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Cyclic O_3 exposure synergizes with aging leading to memory impairment in male APOE ε3, but not APOE ε4, targeted replacement mice

Chunsun Jiang [a], Luke T. Stewart [b], Hui-Chien Kuo [c], William McGilberry [a], Stephanie B. Wall [d], Bill Liang [e], Thomas van Groen [b], Shannon M. Bailey [f], Young-il Kim [a], Trent E. Tipple [d], Dean P. Jones [e], Lori L. McMahon [b], Rui-Ming Liu [a,*]

[a] Department of Medicine, University of Alabama at Birmingham, Birmingham, AL, USA
[b] Department of Cell, Developmental, and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, USA
[c] Department of Biostatistics, University of Alabama at Birmingham, Birmingham, AL, USA
[d] Department of Pediatrics, University of Alabama at Birmingham, Birmingham, AL, USA
[e] Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA
[f] Department of Medicine, Emory University, Atlanta, GA, USA

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ABSTRACT

The etiology of late-onset Alzheimer's disease is unknown. Recent epidemiological studies suggest that exposure to high levels of ozone (O_3) may be a risk factor for late-onset Alzheimer's disease. Nonetheless, whether and how O_3 exposure contributes to AD development remains to be determined. In this study, we tested the hypothesis that O_3 exposure synergizes with the genetic risk factor *APOE ε4* and aging leading to AD, using male apolipoprotein E (apoE)4 and apoE3 targeted replacement mice as men have increased risk exposure to high levels of O_3 via working environments and few studies have addressed *APOE ε4* effects on males. Surprisingly, our results show that O_3 exposure impairs memory in old apoE3, but not old apoE4 or young apoE3 and apoE4, male mice. Further studies show that old apoE4 mice have increased hippocampal activities or expression of some enzymes involved in antioxidant defense, diminished protein oxidative modification, and neuroinflammation following O_3 exposure compared with old apoE3 mice. These novel findings highlight the complexity of interactions between *APOE* genotype, age, and environmental exposure in AD development.

1. Introduction

Alzheimer's disease (AD), a neurodegenerative disease, is a major cause of dementia in the elderly. Despite extensive studies, there is no effective treatment due, in part, to an incomplete understanding of its etiology and pathogenesis. Early-onset (familial) AD, which accounts for less than 5% of AD cases, is attributed to mutations in the genes coding for amyloid precursor protein (APP) or presenilin (PS). The causes for the majority (>95%) of AD cases (sporadic), which occurs after age 65 years (late-onset Alzheimer's disease [LOAD]), however, are known. Human apolipoprotein E (apoE), existing in 3 isoforms (apoE2, apoE3, and apoE4), encoded by 3 distinct alleles ε2, ε3, and ε4, is a major carrier of lipids and cholesterol. Both epidemiologic and animal studies indicate that the *APOE ε4* allele, carried by approximately 15% of the population worldwide (Eisenberg et al., 2010; Singh et al., 2006), is a major genetic risk factor for AD (Farrer et al., 1997; Liu et al., 2013; Raber et al., 2000) and women carrying the *APOE ε4* allele are at highest risk (Altmann et al., 2014; Farrer et al., 1997; Riedel et al., 2016; Ungar et al., 2014). Yet, not all *APOE ε4* carriers, even older women, develop AD, suggesting that other factors, including environmental exposures, must play a role.

Ozone (O_3), a highly reactive oxidant, is one of the most abundant urban pollutants. Unfortunately, over 30% of the U.S. population lives in areas with unhealthy levels of O_3 (American Lung Association State of the Air, 2012). In addition, some workers (e.g., pulp mill and outdoor construction workers) are intermittently exposed to relatively high levels of O_3 through their working environments (Chan and Wu, 2005; Henneberger et al., 2005).

* Corresponding author at: Department of Medicine, University of Alabama at Birmingham, Birmingham, AL 35294, USA. Tel.: (205) 934-7028; fax: (205) 934-1721.
E-mail address: rliu@uab.edu (R.-M. Liu).

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Although the lung is a primary target, several studies, including our own, have shown that O_3 exposure induces oxidative stress in the brain and impairs memory in rats (Hernandez-Zimbron and Rivas-Arancibia, 2015; Rivas-Arancibia et al., 2010) and in genetically predisposed (APP/PS1 double transgenic) mice (Akhter et al., 2015). Importantly, recent epidemiologic studies show a positive correlation between O_3 levels and the incidence of AD (Chen and Schwartz, 2009; Cleary et al., 2018; Gatto et al., 2014; Jung et al., 2015; Wu et al., 2015). Nonetheless, whether O_3 is a culprit for AD and whether O_3 acts alone or synergizes with other risk factors such as *APOE ε4* and aging leading to AD remains to be determined.

Humanized or targeted replacement (TR) apoE4 and apoE3 mice, in which the endogenous murine *APOE* gene is replaced with human *APOE ε4* or *APOE ε3* (common human form) gene, respectively (Knouff et al., 1999), are useful tools to study the mechanisms whereby *APOE ε4* modulates AD pathophysiology. As in humans, the *APOE ε4* allele mainly affects the memory of female mice under unchallenged conditions (Bour et al., 2008; Raber et al., 2000; Rijpma et al., 2013; Villasana et al., 2006). Consequently, most studies have used female apoE4 TR mice and very few have interrogated the effects of *APOE ε4* on male disease susceptibility. As men have increased risk to be exposed to elevated levels of O_3 through their working environment (e.g., outdoor construction and pulp mills), the present studies used male apoE3 and apoE4 TR mice to test the hypothesis that O_3 exposure synergizes with *APOE ε4* and/or aging to cause memory impairment. Moreover, we measured the levels of antioxidants glutathione (GSH) and cysteine (Cys), the activities or expression of enzymes involved in antioxidant defense, protein oxidative modifications, and neuroinflammatory responses to further explore the potential mechanisms underlying O_3-induced memory impairment.

2. Materials and methods

2.1. Animals and ozone exposure

Ten- to 12-week-old male apoE4 and apoE3 TR mice, in which the endogenous murine *APOE* gene is replaced with human *APOE ε4* and *APOE ε3* gene, respectively (Knouff et al., 1999), were purchased from Taconic and aged in UAB animal facility. The *APOE* genotype of the mice was confirmed by PCR and DNA sequencing as described (Hixson and Vernier, 1990). Three- and seventeen-month-old apoE3 and apoE4 mice were exposed to a series of O_3 exposure cycles, consisting of 5 days of O_3 exposure (0.8 ppm, 7 h/d) followed by 9 days of filtered air (FA) recovery, for 8 cycles at the UAB Environmental Exposure Facility, as we have described previously (Akhter et al., 2015; Katre et al., 2011). FA controls were treated identically and were done in parallel to O_3-exposed mice. Fourteen to 16 mice were used for each genotype, age, and treatment group (total 120 mice for 8 groups). A 0.8 ppm O_3 dose was used as rodents are insensitive to O_3 insult because of obligatory nose breathing and other intrinsic factors, and as 0.2–0.3 ppm O_3 is frequently achieved in highly polluted areas, whereas a factor 3 is accepted practice to extrapolate O_3 dose from rodents to human [Air Quality Criteria for Ozone and Related Photochemical Oxidants (Final). US Environmental Protection Agency, Washington, DC. EPA/600/R-05/004aF-cF, 2006] (Mumaw et al., 2016; Ren et al., 2008). A 5-day on and 9-day off exposure protocol was used to mimic what occurs in urban settings wherein several days of elevated O_3 are usually followed by longer periods of "clean" air. Animals were allowed free access to water, whereas food was withheld during exposures to prevent ingestion of chow constituents oxidized by O_3, which could introduce confounders. All procedures involving animals were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham.

2.2. The open field and zero maze tests

After termination of O_3 exposure at the end of 8 cycles, the open field test and then the zero maze test were conducted to assess general activity and anxiety levels of mice as described previously (Akhter et al., 2015, 2018). The time spent in the center or at the sides in the open field test as well as in the closed or open area in the zero maze test was recorded using a camera-driven tracker system (EthoVision XT11, Noldus).

2.3. Water maze test

After the open field and zero maze tests (next day), learning and memory were assessed by the well-established, hippocampus-dependent Morris water maze at UAB behavioral assessment core facility as described previously (Akhter et al., 2015, 2018). During day 1 through day 5 of the training period, the mice were placed in the water basin next to and facing the wall successively in north (N), east (E), south (S), and west (W) positions (4 trials/day/mouse with the intertrial interval of 2 minutes). The hidden platform was placed 0.5 cm below the water surface at the southeast quadrant. All mice were tested on the same day in a counterbalanced order. The escape latencies (from the time mice were placed into the water till they found the platform), swim path lengths (distances), and swim speeds were recorded simultaneously with a camera-driven tracker system (Noldus Ethovision system, version 7.1). On the day 5, probe trials were conducted after water maze tests by removing the platform and recording the time each mouse spent in each quadrant in a 1-minute trial.

