(1, 18) = 3.03$ $P < .1$; Interaction effect: $F(1, 18) = 4.23$ $P = .05$). In addition, DHA: AA ratio was lower following VCD treatment (VCD effect $F(1, 17) = 4.49$ $P < .05$). Although no interaction was detected, DHA: AA ratio was 8% lower in *APOE4* animals but remained constant in *APOE3* regardless of treatment.

To identify whether the observed lower DHA related to deficits in DHA transport, gene expression profiles of key DHA transporters in the brain were assessed. Interestingly, a member of the long-chain acyl-CoA synthetase family *Acsl6* known to be highly abundant in the brain[39] and present in brain microvessels,[40] was reduced by 10% in VCD-treated animals (VCD effect: $F(1, 20) = 4.76$ $P < .05$ Figure 4B). Expression of the predominant fatty acid transport proteins in the BBB endothelial cells, *Fatp4* and *Fatp1*,[41] was also determined. *Fatp4* expression was 14% lower as a result of *APOE4* genotype, with the reduction particularly evident in *APOE4* VCD-treated animals although the interaction did not reach significance (genotype effect: $F(1, 20) = 9.29$ $P < .01$; Interaction effect: $F = 3.35$ $P = .08$ Figure 4C). A similar genotype trend was observed for *Fatp1* ($P = .069$ Figure 3D), while no effect was observed for major facilitator superfamily domain-containing 2a *Mfsd2a* or fatty acid-binding protein 5 *Fabp5* (Figure 4E,F).

3.4 | VCD injections and APOE4 trigger lower synaptic plasticity response

Both *APOE4* genotype and VCD administration led to diminished BDNF levels in the brain (VCD effect: $F(1, 19) = 7.24$ $P < .05$; genotype effect: $F(1, 19) = 5.96$ $P < .05$ Figure 5A). Lower ApoE protein levels were observed in *APOE4* compared to *APOE3* animals (genotype effect: $F(1, 20) = 55.90$ $P < .0001$; Figure 5B). Phosphorylation of mechanistic target of rapamycin (mTOR) was significantly influenced by VCD treatment and genotype (VCD effect: $F(1, 20) = 5.17$ $P < .05$; genotype effect: $F(1, 20) = 5.86$ $P < .05$ Figure 5C). *APOE4* genotype and its interaction with VCD resulted in impaired extracellular signal-regulated kinase (ERK) phosphorylation, with both ERK1 (interaction effect: $F(1,20) = 10.28$ $P < .01$; genotype effect: $F(1, 20) = 6.79$ $P < .05$ Figure 5D) and ERK 2 (interaction effect: $F(1,20) = 9.51$ $P < .01$; genotype effect: $F(1, 20) = 3.19$ $P = .09$ Figure 5D) affected. At the transcriptional level only genotype influenced *mTOR* expression (genotype effect: $F(1, 20) = 6.97$ $P < .05$; Figure 5E), however its upstream activator, *Akt* was downregulated in a VCD*genotype-dependent manner

