

Article

Traffic-Related Emissions Induce Angiotensin II-Dependent Oxidative Stress in the Hippocampus of ApoE-Null Male Mice

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Abstract

Traffic-related air pollution (TRAP) is known to contribute to oxidative stress in the central nervous system (CNS) and has been linked to increased risk of Alzheimer's disease (AD). Alterations in the renin–angiotensin system (RAS), specifically increased angiotensin II (Ang II) signaling via the angiotensin II type 1 (AT₁) receptor, are implicated in increased oxidative stress in the CNS via activation of NADPH oxidase (NOX). As exposure to TRAP may further elevate AD risk, we investigated whether exposure to inhaled mixed gasoline and diesel vehicle emissions (MVE) promotes RAS-dependent expression of factors that contribute to AD pathophysiology in an apolipoprotein E-deficient (ApoE^{−/−}) mouse model. Male ApoE^{−/−} mice (6–8 weeks old) on a high-fat diet were treated with either an ACE inhibitor (captopril, 4 mg/kg/day) or water and exposed to filtered air (FA) or MVE (200 µg PM/m³) for 30 days. MVE exposure elevated plasma Ang II, inflammation, and oxidative stress in the hippocampus, associated with increased levels of Aph-1 homolog B (APH1B), a gamma-secretase subunit, and beta-secretase 1 (BACE1), involved in Aβ production. Each of these endpoints was normalized with ACEi treatment. These findings indicate that TRAP exposure in ApoE^{−/−} mice drives a RAS- and NOX-dependent oxidative and inflammatory response and shifts Aβ processing towards an amyloidogenic profile before overt Aβ deposition, suggesting a potential therapeutic approach for air pollution-induced AD risk.

Keywords: traffic-generated air pollution; central nervous system; oxidative stress; Alzheimer's disease; apolipoprotein E; angiotensin II



Academic Editor: Yasuhiro Yoshida

Received: 2 December 2025

Revised: 6 January 2026

Accepted: 19 January 2026

Published: 25 January 2026

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1. Introduction

Traffic-related air pollutants (TRAP), including those derived from motor vehicle emissions, consist of both gaseous components, such as carbon monoxide, sulfur dioxide, and nitrogen oxides, and particulate matter (PM). These environmental pollutants significantly contribute to central nervous system (CNS) oxidative stress, a key factor in the initiation and progression of neurodegenerative diseases [1–4]. There is also growing evidence that environmental factors such as air pollution may contribute to the pathogenesis of neurodegenerative diseases, including Alzheimer's disease (AD) [5–9]. Long before cognitive symptoms, the brain exhibits elevated nucleic acid oxidation, mitochondrial dysfunction, and redox-sensitive inflammatory signaling [10,11]. While the underlying mechanisms

and causative pollutants have not been fully elucidated, these alterations are thought to contribute to impaired neuronal resilience, shifts towards amyloidogenic processing, and ultimately neuronal loss [10,11]. Importantly, PM-mediated neurotoxicity has been shown to occur in the CNS, particularly the hippocampal CA1 region, a vulnerability that parallels AD, and damage can begin at a young age [12,13], decades before the onset of symptoms.

AD is currently the most prevalent form of dementia and the 7th leading cause of death worldwide as of 2024 [14,15]. AD is the most common cause of dementia, characterized by progressive impairments with memory, language, problem-solving, and critical thinking skills, as well as brain atrophy, synaptic disintegration, and neuronal loss [16,17]. A hallmark feature of AD-related dementia is the histopathological accumulation of insoluble amyloid- β (A β) and microtubule-associated protein tau neurofibrillary tangles in the hippocampus region of the brain [17]. A β is primarily generated through proteolytic cleavage of amyloid- β precursor protein (APP) by β -secretase1 (BACE1), followed by subsequent cleavage by γ -secretase. Processing of APP by BACE1 promotes the pathological amyloidogenic pathway, leading to insoluble A β accumulation in the brain parenchyma and cerebral vasculature decades before the onset of symptoms [18,19]. Currently, there is no cure for AD, and available treatments are limited to either palliative pharmacotherapies for symptomatic relief or recently approved anti-amyloid monoclonal antibodies with uncertain efficacy [20–22]. Given the profound disability associated with AD and a lack of effective treatment options, it is essential to identify the health and environmental risk factors for AD to inform the development of therapeutic options.