2.4. Tissue collection

After memory tests, mice were euthanized by overdosing with isoflurane, followed by bilateral thoracotomy; blood was withdrawn from the heart, followed by transcardial perfusion (Liu et al., 2011). The brain was dissected sagittally into right and left hemispheres with the right hemisphere fixed in 10% phosphate buffered formalin and the left hemisphere dissected and the hippocampus and cerebral cortex frozen in liquid nitrogen immediately for subsequent biochemical analyses.

2.5. High performance liquid chromatography (HPLC) analyses of low molecular weight antioxidants in the hippocampus

The hippocampus was homogenized with 5% perchloric acid/0.2 M Boric acid/10uM γ-glutamylglutamate (internal standard) solution (100 μL buffer/10 mg tissue) as described previously (Jones and Liang, 2009). GSH, glutathione disulfide, Cys, and cystine levels in the hippocampus were measured using a well-established high performance liquid chromatography method (Jones and Liang, 2009), calculated based on the standard curves run simultaneously with samples, and normalized by protein content. The total amount of GSH or Cys was calculated based on the following equation: total [GSH]/[Cys] = [GSH]/[Cys] + 2 × [glutathione disulfide]/[cystine].

2.6. Analyses of enzyme activities

Total activity of superoxide dismutase (SOD) in hippocampal homogenates was measured using a kit from Sigma (catalog number 19160-1KT), following the manufacturer's protocol. Catalase activity was determined using a fluorescence-based method as described previously (Ando et al., 2008). Activities of thioredoxin8 1 (Trx1) and thioredoxin reductase 1 (TXNRD1) were assessed under nonreducing conditions using a Kit from Cayman (catalog number 11526) as we have described before (Li et al., 2016). The

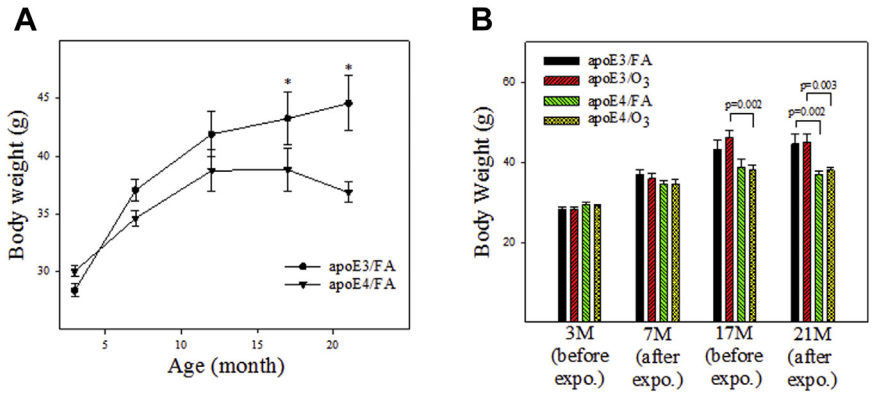

Fig. 1. Effects of age and O_3 exposure on body weights of male apoE3 and apoE4 TR mice. (A) Age-dependent increases in body weights of unchallenged apoE3 and apoE4 male mice. *, Significantly different from same ages of apoE4 mice ($p < 0.05$; n = 13–16). (B) Effects of O_3 exposure on body weights of apoE3 and apoE4 male mice. Body weights were recorded at before and after 8 cycles of filtered air (FA) or O_3 exposure (n = 13–16). Abbreviations: O_3, ozone; apoE, apolipoprotein E; TR, targeted replacement.

activity of GSH peroxidase (GPx) was assessed after the protocol described previously (Flohe and Gunzler, 1984).

2.7. Western analyses of specific proteins

For Western analysis of the abundances of specific proteins, the mouse hippocampus was homogenized in a buffer containing 10 mM Tris-HCl, pH 7.4, 250 mM sucrose, 0.1% Triton X-100, 0.1 mM of diethylenetriaminepentaacetic acid, and protease inhibitor cocktail. Diethylenetriaminepentaacetic acid was used to block adventitious iron-mediated sample auto-oxidation. For analysis of proteins modified by 4-hydroxynonenal (4-HNE), a lipid peroxidation product, or by GSH (glutathionylation), equal amounts of proteins (50 μg) from each sample were subjected to 10% polyacrylamide gel electrophoresis using nonreducing buffers, and the proteins were transferred to nitrocellulose membrane using Trans-blot Turbo system (Bio-Rad) at

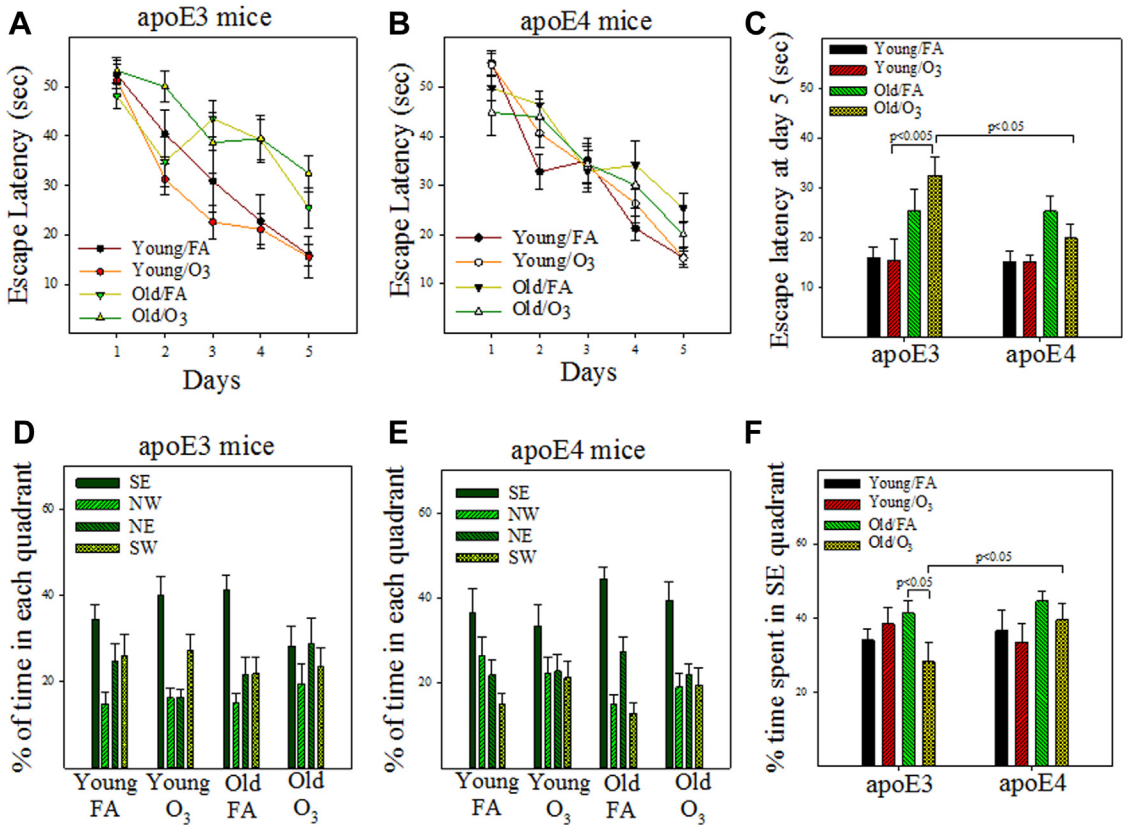

Fig. 2. Effect of age and O_3 exposure on memory of male apoE3 and apoE4 TR mice. Memory function was assessed by Morris Water Maze (MWM) as described in the Materials and methods section. (A and B) Time-dependent changes in escape latencies of filtered air (FA)- and O_3-exposed young and old apoE3 (A) and apoE4 (B) mice. (C) Escape latencies at the day 5 of MWM test. (D-F) Probe trials were performed 24 hours after MWM test and the percentage of time spent in southeast (SE), north west (NW), north east (NE), and south west (SW) quadrants are presented in D (apoE3) and E (apoE4) panels while the times spent in SE (the correct) quadrant are summarized in panel F. Statistical analyses were performed using general linear models with repeated measures and 3 fixed factors (age, exposure, and genotype) as described in the Material and method section. The *p*-values are from *Tukey's* Honestly Significant Difference (HSD) post hoc analyses (n = 13–16). Abbreviations: O_3, ozone; apoE, apolipoprotein E; TR, targeted replacement.