TABLE 1 Brain fatty acid composition of experimental animals

	APOE3		*APOE4*				
Fatty acid	**HF Sham**	**HF VCD**	**HF Sham**	**HF VCD**	**Genotype *P* value**	**VCD *P* value**	**Interaction *P* value**
Total n-3 PUFA	13.30 ± 0.20	11.60 ± 0.70	13.20 ± 0.30	11.80 ± 0.50	0.964	**0.004**	0.805
20:5 n-3 (EPA)	0.02 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	0.03 ± 0.01	0.892	0.315	0.978
22:6 n-3 (DHA)	13.20 ± 0.20	11.20 ± 0.50	13.10 ± 0.30	11.70 ± 0.50	0.650	**>0.001**	0.519
Total n-6	14.20 ± 0.20	12.60 ± 0.40	14.80 ± 0.90	13.20 ± 0.60	0.274	**0.011**	0.979
20:4 n-6 (AA)	9.37 ± 0.21	8.10 ± 0.39	8.95 ± 0.25	8.67 ± 0.50	0.860	0.058	0.221
DHA:AA	1.40 ± 0.03	1.39 ± 0.05	1.48 ± 0.01	1.36 ± 0.03	0.619	**0.049**	0.134
Total SFAs	38.70 ± 0.40a	35.50 ± 0.90b	36.60 ± 0.60	36.90 ± 1.20	0.689	0.098	**0.054**
16:0	17.60 ± 0.50a	14.70 ± 0.70b§	16.20 ± 0.50	17.30 ± 0.90§	0.406	0.207	**0.010**
18:0	20.10 ± 0.30	18.90 ± 0.50	19.30 ± 0.20	18.30 ± 0.60	0.138	**0.025**	0.802
20:0	0.31 ± 0.02	0.41 ± 0.03	0.28 ± 0.02	0.33 ± 0.03	0.053	**0.014**	0.343
22:0	0.15 ± 0.05a	0.38 ± 0.05b	0.26 ± 0.01	0.19 ± 0.06	0.165	0.552	**0.007**
Total MUFAs	25.40 ± 0.40a	30.50 ± 0.80b	27.30 ± 0.90	27.90 ± 1.40	0.448	**0.021**	**0.013**
18:1 n-9	18.20 ± 0.50	21.20 ± 0.80	19.30 ± 0.60	19.30 ± 1.20	0.684	0.091	0.084
20:1 n-9	2.03 ± 0.18a	3.04 ± 0.20b	2.28 ± 0.20	2.21 ± 0.21	0.166	**0.028**	**0.013**
24:1 n-9	1.37 ± 0.09a	1.96 ± 0.18b§	1.56 ± 0.11	1.14 ± 0.24§	0.080	0.641	**0.008**

Note: (n = 5/6 per group). Data are % of total fatty acids and mean value ± S.E.M. Two-way ANOVA. Letters a and b denote significant difference between intervention while § denotes significant genotype effect as analyzed via post hoc. Bold values denote significant values. The full table can be found in supplementary data (Table S3).

Abbreviations: DHA:AA, Docosahexaenoic acid to Arachidonic acid ratio; MUFAs, Monounsaturated fatty acids; PUFA, Polyunsaturated fatty acid; SFAs, Saturated fatty acids; 20:5 n-3 EPA, Eicosapentaenoic acid; 22:6 n-3 DHA, Docosahexaenoic acid; 20:4 n-6 AA, Arachidonic acid; 16:0, Palmitic acid; 18:0, Stearic acid; 20:0, Eicosanoic acid; 22:0, Docosanoic acid; 18:1 n-9, Oleic acid; 20:1 n-9, 11-Eicosenoic acid; 24:1 n-9, Nervonic acid.