Currently, the most prominent risk factor for AD is aging; however, environmental exposure to TRAP and genetic predisposition are also correlated with AD [23,24]. The apolipoprotein (ApoE) allele exists in humans in several isoforms: ApoE2, ApoE3, and ApoE4, with ApoE3 being the most common isoform [25]. Of these, ApoE4 is considered the most significant genetic risk factor for AD, and those carrying more than one ApoE4 allele are at a much higher risk. ApoE is primarily expressed in astrocytes in the brain, where it functions in lipid metabolism and the efflux of substances from the brain to the blood, including A β peptides [25]. As such, carriers of the ApoE4 allele frequently exhibit altered lipid homeostasis and decreased ability to clear A β from the brain compared to carriers of ApoE2 and ApoE3 [25]. Carriers of the ApoE4 allele also have increased neuroinflammation and oxidative stress, which is further exacerbated with exposure to environmental pollutants such as TRAP [23,25]. For example, TRAP PM exposure is associated with increased A β accumulation and phosphorylated tau in humans, including children, with carriers of the ApoE4 gene being at an increased risk of developing AD pathologies with exposure [23].

While the mechanisms of PM air pollutant toxicity have been investigated in AD mouse models [26–28], the role of the ApoE gene in these pathological outcomes has not been fully characterized. ApoE-null mice (ApoE^{−/−}) are traditionally used as a model for hyperlipidemia and atherosclerosis, but they are also valuable for studying neurodegenerative diseases [29], given the established links between hyperlipidemia, diet-induced obesity, and neurodegenerative diseases [30]. Moreover, ApoE^{−/−} mice have been reported to exhibit disruption of blood–brain barrier (BBB) integrity by 8 weeks of age, spatial memory deficits as early as 3 months of age, and impacts on short-term memory at 6–8 months of age, as determined by the Morris water maze [30]. While these mice do not readily accumulate A β , when injected with synthetic A β , ApoE^{−/−} mice have been shown to have reduced A β clearance compared to wild-type mice [29]. This prior work highlights altered A β homeostasis in ApoE^{−/−} mice, although the mechanisms governing A β efflux and transport were not assessed in the present study. Our laboratory previously reported that ApoE^{−/−} mice on a high-fat diet exposed to mixed vehicle emissions (MVE) display

increased BBB disruption, oxidative stress, and markers of neuroinflammation compared to those exposed to filtered air [31,32]. As such, we utilized the same animal model, diet, and exposure conditions in the current study to investigate the outcomes of MVE exposure on mechanistic pathways that may contribute to AD pathogenesis.

The renin–angiotensin system (RAS) is a well-characterized hormonal signaling system that regulates vascular tone and fluid balance, and accumulating evidence suggests that RAS also influences cognitive health [33,34]. Angiotensin II (Ang II), the primary active peptide of the RAS, is generated through the conversion of angiotensinogen (Agt) to Ang I by renin, and subsequent processing via angiotensin-converting enzyme (ACE). In addition to systemic RAS signaling, tissue-specific RAS signaling has been identified in the CNS. In the brain, RAS signaling is primarily mediated by astrocyte-derived Agt, which is processed to Ang II by CNS-derived renin and ACE [34]. Ang II exerts its effects in the brain through interaction with its primary receptors, Ang II receptor types 1 (AT₁) and 2 (AT₂), which are differentially expressed in the CNS during development [35]. While Ang II-AT₂ interactions are considered neuroprotective, AT₂ receptors are significantly downregulated after development and further reduced with age [36]. Conversely, excessive Ang II-AT₁ signaling in the CNS is associated with increased oxidative stress [37–39], cognitive impairment [40], and A β production and clearance [41,42]. In agreement with this, the use of pharmaceutical AT₁ receptor inhibitors is reported to reduce deleterious CNS effects in humans and animal models [42–45]. Previous studies from our laboratory show that RAS signaling is increased in wild-type mice exposed to MVE [46,47]; however, data are currently lacking on the effects of MVE on CNS oxidative stress, amyloid processing, and RAS signaling in ApoE^{−/−} mice. Therefore, this study tested the hypothesis that exposure to traffic-generated air pollution (MVE) in ApoE^{−/−} mice leads to increased oxidative stress and amyloid processing, both of which are associated with RAS signaling.

2. Materials and Methods

2.1. Animals, Treatment, and Inhalation Exposure Protocol

All procedures were approved by the Lovelace Biomedical Research Institute's Animal Care and Use Committee (IACUC) Protocol #08-072, originally approved 29 April 2009, and conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Six–eight-week-old male apolipoprotein null mice (ApoE^{−/−}) were fed a high-fat “Western” diet (HF, 21.2% fat content by weight, 1.5 g/kg cholesterol content, TD88137 custom research diet, Harlan Teklad, Indianapolis, IN, USA), beginning 30 days before treatment/exposure. Mice were randomly assigned to receive either angiotensin-converting enzyme inhibitor (ACEi), captopril (4 mg/kg/day), or no treatment (control) in their drinking water. Mice were individually caged, and daily water intake was measured using graduated low-drip water bottles. Captopril concentrations were adjusted based on daily water intake to achieve a targeted 4 mg/kg/day dose throughout the study. GC/MS was used to verify the duration of the captopril stability in water at room temperature before the initiation of the study. Mice from each treatment group were then randomly assigned for inhalation exposure to either filtered air (FA: $n = 10$ FA+ACEi; $n = 10$ FA+control) or a 200 $\mu\text{g PM}/\text{m}^3$ mixture of gasoline and diesel engine emissions (MVE: $\sim 50 \mu\text{g PM}/\text{m}^3$ gasoline engine + 150 $\mu\text{g PM}/\text{m}^3$ diesel engine emissions; $n = 10$ MVE+ACEi; $n = 10$ MVE+control) for 6 h/day, 7 days/week, for 30 days, as previously described [32]. The MVE was generated using a 1996 model 4.3 L General Motors V-6 engine (USA) equipped with a stock exhaust system, fueled with conventional unleaded, non-oxygenated, non-reformulated gasoline blended with a diesel generator, fueled with a diesel blend (300 ppm sulfur content), operated on a steady state load condition. The emissions from both systems were combined into a