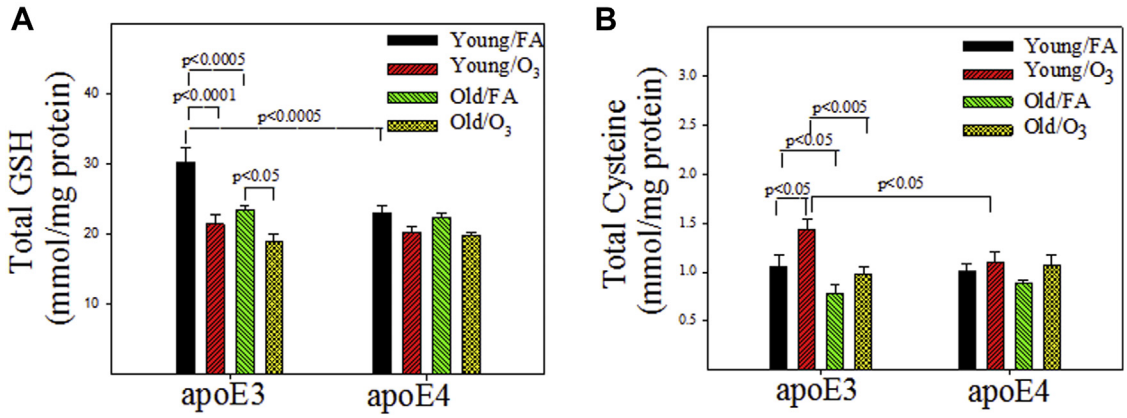

Fig. 3. Effect of age, APOE genotype, and O_3 exposure on the concentrations of total GSH and cysteine in the hippocampus. The amounts of GSH, glutathione disulfide (GSSG), cysteine, and cystine in the mouse hippocampus were measured by HPLC and normalized by protein as described in the Materials and methods section. The total amounts of GSH (A) and cysteine (B) were calculated according to the equation: [GSH]/[cysteine] = [GSH]/[cysteine] + 2 × [GSSG]/[cysteine]. The results were analyzed by 3-factor ANOVA, and the *p*-values are from Tukey post hoc analyses (n = 8). Abbreviations: O_3, ozone; apoE, apolipoprotein E; GSH, glutathione; ANOVA, analysis of variance; HPLC, high performance liquid chromatography.

room temperature for 2 hours. The membranes were then blocked with 5% milk and probed with anti 4-HNE antibody (Alpha Diagnostic, HNE-11S, 1:1000 dilution). The membranes were then stripped with a mild stripping buffer containing no reducing agent and reprobed with anti-GSH antibody (ViroGen; 101-A-100, 1:1000 dilution). For analyses of other proteins, 50 μg of proteins were subjected to 4%–20% gel electrophoresis under reducing conditions and proteins transferred to nitrocellulose membranes as described previously. The membranes were probed with antibodies to glial fibrillary acid protein (GFAP) (Sigma; G9269, 1:1000 dilution), tumor necrosis factor alpha (TNFα) (Santa Cruz; sc 52746, 1:500 dilution), glutaredoxin 1 (Abcam; ab45953, dilution 1:1000 dilution), and glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz, sc 47724, 1:2000 dilution). Semiquantifications of band intensities were performed using the ImageJ software and protein loading normalized by glyceraldehyde 3-phosphate dehydrogenase.

2.8. Immunostaining of activated astrocytes and microglia in the hippocampus

Activated astrocytes were revealed by immunostaining of brain tissue sections with anti-GFAP antibody (Sigma; G9269, 1:500 dilution) and anti-aldehyde dehydrogenase 1 family member L1 antibody (Aldh1L1) (Abcam; ab87117, 1:500 dilution). Microglia were revealed with antibodies to ionized calcium-binding adaptor molecule 1 (Iba1) (Abcam; ab178847, 1:1000 dilution) and to classification determinant

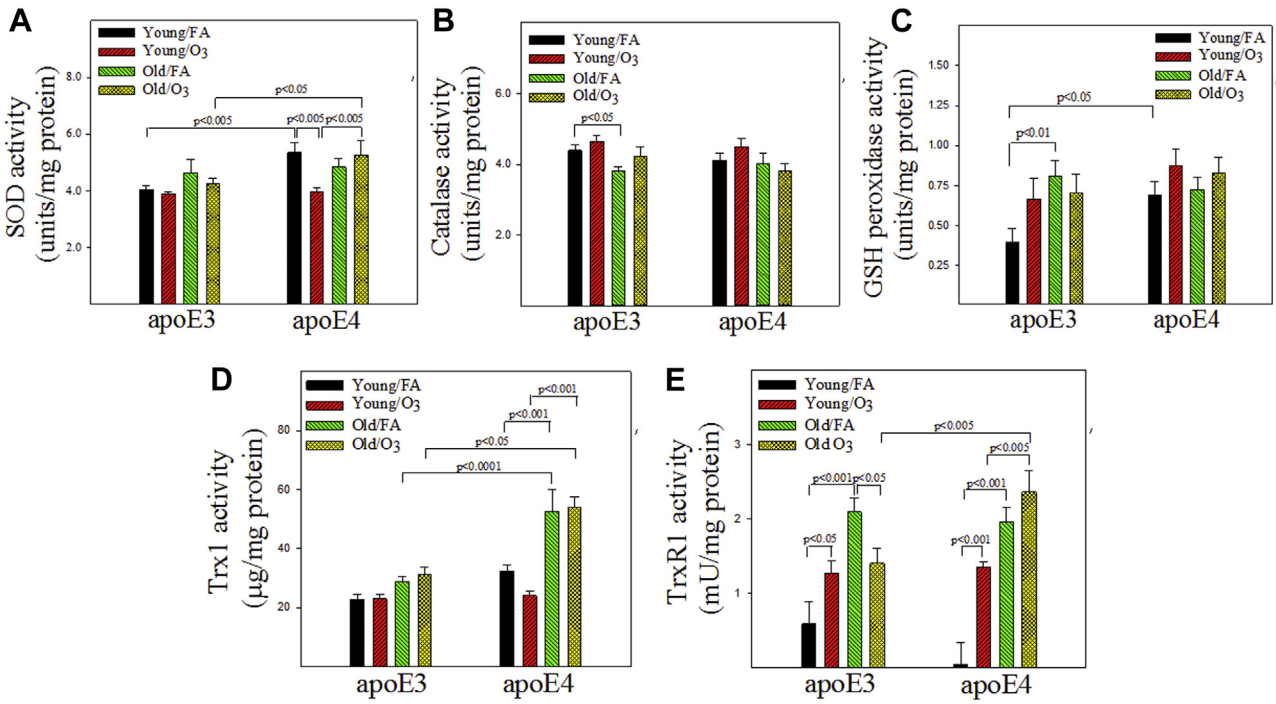

Fig. 4. Effect of age, APOE genotype, and O_3 exposure on the activities of antioxidant enzymes in the hippocampus. The activities of total superoxide dismutase (SOD) (A), catalase (B), thioredoxin 1 (Trx1) (C), thioredoxin reductase 1 (TXNRD1) (D), and glutathione peroxidase (GPx) (E) in mouse hippocampus tissue were measured as described in the Materials and methods section. The results were analyzed by 3-factor ANOVA, and the *p*-values are from Tukey post hoc analyses (n = 6–10). Abbreviations: O_3, ozone; apoE, apolipoprotein E; ANOVA, analysis of variance.