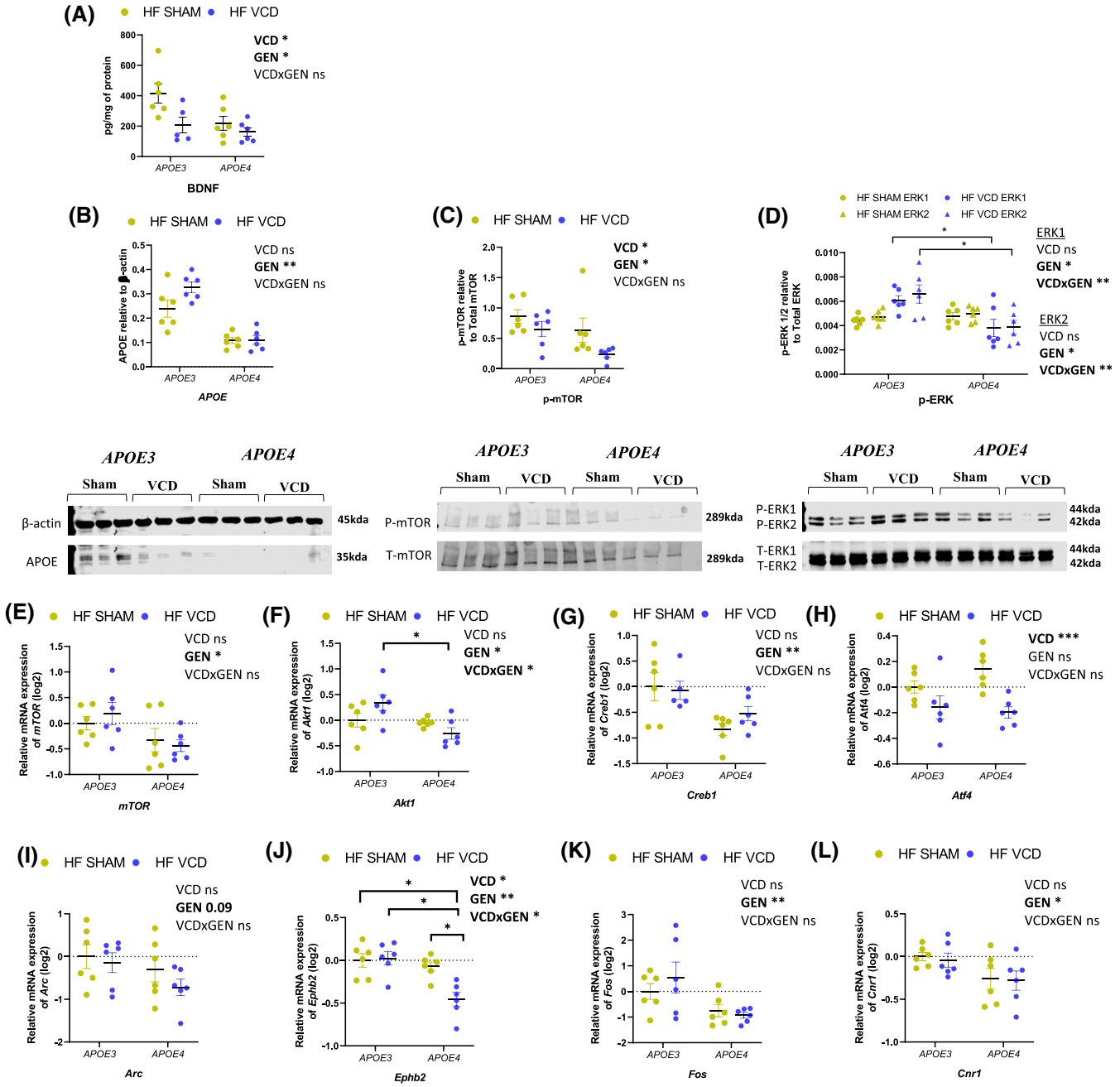

FIGURE 5 Selective impact of genotype and VCD on synaptic plasticity-related genes and proteins; A, ELISA revealed a reduction in BDNF protein levels as a result of VCD insult and APOE genotype; B, ApoE protein was lower in the cortex of *APOE4* animals; C, p-mTOR was lower in *APOE4* VCD-treated animals; D, p-ERK was reduced as a result of *APOE4* genotype and VCD*genotype interaction and particularly reduced in *APOE4* VCD-treated animals; representative western blots are given below each graph (n = 6); E-L, Hippocampal expression profiles relating to synaptic plasticity (n = 6); E-F, in which genotype and VCD-mediated reductions in *mTOR* and *Akt*; G-I, resulted in diminished *Creb1*, *Atf4*, *arc*; J-L) Further dysregulation of *Ebpb2, Fos, and Cnr1* were also observed. Data are presented as the mean ± S.E.M $*P < .05$, $**P < .01$, $***P < .001$, $****P < .001$

(interaction effect: $F(1, 20) = 5.49$ $P < .05$; genotype effect: $F(1, 20) = 7.31$ $P < .05$ Figure 5F). VCD and *APOE4* genotype resulted in further synaptic plasticity-related signaling deficits (Figure 5H-M). The transcription factor cAMP responsive element-binding protein 1 *(Creb1)* was downregulated in *APOE4* animals (genotype effect: $F(1, 19) = 11.56$ $P < .01$; Figure 5G) while its fellow CREB family member, the activating transcription factor 4 *(Atf4)* was downregulated in response to VCD treatment (VCD effect F 1, 20) = 14.95 $P < .001$; Figure 5H). Interestingly, the downstream target activity-regulated cytoskeleton-associated protein (*Arc)* was nominally reduced ~2-fold in *APOE4* VCD-treated animals,

although this did not reach significance. Ephrin type-B receptor 2 (*Ephb2)* was significantly impacted by the interactive impact of *APOE4**VCD, resulting in a ~30% reduction in *APOE4* VCD-treated animals compared to *APOE4* sham or *APOE3* counterparts (interaction: $F(1, 20) = 7.67$ $P < .05$; VCD effect: $F(1, 20) = 5.97$ $P < .05$; genotype effect: $F(1, 20) = 14.05$ $P < .01$; Figure 5J). In addition to this, further genotype effects were apparent for *Fos* (genotype effect: $F(1, 20) = 9.23$ $P < .05$ Figure 5K) and the cannabinoid receptor *Cnr1* (genotype effect: $F(1, 20) = 6.84$ $P < .05$ Figure 5L).FIGURE 5 (*Continued*)