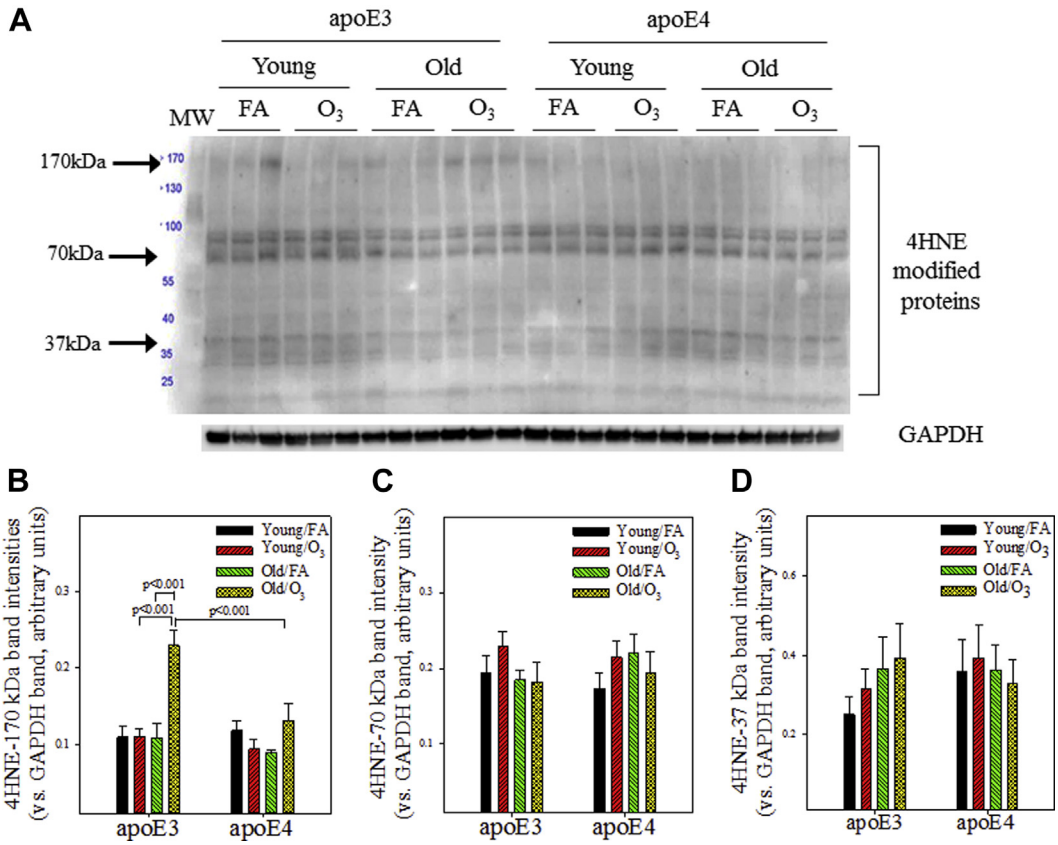

Fig. 5. Effect of age and O_3 exposure on protein oxidative modifications and glutaredoxin 1 protein expression in the hippocampus of male apoE3 and apoE4 TR mice. (A-D) 4-HNE–modified proteins; (E-H) GSH-modified proteins (glutathionylation). Top panels (A and E): representative Western blots; bottom panels (B-D and F-H): semi-quantifications of the intensities of the bands as indicated by arrows after normalized to corresponding GAPDH band using the ImageJ software. (I and J) Western blots of glutaredoxin 1 (Glrx1) protein in the mouse hippocampus. GAPDH was used to normalize sample loading. The quantitative results are from 6 samples per group. Statistical analyses were performed by 3-factor ANOVA, and the *p*-values are from Tukey post hoc analyses (n = 6). Abbreviations: O_3, ozone; apoE, apolipoprotein E; GSH, glutathione; ANOVA, analysis of variance; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; 4-HNE, 4-hydroxynonenal; TR, targeted replacement.

11b (CD11b) (Abcam; ab133357, 1:200 dilution). The numbers of activated astrocytes and microglia were counted in the hippocampus and results expressed as numbers of cells/mm^2.

2.9. Analysis of amyloid beta peptide deposition in the hippocampus

Brain amyloid beta (Aβ) deposits (plaques) were assessed by immunohistochemical staining techniques using anti-mouse Aβ1-42 monoclonal antibody (Signet-39142). For the measurement of hippocampal Aβ42 and Aβ40, the mouse hippocampus was homogenated in a cell extraction buffer (FNN0011, Invitrogen) containing protease inhibitor cocktail and then extracted with guanidine hydroxide. After centrifugation, the amounts of Aβ42 and Aβ40 in the supernatant were quantified using the ELISA kits from Invitrogen, following the protocol provided by the manufacturer.

2.10. Statistical analysis

Data are expressed as mean ± standard deviations. T-tests were performed for the data presented in Fig. 1A to compare body weights between unchallenged apoE3 and apoE4 mice at same age. Body weights were also compared by analysis of variance with 2 fixed factors (genotype and exposure) (2-factor ANOVA) at the ages indicated (Fig. 1B). For the memory function data presented in Fig. 2A and B, SAS, version 9.4, was used for general linear model analysis with repeated measures to determine if changes in escape latencies over 5 days are different by experiment factors: genotype (apoE3 vs. apoE4), age (old vs. young), and exposure (FA vs. O_3). The comparison was repeated for the evaluation of differences in escape latencies between 2 groups at the same level of genotype, age, or treatment. Because memory deficits usually become evident at day 5, we have also compared the escape latencies at day 5 using ANOVAs with 3 fixed factors (age, exposure, and genotype) (3-factor ANOVA), followed by *Tukey's* Honestly Significant Difference *post hoc* analysis. For the data presented in Figs. 3–7 and in the Supplementary figures, 3-factor ANOVA and Honestly Significant Difference analyses were performed. The *p* values presented in the figures are from post hoc analysis. Sample sizes are included in figure legends for each experimental measure.

3. Results

3.1. Effects of age and O_3 exposure on the body weights of male apoE3 and apoE4 TR mice

Mouse body weights were determined at 3, 7, 12, 17, and 21 months of age and every week during the O_3 exposure period. Results show that, under unchallenged conditions (FA exposure), male apoE3 mice have significantly greater body weights than male apoE4 mice at 17 and 21 months of age (Fig. 1A). Two-factor ANOVA further identifies a significant effect of genotype, but not O_3 exposure, on the body weights at 17 and 21 months of age (Table 1 and Fig. 1B). O_3 exposure also has no immediate effect on body weights, measured right after each exposure cycle (data not shown).

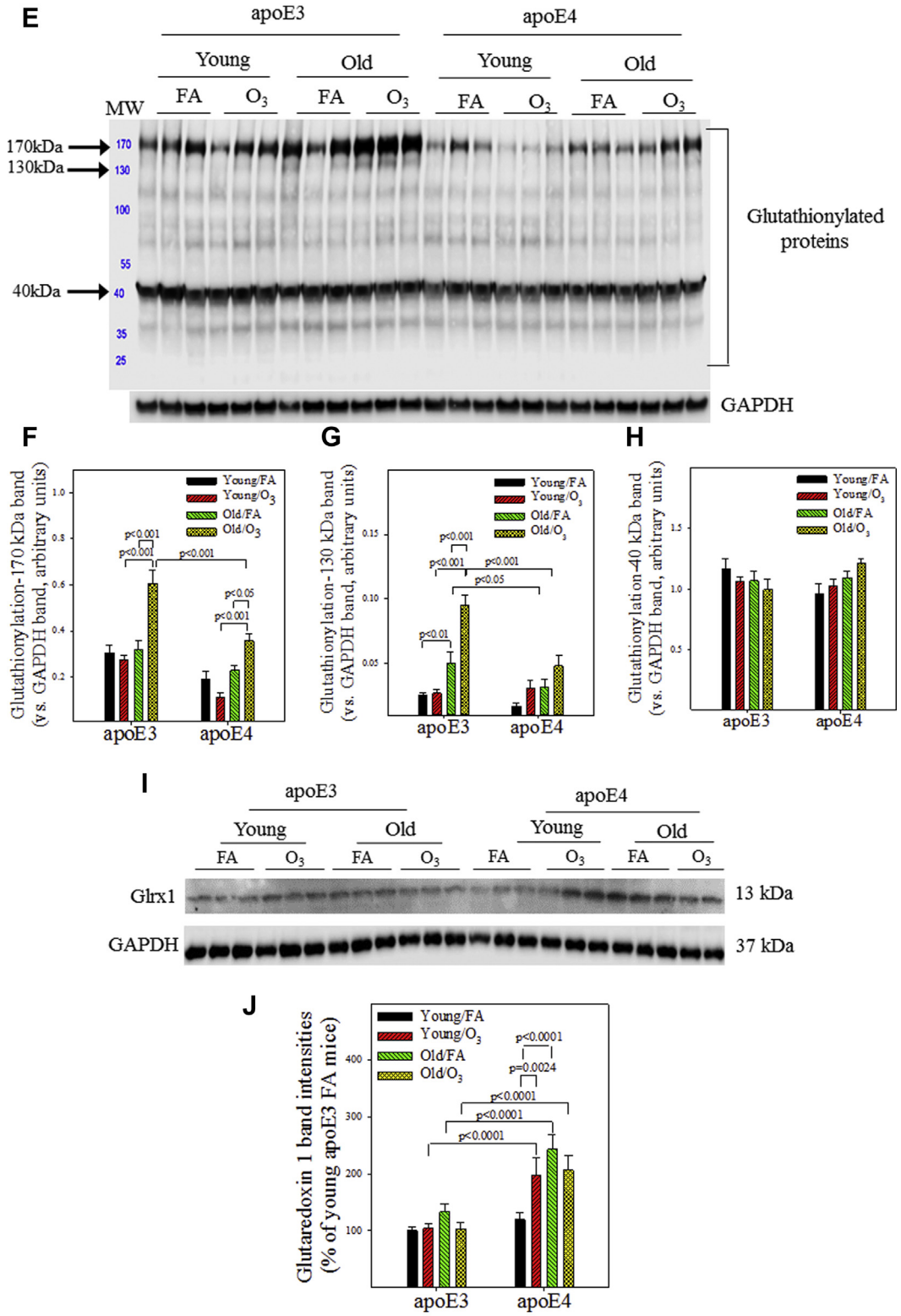

Fig. 5. (*continued*).

3.2. Effect of age and O_3 exposure on memory of male apoE3 and apoE4 TR mice

To test whether O_3 exposure synergizes with *ApoE ε4* and aging to impair memory, we exposed 3- and 17-month-old male apoE4 mice to a cyclic O_3 exposure protocol, which mimics human exposure scenarios and compared the results to age-matched male apoE3 mice. Statistical analyses (performed using a general linear model with repeated measures) show that the trends of escape latency decline over 5 days of water maze test are significantly different overall among 8 groups [$F_{(28,416)} = 2.21$; $p = 0.0007$]. Subgroup analyses identify a significant difference in the

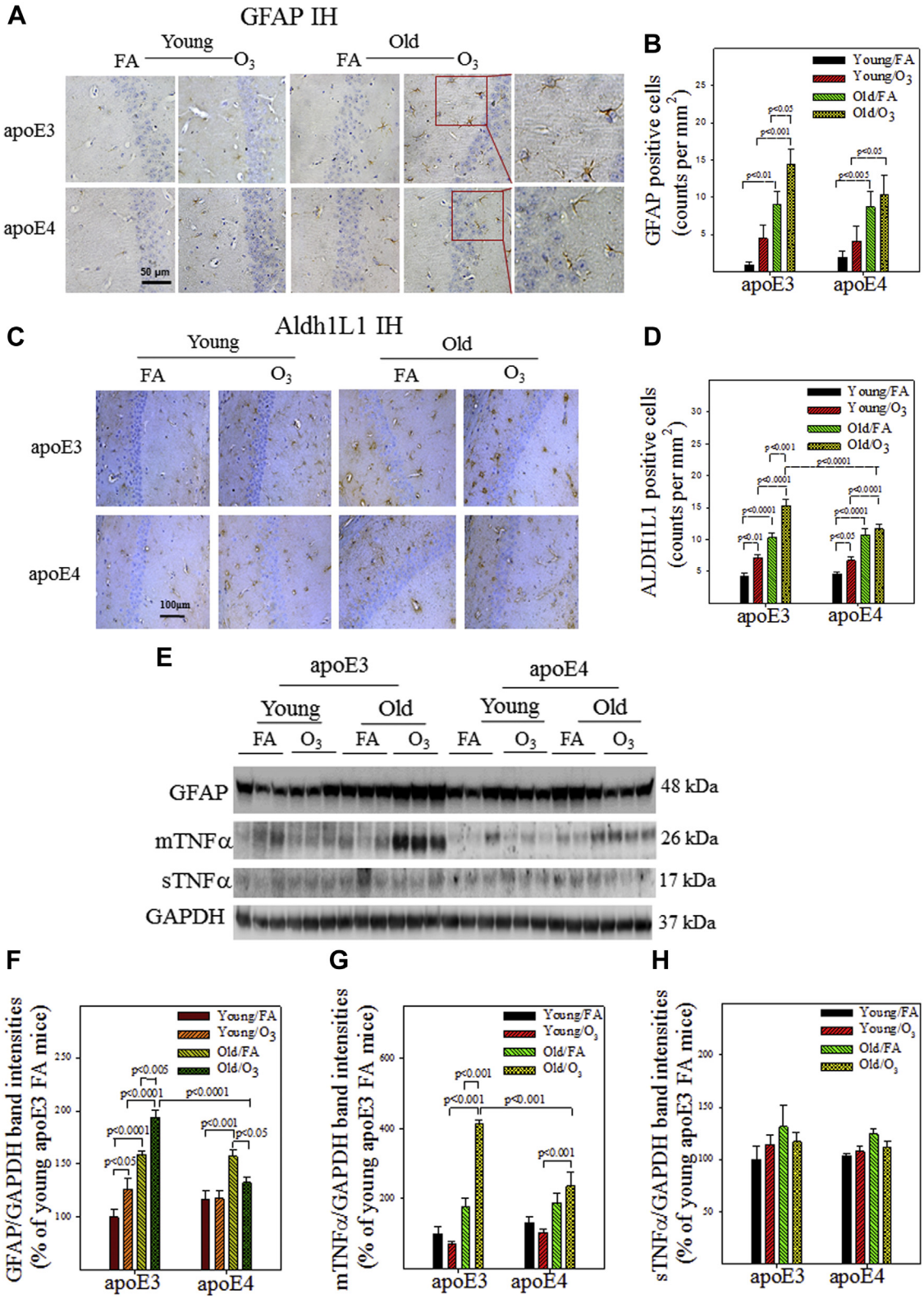

Fig. 6. Effects of age and O_3 exposure on inflammatory responses in the hippocampus of male apoE3 and apoE4 TR mice. (A and B) Representative pictures of hippocampal immunostaining and quantitative data of GFAP-positive cells. (C and D) Representative pictures of hippocampal immunostaining and quantitative data of Aldh1L1-positive cells. (E-H) Representative Western blot pictures of GFAP, mTNFα, and sTNFα proteins as well as semiquantification of the band intensities. GAPDH was used to normalize sample loading. (I and J) Representative immunostaining and quantification of Iba1-positive microglia in the hippocampus. (K and L) Representative immunostaining and quantification of CD11b-positive microglia in the hippocampus. The results were compared between 8 groups by 3-factor ANOVA and the *p*-values are from Tukey post hoc analyses (n = 6–7). Abbreviations: O_3, ozone; apoE, apolipoprotein E; Aldh1L1, anti-aldehyde dehydrogenase 1 family member L1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mTNFα, membrane-bound TNFα; sTNFα, soluble TNFα; GFAP, glial fibrillary acid protein; ANOVA, analysis of variance; 4-HNE, 4-hydroxynonenal; TR, targeted replacement.

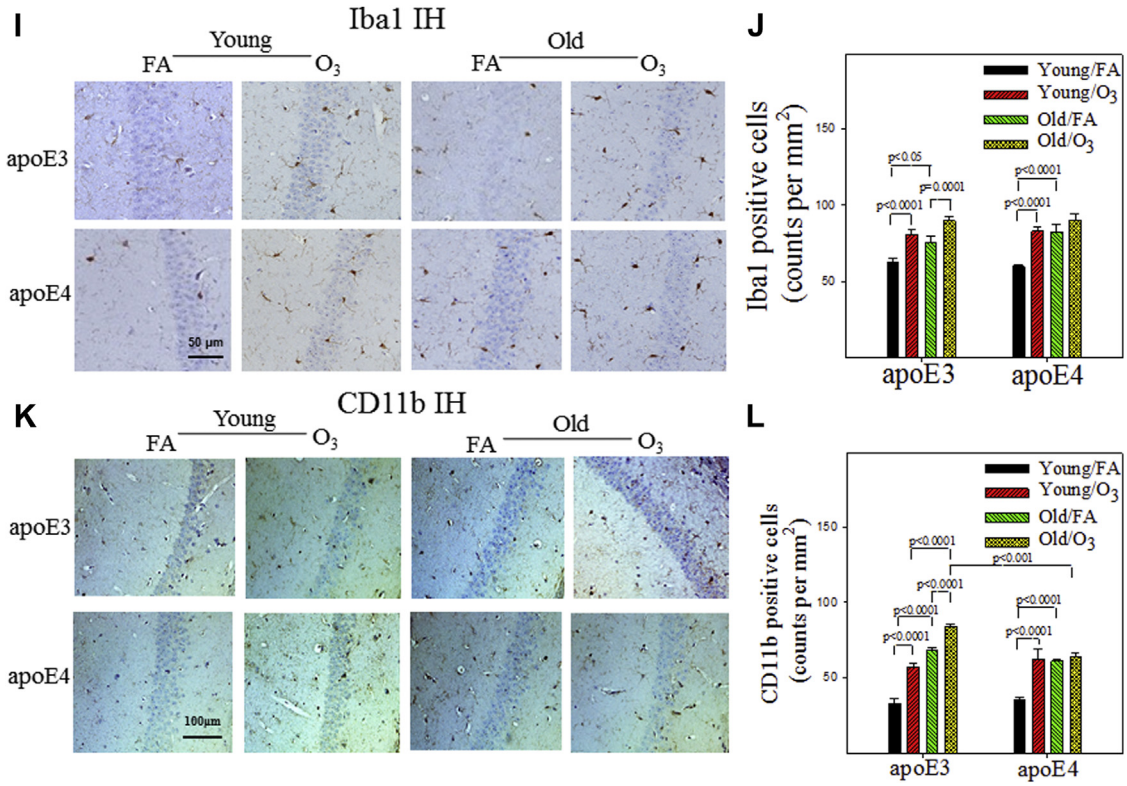

Fig. 6. (*continued*).

trends of escape latency decline between FA- and O_3-exposed old apoE3 mice ($F_{(4, 112)} = 2.27$; $p = 0.0332$; treatment effect, $p = 0.1146$; day effect, $p = 0.0005$). There is no significant difference in the trends between the other groups (Fig. 2A and B). As memory deficits usually become evident at day 5, we also analyzed the day 5 escape latency data using 3-factor ANOVA. Statistical analyses reveal significant effects of genotype and age on day 5 escape latencies (Table 2, Fig. 2C). Post hoc analyses further show that the escape latencies at day 5 are significantly increased in O_3-exposed old apoE3 mice when compared with O_3-exposed young apoE3 mice and O_3-exposed old apoE4 mice (Fig. 2C). Statistical analyses of probe trial data also show that O_3-exposed old apoE3 mice spend significantly shorter time in the southeast (correct) quadrant than do FA-exposed old apoE3 mice and O_3-exposed old apoE4 mice (Fig. 2D–F). These data suggest that O_3 exposure synergizes with age, leading to impairment of learning/memory in male apoE3, but not male apoE4, mice.