4 | DISCUSSION

Several studies report that female carriers of the *APOE4* genotype are at a higher risk of AD,[8] however little is known as to how this predisposition manifests.[17] Here, we sought to determine whether the cumulative *APOE4**menopause effect contributes toward this greater risk. With VCD treatment emerging as a robust model to assess the implications of menopause in neurological disease,[23] we investigated for the first time the impact of VCD-mediated ovarian failure in the *APOE*-TR mouse model, focusing on cognition, brain fatty acid profiles, and synaptic plasticity-related signaling. Deficits in spatial learning and memory were related to the *APOE4* genotype, with an additive effect of menopause and *APOE4* carrier status on recognition memory. Furthermore, the VCD insult lowered brain DHA status while both factors influenced synaptic plasticity-related signaling genes and proteins.

Unlike previously published data by us and others reporting a strong impact of *APOE4* genotype on body weight gain in male mice,[42,43] no effect of *APOE* genotype nor VCD injections was observed in our female mice. This observation is in agreement with previous reports indicating limited impact of menopause[44] and *APOE4*[45] on body weight gain in female mice.

Y-maze and Barnes maze revealed *APOE4*-dependent deficits in spatial learning and memory. Deficits in spatial memory performance mediated via *APOE4* genotype have been reported in several studies.[46-48] Interestingly, the extent of the deficit appears to be greater in *APOE4* female mice or absent in *APOE4* male mice.[46] No exacerbation of spatial deficits were detected as a result of menopause in agreement with reports indicating that the impact on spatial memory is subtle,[49] and dependent upon age, which represents a key factor in the extent of decline.[50] Conversely, recognition memory was significantly influenced by the menopause mimic VCD with the effect restricted to *APOE4* animals. Impairment of recognition memory has been previously reported in chronic ovariectomized C57BL/6 mice,[51] and aged rhesus monkeys,[52] suggesting that the medial temporal lobe and surrounding cortical areas are particularly sensitive to ovarian failure. Interestingly, we report that this effect was exacerbated by the *APOE4* genotype, highlighting this specific brain area as a focus for future endeavors. Together, the behavioral test results indicate both genotype- and genotype*VCD-mediated cognitive deficits and show how the combination of factors result in broader cognitive impairment.

Reduced DHA intake and status are associated with neuropathology, cognitive decline, and higher AD risk.[15,53,54] Both *APOE4* and ovarian function impact n-3 PUFA and DHA homeostasis.[55-58] To the best of our knowledge, the combined impact of both ovarian failure and *APOE4* genotype on brain DHA levels has not been previously explored. Here, brain DHA levels were equally diminished in both genotypes following VCD treatment. Interestingly, we observed lower brain ApoE levels in both *APOE4* groups, independent of VCD insult, suggesting DHA concentration was uncoupled from ApoE protein levels. This contrasts a previous report indicative of ApoE involvement in brain DHA levels,[55] however as previously mentioned, this may be sex-dependent and more prominent in male mice. Although this is the first time brain fatty acid levels have been analyzed in an *APOE*-TR VCD model, results from OVX models demonstrate similar reductions in DHA,[59] indicating a clear relationship between reproductive hormones and brain DHA maintenance. Surprisingly, the reduction in DHA did not translate to an observable behavioral deficit in *APOE3*-treated animals suggesting that protection/tolerance is conferred by *APOE3,* perhaps via a compensatory mechanism. For example, the DHA:AA ratio in *APOE4* VCD-treated animals was more extensively reduced, offering one possible explanation for the greater resilience shown by their *APOE3* counterparts. Indeed, the importance of DHA:AA as a determinant of neuro-inflammatory status, given that AA and DHA are precursors of potent pro-inflammatory eicosanoids[60] and specialized pro-resolving mediators, respectively,[12,61] offers a plausible mechanistic basis. Furthermore, in *APOE3* the VCD-mediated fluctuations of higher total MUFA and lower total SFA (absent in *APOE4*) may have influenced cognition to some extent. Higher MUFA intake has been previously shown to improve the brain function while lower MUFA brain levels are associated with AD, aging, and depression.[62] A number of pathways have been proposed to explain the benefits of MUFAs including the maintenance of membrane flexibility[63] and the actions of MUFA's as anti-inflammatory and antioxidant derivatives[64] which have been shown to modulate the neuroinflammation in ApoE KO mice.[65]