No significant difference is observed in swimming speeds between O_3- and FA-exposed mice, apoE3 or apoE4, although swimming speeds are slightly higher in old versus young mice (Supplementary Fig. 1A and B). Three-factor ANOVA of the data from open field and zero maze tests identifies significant effects of genotype and age, but not O_3 exposure, on anxiety levels (Table 2, Supplementary Fig. 2). Post hoc analyses further show that, compared with age-matched male apoE3 mice, both young and old male apoE4 mice have higher levels of fear/anxiety, as indicated by significantly less time spent in the center in the open field test (Supplementary Fig. 2B) and in the open area in the zero maze

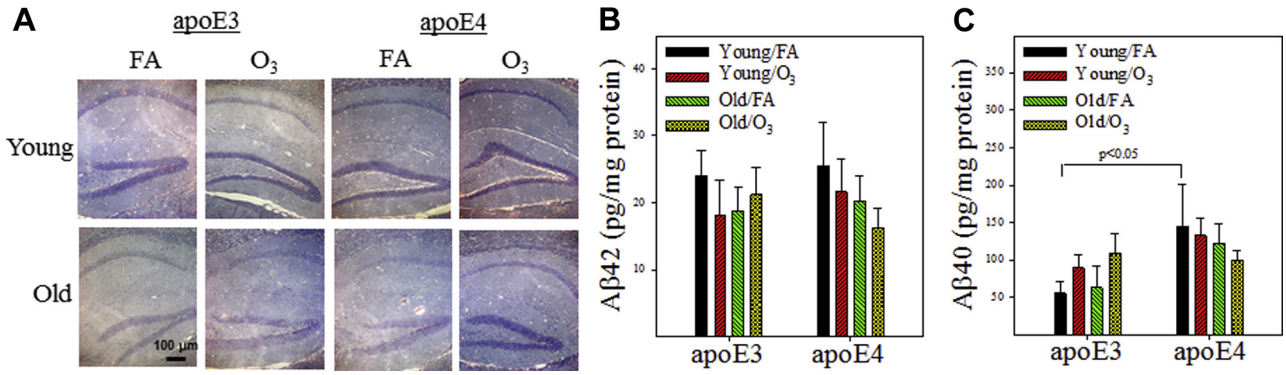

Fig. 7. Effect of age and O_3 exposure on Aβ deposition in the hippocampus of apoE3 and apoE4 TR male mice. (A) Representative immunostaining pictures of Aβ plaque in the hippocampus of mice; (B and C) The amounts of guanidine-soluble Aβ42 and Aβ40 in the hippocampus were determined by ELISAs. The results were analyzed by 3-factor ANOVA, and the *p*-values are from Tukey post hoc analyses (n = 8). Abbreviations: O_3, ozone; apoE, apolipoprotein E; TR, targeted replacement; Aβ, amyloid beta; ANOVA, analysis of variance.

Table 1
Two-factor ANOVA of body weights at different ages

Ages	Genotype		O_3		Genotype × O_3	
	F	*p* Value	*F*	*p* Value	*F*	*p* Value
3 mo	2.975	0.091	0.254	0.617	0.058	0.811
7 mo	3.017	0.089	0.309	0.581	0.243	0.625
17 mo	12.037	0.001	0.374	0.543	1.079	0.303
21 mo	20.309	<0.001	0.265	0.608	0.032	0.859

Key: ANOVA, analysis of variance; O_3, ozone.

test (Supplementary Fig. 2D). There is no effect of age on the anxiety level of apoE4 mice (Supplementary Fig. 2C and D). Age effect on anxiety levels in apoE3 mice is uncertain as old apoE3 male mice spend less time in the center in the open field test (higher anxiety) (Supplementary Fig. 2B) but more time in the open area in the zero maze test (less anxiety) (Supplementary Fig. 2D), compared with young apoE3 mice. O_3 exposure alone does not increase fear/anxiety levels in young or old mice of either genotype. Together, the results suggest that the increased escape latency in the water maze test and the decreased retention time in the correct quadrant in the probe trial in O_3-exposed old apoE3 mice are not due to a decline in motor activity or an increase in anxiety level.

3.3. Effects of age, APOE genotype, and O_3 exposure on the concentrations of total GSH and Cys in the hippocampus

GSH is the most abundant intracellular free thiol and an important antioxidant, whereas Cys is a rate-limiting substrate for GSH synthesis. To explore the mechanism whereby O_3, a highly reactive oxidant, impairs memory of old apoE3 mice, we measured the total amounts (reduced and oxidized) of GSH and Cys in the hippocampus by high performance liquid chromatography. Three-factor ANOVA reveals significant effects of genotype, O_3 exposure, and age on GSH levels as well as O_3 exposure and age on Cys levels (Table 3). There are significant interactions between genotype and age on both GSH and Cys levels (Table 3). Post hoc analyses further show that the basal level of GSH in young apoE4 mice is significantly lower than that in young apoE3 mice; apoE3, not apoE4, mice, however, experience significant age-related and O_3 exposure–related decline in GSH concentrations (Fig. 3A). Cys levels decrease with increased age in apoE3, not apoE4, mice (Fig. 3B), whereas O_3 exposure increases Cys level in young apoE3 mice (Fig. 3B).

3.4. Age-dependent and O_3 exposure–dependent changes in the activities of enzymes involved in antioxidant defense in the hippocampus of male apoE3 and apoE4 TR mice

SOD and catalase are responsible for the reduction of superoxide and hydrogen peroxide, respectively, whereas GPx reduces hydrogen peroxide and lipid peroxide. In addition to their role in hydroperoxide reduction, Trx1 and TXNRD1 also contribute to protein thiol maintenance by reducing protein disulfide bonds formed during oxidative stress. Thus, we measured the activities of these enzymes in the hippocampus to further explore the mechanism underlying O_3-induced memory impairment in old apoE3 mice. Three-factor ANOVA show significant effects of genotype on SOD activity, age on catalase activity, genotype and age on Trx1 activity, as well as age and O_3 exposure on TXNRD1 activity (Table 3). There are significant interactions between genotype, age, and exposure on SOD, Trx1, and TXNRD1 activities (3 factors or 2 factors interact) (Table 3). Post hoc analyses further show that basal SOD activity in FA-exposed mice is significantly higher in young apoE4 mice compared with young apoE3 mice and remains elevated even after O_3 exposure (Fig. 4A). Catalase activity is decreased with increased age in apoE3, but not apoE4, mice (Fig. 4B). GPx activity is significantly higher in young apoE4 and old apoE3 mice compared with young apoE3 mice under unchallenged condition (Fig. 4C). Trx 1 activity is significantly higher in old apoE4 mice when compared with other groups, with or without O_3 exposure (Fig. 4D). TXNRD1 activity, on the other hand, is increased with age in both apoE3 and apoE4 mice and in O_3-exposed young mice. O_3 exposure, however, significantly reduces TXNRD1 activity in old apoE3, but not old apoE4, mice (Fig. 4E). Together, the results suggest that some antioxidant defense mechanisms are upregulated in the hippocampus of male apoE4 mice, especially old apoE4 mice.

3.5. Age-dependent and O_3 exposure–dependent protein oxidative modifications in the hippocampus of male apoE3 and apoE4 TR mice

To further assess oxidative stress responses in these mice, we determined the levels of proteins that are modified by 4-HNE, a lipid peroxidation product, and by GSH (glutathionylation) in the hippocampus. Three-factor ANOVA reveals significant effects of O_3 exposure and age on the levels of 4-HNE–modified proteins as well as effects of genotype, O_3 exposure, and age on the levels of glutathionylated proteins (Table 4). Post hoc analyses further show that the levels of 4-HNE–modified proteins at 170 kDa size are significantly higher in the hippocampus of O_3-exposed old apoE3 male mice when compared with other groups of mice (Fig. 5A and B). There is no significant difference in the amounts of 4-HNE–modified proteins at sizes of 70 kDa or 37 kDa (Fig. 5A, C and D). The levels of glutathionylated proteins at 170 kDa and 130 kDa are significantly higher in O_3-exposed old apoE3 mice when compared with mice in other groups (Fig. 5E–G). A smaller but statistically significant increase in 170 kDa glutathionylated proteins is also detected in O_3-exposed old apoE4 mice when compared with FA-exposed old apoE4 mice and young O_3-exposed apoE4 mice (Fig. 5E and F).

To explore possible mechanisms underlying differences in protein glutathionylation, we assessed hippocampal expression of glutaredoxin 1 (Glrx1), an enzyme that catalyzes the reduction of

Table 2
Three-factor ANOVA of day 5 escape latency, probe trial, open field, and zero maze tests

	Genotype		O_3		Age		Genotype × O_3		Genotype × age		O_3 × age		Genotype × age × O_3	
	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*	$F_{(1, 104)}$	*p*
Day 5 escape latency	8.48	0.0044	0.34	0.5605	6.43	0.0127	2.14	0.1468	3.55	0.0625	0.05	0.8277	1.24	0.2678
Probe trial	0.25	0.6211	3.40	0.0679	2.09	0.1517	0.13	0.7159	3.81	0.0537	1.47	0.2284	2.94	0.0895
OF test (at the side)	43.6	<0.001	0.53	0.4681	12.2	0.0007	0.22	0.6418	2.49	0.1179	0.00	0.9445	0.05	0.8243
ZM test (in open area)	39.1	<0.001	0.37	0.5441	13.3	0.0004	0.45	0.5062	0.39	0.5349	0.27	0.6054	5.27	0.0237

Key: ANOVA, analysis of variance; O_3, ozone; OF, open field; ZM, zero maze.

Table 3
Three-factor ANOVA of antioxidants in the hippocampus

	Genotype		O_3		Age		Genotype × O_3		Genotype × age		O_3 × age		Genotype × age × O_3	
	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p	$F_{(1, 63)}$	p
Total GSH	10.12	0.0024	36.8	<0.001	10.1	0.0025	2.36	0.1299	4.31	0.0425	1.12	0.2955	1.59	0.2120
Total Cysteine	0.33	0.5706	9.90	0.0026	10.6	0.0019	1.04	0.3124	4.39	0.0407	0.04	0.8496	0.89	0.3505
SOD activity	8.65	0.0044	2.97	0.0890	3.91	0.0519	0.25	0.6204	0.04	0.8331	3.14	0.0805	5036	0.0234
Catalase activity	1.08	0.3011	2.13	0.1488	9.06	0.0036	0.63	0.4312	0.15	0.6965	0.48	0.4920	1.52	0.2217
Trx1 activity	22.6	<0.001	0.35	0.5538	28.9	<0.001	1.21	0.2760	8.31	0.0056	0.61	0.4375	0.15	0.7017
TXNRD1 activity	0.97	0.3300	8.49	0.0052	49.7	<0.001	8.58	0.0050	5.26	0.0258	8.00	0.0066	1.30	0.2586
GPx	3.62	0.0644	2.48	0.1229	2.35	0.1331	0.19	0.6663	2.67	0.1100	2.68	0.1097	1.09	0.3032

Key: ANOVA, analysis of variance; O_3, ozone; GSH, glutathione; SOD, superoxide dismutase; Trx1, thioredoxin8 1; TXNRD1, thioredoxin reductase 1; GPx, GSH peroxidase.

glutathionylated proteins. Three-factor ANOVA reveals significant effects of genotype and age on hippocampal Glrx1 protein levels and an interaction between genotype, exposure, and age (Table 4). Post hoc analyses further show that O_3 exposure significantly increases hippocampal Glrx1 protein levels in young apoE4, but not young apoE3, male mice (Fig. 5I and J), and that Glrx1 protein levels are significantly higher in old apoE4 than in old apoE3 male mice, with or without O_3 exposure (Fig. 5I and J). Together, the data suggest that upregulation of some antioxidant defense mechanism may render male apoE4 mice higher resistance to O_3-induced oxidative stress.

3.6. Effect of age and O_3 exposure on neuroinflammation in the hippocampus of male apoE3 and apoE4 TR mice

Astrocytes and microglia play important role in neuroinflammatory response. To assess astrocyte and microglia activation, brain tissue sections were immunostained with antibodies to GFAP and Aldh1L1, 2 astrocyte markers, or with antibodies to Iba1 and CD11b, 2 microglia markers. Hippocampal proteins of GFAP and TNFα were also assessed by Western blotting. Three-factor ANOVA reveals significant effects of genotype, age, and exposure on astrocyte activation and TNFα protein expression (Table 5). Post hoc analyses further show that there are significant age-dependent increases in the numbers of GFAP (Fig. 6A and B) or Aldh1L1 (Fig. 6C and D) positive cells in FA-exposed apoE3 and apoE4 mice. O_3 exposure, however, further promotes astrocyte activation in aged apoE3, but not aged apoE4, male mice (Fig. 6A–D). Western blotting data further show that the amount of GFAP protein increases with age in both apoE3 and apoE4 mice (Fig. 6E and F). O_3 exposure, on the other hand, further increases GFAP expression in old apoE3, not apoE4, mice. Western blotting data also show that the amounts of membrane-bound TNFα increase more in O_3-exposed old apoE3 mice compared with O_3-exposed old apoE4 mice, although there is no significant difference in the amounts of soluble TNFα (sTNFα) between any groups (Fig. 6E, G and H). Three-factor ANOVA also reveals significant effects of genotype, age, and exposure on the number of Iba1- or CD11b-positive cells and an interaction between 3 factors (Table 5). Post hoc analyses show that the numbers of Iba1- and/or CD11b-positive cells increase with age in the hippocampus of FA-exposed apoE3 and apoE4 mice (Fig. 6I–L). O_3 exposure, on the other hand, significantly increases Iba1- and/or CD11b-positive cell counts in young apoE3 and apoE4 mice as well as in old apoE3 mice (Fig. 6I–L). Together, the results suggest that astrocytes and microglia are activated without challenge in both apoE3 and apoE4 male mice; O_3 exposure, however, further activates these cells and increases inflammatory response in old apoE3, but not old apoE4, male mice.

3.7. Hippocampal amyloid load in O_3-exposed male apoE3 and apoE4 TR mice

The amyloid cascade theory of AD remains controversial. To elucidate whether memory impairment in O_3-exposed old apoE3 mice was associated with an increase in brain Aβ load, we measured the amounts of Aβ42 and Aβ40 as well as Aβ plaques in the hippocampus of apoE3 and apoE4 male mice by ELISAs and immunohistochemical staining. The results show no obvious Aβ plaque in the brain of any group of mice (Fig. 7A). There is also no significant difference in the amounts of Aβ42 between any groups, although there is a trend of increase in the amounts of Aβ40 in FA-exposed young apoE4 mice when compared with FA-exposed young apoE3 mice (Fig. 7B and C). O_3 exposure has no significant effect on brain Aβ load in either genotype or aged mice.

4. Discussion

The etiology of LOAD, which accounts for >95% of AD cases, is unknown. Accumulated evidence suggests that LOAD results from complex interactions between genetic and environmental risk factors plus aging. The *APOE ε4* allele is a major genetic risk factor for LOAD with women *APOE ε4* carriers at greatest risk (Farrer et al., 1997; Liu et al., 2013; Raber et al., 2000). Which environmental factor(s) contributes to the onset of LOAD, however, is unknown. Recent epidemiological studies show that exposure to high levels of O_3 is associated with increased incidence of AD (Chen and Schwartz, 2009; Cleary et al., 2018; Gatto et al., 2014; Jung et al., 2015; Wu et al., 2015). Nonetheless, whether O_3 is a culprit for AD and whether O_3 acts alone or synergizes with other risk factors

Table 4
Three-factor ANOVA of protein oxidative modifications in the hippocampus

	Genotype		O_3		Age		Genotype × O_3		Genotype × age		O_3 × age		Genotype × age × O_3	
	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p
4-HNE 170 kDa band	3.21	0.0808	13.1	0.0008	6.96	0.0118	3.95	0.0537	8.82	0.0050	22.0	<0.001	2.39	0.1303
GSH 130 kDa band	16.3	0.0002	19.9	<0.001	52.4	<0.001	0.78	0.3837	12.9	0.0009	6.72	0.0133	5.94	0.0194
GSH 170 kDa band	27.1	<0.001	10.5	0.0024	40.7	<0.001	0.91	0.3447	3.08	0.0868	28.2	<0.001	5.02	0.0306
Glrx1 Western blot	61.8	<0.001	0.12	0.7350	7.68	<0.001	2.36	0.1324	5.30	0.0266	6.07	0.0182	4.64	0.0372

Key: 4-HNE, 4-hydroxynonenal; ANOVA, analysis of variance; O_3, ozone; GSH, glutathione; Glrx1, glutaredoxin 1.

Table 5
Three-factor ANOVA of neuroinflammation markers in the hippocampus

	Genotype		O_3		Age		Genotype × O_3		Genotype × age		O_3 × age		Genotype × age × O_3	
	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p	$F_{(1, 40)}$	p
GFAP														
Western blot	7.47	0.0093	3.54	0.0674	80.1	<0.001	17.76	<0.001	12.41	0.0011	0.64	0.4302	2.94	0.0942
IH	0.14	0.7149	21.6	<0.001	45.1	<0.001	0.36	0.5493	0.18	0.6712	7.84	0.0075	0.05	0.8320
TNFα protein	4.77	0.0349	18.3	<0.001	90.2	<0.001	12.81	<0.001	10.58	0.0023	25.27	<0.001	6.87	0.0123
ALDH1L1 IH	2.68	0.1081	30.1	<0.0001	159	<0.0001	5.52	0.0230	2.23	0.1415	0.25	0.6205	2.68	0.1081
Iba1 IH	1.40	0.2439	65.3	<0.0001	26.8	<0.0001	1.01	0.3209	5.06	0.0301	10.8	0.0021	6.71	0.0133
CD11b IH	4.62	0.0367	64.4	<0.0001	101	<0.0001	1.19	0.2815	14.9	0.0003	13.4	0.0006	3.15	0.0822

Key: ANOVA, analysis of variance; IH, immunohistochemistry; O_3, ozone; GFAP, glial fibrillary acid protein; TNFα, tumor necrosis factor alpha; Aldh1L1, anti-aldehyde dehydrogenase 1 family member L1; Iba1, ionized calcium-binding adaptor molecule 1.

leading to AD remain to be determined. In this study, we tested the hypothesis that O_3 exposure synergizes with *APOE ε4* and aging leading to the development of AD, using male apoE3 and apoE4 TR mice. Our results show, surprisingly, that O_3 exposure synergizes with aging, leading to memory impairment in male apoE3, but not apoE4, mice. These novel findings reveal a complex interaction between *APOE* genotype, aging, and environmental exposure in AD pathophysiology.