Although the mechanism by which DHA enters the brain (passive/active) remains to be fully determined, current evidence points out it is likely to be in part mediated by a small number of specific binding/transporter proteins. MFSD2A, ACSL6, FABP5, and the FATPs 1 and 4 are expressed at

the BBB[40] and have been previously associated with DHA transport into the brain.[17] Of the DHA transporters measured, *Acsl6* described from knockout studies as being a key mediator of neuroprotective DHA within the brain[39] and believed to be critical in maintaining brain DHA levels,[66] was downregulated in response to VCD treatment. *Acls6* may, therefore, account for the reduction in DHA observed and should be a focus for future studies evaluating the impact of menopause on brain DHA levels. *Fatp1,* but particularly *Fatp4* displayed genotype deficits; these fatty acid transporters have been shown to bind DHA and facilitate its transport across the endothelial cell membrane.[67] This lower expression may in part explain the genotype reductions in DHA observed by other groups particularly if exacerbated by age. Previous work has indicated that blood-brain barrier transporter protein cell localization and membrane shedding are influenced by *APOE* and neuropathology. This could also influence the capacity to uptake DHA into the brain.[68,69] Moreover, as previously mentioned the change may be independent of transport and instead relate to metabolic disturbances which lead to greater β-oxidation of DHA.[70] This is conceivable given the role estrogens play in bioenergetic systems within the brain.[71,72]

Given the role of ApoE protein in neurite outgrowth[73] and neuronal repair processes,[74] *APOE4*-specific reductions in spatial memory might be expected in light of the diminished protein levels observed in this experimentation. Further investigation may be warranted to establish if these lower APOE4 levels are constant across sexes given that spatial memory deficits appear to be *APOE4* female specific.[46] As with human menopause, VCD treatment is associated with increased FSH levels. Research evaluating the impact of elevated FSH on cognition is limited, however in OVX mice a reduction of all gonadotropins, appears equally as effective at preserving cognition as providing estrogen.[75] Additionally, in humans FSH:estradiol has been used as a predictor of MCI.[76] Moreover, estrogen is a well-established regulator of synaptic plasticity in key areas of the brain including the hippocampus.[77]

In this study, we observed that both the menopause mimic and *APOE4* genotype influenced synaptic signaling in the in the brain. First, the brain neurotrophic factor BDNF, described to be multifaceted and considered a flexible hub for synaptic plasticity and cognitive functioning[78] was reduced as a result of *APOE4* genotype and VCD treatment. Such observation is in agreement with previous studies focusing on *APOE4*[79] and VCD treatment separately.[80] Interestingly BDNF is known to influence both mTOR and ERK activation.[81] mTOR signaling was impaired by the *APOE4**VCD combination. To the best of our knowledge, such an effect has not been previously reported in *APOE4* mice, but the effect of menopause has been observed in OVX models.[82-84] mTOR activation, likely triggered via the PI3K-AKT pathway, is critical for neuronal cell survival, and is intriguingly associated with maintenance of glucose homeostasis, with similar deficits established in the diabetic rat brain,[85] thus highlighting potential metabolic/bioenergetic disturbances in the *APOE4* VCD-treated brain. Furthermore, AKT and mTOR activation in AD mouse models ameliorates deficits in synaptic plasticity improving associated learning and memory.[86,87] It is, therefore, possible that the impaired object recognition performance observed in this study stems from impairment of PI3K-AKT-mTOR signaling, which is already known to be estrogen and ER sensitive.[77] ERK signaling represents another important cascade which is similarly intertwined with BDNF and synaptic plasticity.[88] ERK activation was found to be reduced as a result of *APOE4* genotype and the interactive impact of VCD and genotype. This is in line with Yong and colleagues who reported diminishing ERK phosphorylation in aging female *APOE4*-TR mice.[89] Interestingly both Yong and our group show a reduction in brain APOE protein levels in the *APOE4* female mice, highlighting how this may be coupled to ERK phosphorylation. Additionally, ERK activation has been reported in OVX studies.[90,91] Given DHA's proposed influence upon both PI3K-AKT-mTOR[92] and ERK signaling pathways,[93] the altered DHA/PUFA status associated with VCD treatment, and the subsequent reduction in BDNF may offer a plausible mechanistic basis for their disruption, although further confirmatory experiments are required. As discussed mTOR and ERK are central components of synaptic plasticity-related signaling and are involved in various down-stream pathways explaining the diminished transcription of the CREB family transcription factors (*Creb1* and *Atf4*), both of which have integral roles in synaptic plasticity.[94,95] Further down-stream effects reflected previous observations with *Arc* expression nominally reduced in *APOE4*, particularly VCD-treated animals. Referred to as the master organizer of long-term synaptic plasticity,[96] *Arc* has been reportedly regulated by both *APOE4* and estrogen with both MAPK and PI3K pathways implicated.[97,98] We also noted diminished *Ephb2* expression as a result of the *APOE4**VCD interaction. Interestingly, *Ephb2* plays a fundamental role in learning and memory, with implications ranging from synapse maintenance and synaptogenesis to AMPA and NMDA receptor expression, localization, and function.[99-103] Indeed, increased *Ephb2* expression has been found to compensate for AD-related NMDA receptor impairment.[104] *Ephb2,* therefore, offers additional mechanistic basis for the cognitive impairment associated with *APOE4**VCD. Further to this, *APOE4* genotype led to a downregulation of *Fos* an indicator for neuronal activation,[105] and may be linked to the altered ERK signaling profile to which it is known to be influenced.[106] Finally, with implications in hippocampal synaptic plasticity, adult neurogenesis, and subsequent memory consolidation we