APOE ε4 is a major genetic risk factor for AD and predominantly impacts the susceptibility of women (Altmann et al., 2014; Farrer et al., 1997; Riedel et al., 2016; Ungar et al., 2014). Animal studies using apoE TR mice have also shown that female, not male, apoE4 mice experience memory loss under unchallenged condition (Bour et al., 2008; Raber et al., 2000; Rijpma et al., 2013; Villasana et al., 2006). Consequently, most studies have used female mice to address the effects of *APOE ε4* on neuropathophysiology. Whether and how *APOE ε4* affects the response of males to challenges has received very little attention. The results from the present study show, unexpectedly, that *APOE ε4* protects old male mice from O_3-induced memory loss. Whether this is a sex-specific response warrants further investigation. Interestingly, similar to our results, Peris-Sampedro at al. found that exposure to an organophosphate pesticide chlorpyrifos impaired spatial memory in male apoE3, not male apoE4, TR mice (Peris-Sampedro et al., 2015). Using different exposure strategy, the same group further showed that postnatal chlorpyrifos exposure impaired spatial memory in apoE3 but not apoE4 female mice (Basaure et al., 2019). Together, the results suggest that interaction between *APOE ε4* and environmental exposure is genotype, sex, and age dependent. It should be stressed that a recent epidemiologic study showed that *APOE ε4* carriers were more sensitive than non–*APOE ε4* carriers to O_3-induced cognitive decline (Cleary et al., 2018). However, as no sex-stratified data were reported, it was unclear whether the synergy was sex-related or not (Cleary et al., 2018). So far, no study has addressed the sex-dependent interaction between *APOE ε4* and O_3 exposure in AD pathophysiology in either human population or experimental animal models. More studies are warranted to answer these important questions.

The mechanism underlying selective effect of O_3 exposure on memory of old male apoE3 mice is unclear. Given that O_3 is a highly reactive oxidant, we assessed age-related and O_3 exposure–related changes in the levels of antioxidants and oxidative stress in the hippocampus of these mice. Our data show that O_3-exposed old apoE3 mice have significantly increased protein oxidative modifications than any other groups, consistent with memory impairment data. Our results further show that, although the basal level (FA exposure) of GSH is higher in young apoE3 mice compared with young apoE4 mice, both GSH and Cys concentrations decline with age and with O_3 exposure in apoE3, not apoE4, mice. Moreover, young male apoE4 mice have higher basal levels of SOD and GPx activities compared with young male apoE3 mice, whereas old apoE4 mice have higher activities of SOD, Trx1, and TXNRD1 as well as increased expression of Glrx1 compared with other groups, with or without O_3 exposure. These data suggest that decreases in GSH and Cys levels at old age may render male apoE3 mice highly sensitivity to O_3-induced oxidative stress and memory impairment. ApoE4 mice, which have lower GSH since young age, have developed some other compensatory mechanisms to combat O_3-induced damaging effects. It should be stressed that, besides Trx and Glrx, other enzymes such as sulfiredoxin also play a critical role in maintaining protein thiol homoeostasis (Mishra et al., 2015; Ramesh et al., 2014). Whether the expression/activity of this protein is altered in apoE3 and/or apoE4 mice and contributes to increased resistance of apoE4 mice to O_3-induced oxidative stress remains to be determined. It should also be pointed out that although it has been reported that oxidative stress level is higher and antioxidant levels are lower in *APOE ε4*–positive AD patients compared with non-*APOE ε4* AD patients, none of these studies have addressed sex difference (Duits et al., 2013; Glodzik-Sobanska et al., 2009; Kharrazi et al., 2008; Ramassamy et al., 2000). The studies showing apoE4 TR mice have decreased antioxidant levels and increased sensitivity to oxidative challenge than apoE3 TR mice were all conducted with young female mice (Graeser et al., 2011; Persson et al., 2017; Villasana et al., 2016). Collectively, the data from this laboratory and from others suggest that *APOE ε4*–associated decline in the antioxidant levels may be age- and sex-dependent. Our results also suggest that oxidative modifications of the proteins that are critical for cell signaling and/or neuron survival may contribute to O_3-induced memory impairment in old apoE3 male mice. Future studies are warranted to identify these redox-sensitive proteins that contribute to increased sensitivity of old apoE3 mice to O_3-induced memory impairment.

Besides oxidative stress, other aging- and/or genotype-related factors may also contribute to the increased sensitivity of old apoE3 male mice to O_3-induced memory impairment. One of these factors is inflammation. Neuroinflammation contributes importantly to neuronal damage, whereas astrocytes and microglia are the key players in neuroinflammatory responses. Our data show that both astrocytes and microglia are activated with increased age in apoE3 and apoE4 mice. O_3 exposure, however, further activates astrocytes and microglia in old apoE3, but not old apoE4, male mice. Our data are consistent with previous publications using non-O_3 stimuli, which showed that apoE3 mice exhibited greater astrocyte activation on lipopolysaccharides challenge compared with apoE4 mice (Maezawa et al., 2006; Ophir et al., 2003). The mechanism underlying the augmented response of astrocytes and microglia in old apoE3 male mice after O_3 exposure is unclear at moment. As oxidative stress plays a critical role in inflammatory responses, it is speculated that increased oxidative stress likely contributes to augmented inflammatory response in

old apoE3 mice. It should be mentioned, however, that some studies have shown increased inflammatory responses in apoE3 mice compared with apoE4 mice, although opposite observation has also been reported (Belinson and Michaelson, 2009; Liu et al., 2013; Maezawa et al., 2006; Ophir et al., 2003; Zhu et al., 2012). Therefore, it is possible that augmented inflammatory response drives excessive oxidative stress and thus memory impairment in O_3-exposed old apoE3 mice. Although we could not exclude this possibility, we believe that increased oxidative stress is more likely the driver of augmented inflammatory response and memory loss in old apoE3 mice as O_3 is highly reactive oxidant. More studies are needed to address this issue.

Increased production and deposition of Aβ, due to APP and PS1/PS2 gene mutation, is believed to attribute importantly to the neuropathophysiology in familial AD, although the amyloid cascade theory of AD remains controversial. ApoE ε4 has been shown to increase brain Aβ load in familial AD model mice (apoE4 × APP mice) (Chan et al., 2016; Tachibana et al., 2019; Youmans et al., 2012); very few studies, however, demonstrate Aβ plaques in the brain of apoE4 TR only mice. In a previous study, Sullivan et al. showed Aβ deposition in cerebral vessels in apoE4 TR mice (Sullivan et al., 2008). In a recent study, Zhang et al. showed a positive staining for Aβ42 in the cortex of old (10 months) female, but not male, apoE4 TR mice (Zhang et al., 2019). No Aβ plaques were detected in these studies. In this study, we show a slight increase in the amount of Aβ40 in the hippocampus of male apoE4 mice with no Aβ plaque. Nonetheless, the results from this study suggest that memory impairment observed in O_3-exposed old male apoE3 mice is not caused by increased brain amyloidosis.

Obesity affects cognitive function through multiple mechanisms (Solas et al., 2017; Sripetchwandee et al., 2018; Toda et al., 2014). Consistent with previous reports (Huebbe et al., 2015), our data show that apoE3 male mice gain more body weight with increased age compared with apoE4 male mice. However, O_3 exposure has no significant effect on age-dependent body weight gain in either genotype. Therefore, it is suggested that O_3 exposure impairs memory in old apoE3 male mice not by increasing body weight. Our data also show that apoE4 mice have an increased anxiety level compared with apoE3 mice, which is consistent with the results reported by others (Meng et al., 2017; Villasana et al., 2016). O_3 exposure, however, has no significant effect on anxiety levels of either genotype of mice, suggesting that O_3 exposure impairs memory of old apoE3 mice not by increasing their anxiety.

In summary, the results from this study show, for the first time, that cyclic O_3 exposure impairs memory in old apoE3, not old apoE4 or young apoE3 and apoE4, male mice. In other words, *APOE ε4*, a well-established genetic risk for AD, protects old male mice from O_3-induced memory impairment. This is associated with upregulation of some antioxidant defense mechanisms in the hippocampus of apoE4 mice. The results from this study suggest complex interactions between *APOE* genotype, age, and environmental exposure in AD pathophysiology.

Disclosure

The authors declare they have no actual or potential competing financial interests.

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Appendix A. Supplementary data

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