assessed endocannabinoid, specifically *Cnr1* regulation.[107] An *APOE4*-dependent reduction in *Cnr1* expression was observed, CNR1 receptor antagonism and subsequent endocannabinoid dysregulation has been associated with deficits in learning and memory.[108] Recently CNR1 activation has been reported to be neuroprotective conferring specific improvement in spatial memory,[109] therefore, diminished CNR1 may also contribute to the spatial memory deficits associated with *APOE4*. Research connecting APOE and endocannabinoid signaling, particularly within the brain is surprisingly limited, and warrants further investigation in light of these results.

As with all animal models some limitations associated with the model system should be mentioned. First, although well-established,[23] particularly in cognitive and cardiology research[24,110,111] and believed to be non-toxic/ovarian follicle specific,[24,112] the VCD menopause induction may introduce as yet unidentified off-target impacts. The timing of the VCD and cognitive assessment (ie, middle-aged animals) could have resulted in some sham animals being spontaneously acyclic. However, given that VCD was introduced before natural ovarian failure is known to occur in C57BL/6 mice,[25,113] and that we observed primordial follicles in sham animals (Figure S1), the risk of this is low, consistent with the increase in FSH levels in only VCD-treated animals.[114,115] Furthermore, even if some animals had become partially senescent over the later stage of the assessment period, the VCD-treated animals would have undergone the process much earlier and thus would have been exposed to the detrimental effects of ovarian failure for a longer duration of time.

5 | CONCLUSION

Despite its well-established impact on late-onset AD risk, the etiological basis of the *APOE4* genotype-associated cognitive deficits and neuropathology remains elusive. Sex, and menopausal status remain overlooked factors that likely influence the progression of neurological diseases such as AD. Here we provide evidence of menopause-related risk and suggest a greater sensitivity in *APOE4* carriers, with *APOE4* carriers displaying greater cognitive impairment and more extensive deficits in synaptic plasticity-related signaling.

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CONFLICT OF INTEREST

There are no actual or potential conflict of interest.

AUTHOR CONTRIBUTIONS

A.M. Minihane, D. Vauzour, and M.G Pontifex conceptualized and designed the experiments and analytical approaches. D. Vauzour provided the Home Office Animal Licence; M. G. Pontifex, A. Martinsen, and G. Harden, performed the animal research and subsequent sample processing; M G Pontifex, R. Saleh, and N. Tejera performed the fatty acid analysis; M. G Pontifex performed all other analysis and analyzed the data; M. G Pontifex, D. Vauzour, and A. M. Minihane wrote the manuscript with contributions from all authors; M. Muller and C. Fox critically revised the manuscript. All authors approved the final manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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