2 m³ mixing chamber and characterized for the chemical composition, as previously reported [31,32,48,49]. Mice were housed within an AAALAC-approved rodent housing facility throughout the study, which maintained 40–60% relative humidity and a constant temperature (20–24 °C), and monitored daily for health status. All animals had access to chow and water ad libitum throughout the study period, except during the daily exposures when chow was removed to minimize the ingestion of PM.

2.2. Tissue Collection

All study animals were sacrificed 14–16 h after the final assigned exposure; anesthetized with Euthasol (0.1 mL per 30 g mouse), followed by euthanization by exsanguination. The brains were then carefully dissected, meninges gently removed, weighed, snap-frozen in liquid nitrogen, and stored at −80 °C. Whole blood was collected in a heparinized syringe and centrifuged for 10 min at 3800× g at 4 °C to separate plasma, which was aliquoted and stored at −80 °C until assayed.

2.3. Immunofluorescent Staining of Brain Tissue

Brains were thawed on ice and fixed in Histochoice (VWR, Irving, TX, USA) at 4 °C overnight, embedded in Tissue Freezing Medium (TBS, IMEB Inc., San Marcos, CA, USA), and frozen at −80 °C until sectioning. Frozen brain sections were cut (coronal plane) with a 10 µm thickness between Bregma 0 and Bregma 2.12 mm, and were prepared for either single or double immunofluorescence, as previously described by our laboratory [50], using the following primary antibodies: rabbit anti-AGTR-1 pAb (1:1000, Novus, Centennial, CO, USA, NBP1-77078AF555), mouse anti-beta amyloid mAb (1:500, Novus, NBP2-13075AF488), mouse anti-BACE1 mAb (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-33711), mouse anti-8-OHdG mAb (1:1000, Santa Cruz Biotechnology, Dallas, TX, USA, sc-66036), sheep anti-Von Willebrand Factor pAb (1:1000, Abcam, Waltham, MA, USA, Ab11713), and rabbit anti-IL-1β pAb (1:1000, Abcam, ab9722). Primary antibodies were conjugated with appropriate secondary antibodies at a 1:2 (primary: secondary) concentration, using Alexa Fluor 555 donkey anti-rabbit (Invitrogen, Carlsbad, CA, USA, A31572), Alexa Fluor 555 goat anti-mouse (Invitrogen, A21422), or Alexa Fluor 488 donkey anti-sheep (Invitrogen, A11015). The negative controls were processed using only secondary antibodies to ensure there was no non-specific binding (Supplemental Figure S1). For each brain sample, two slides with two brain sections per slide were used for analysis [31], with an $n = 3$ brains per group analyzed by a blinded participant. Slides were imaged under fluorescent microscopy at 40× with the appropriate excitation/emission filter, digitally recorded, and analyzed with image densitometry with ImageJ software, version 1.51n (NIH, Bethesda, MD, USA). The fluorescence in the CA1 area of the hippocampus was analyzed using beacons to quantify the specific region for consistency across sections and slides.

2.4. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

Gene expression of cerebral APP, BACE1, Aph1B, p47^{phox}, gp91^{phox}, ACE1, and AT₁ receptor was analyzed using the appropriate forward and reverse primers (Table 1), as previously described [46]. Briefly, RNA was isolated from midbrain cerebral tissue (~20 mg of tissue) using a Tissue Lyser system and AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Germantown, MD, USA), following the manufacturer's protocol. RNA quality and concentration were measured on a Cytation 3, using a Take3 plate (BioTek, Winooski, VT, USA). Real-time RT-qPCR was detected on a BIORAD CFX96 Touch Real-Time PCR system (Hercules, CA, USA) and analyzed using $\Delta\Delta C_T$, as previously described by our laboratory [31]. Samples were removed from analyses if the standard error of the mean (SEM) normalized gene expression for the triplicate C_T value exceeded the 20% threshold.

Table 1. Mouse primer sequences used for real-time RT-qPCR analyses.

Gene	Primer Sequences Accession #
<i>APP</i>	FP: 5'-GACCACTCGACCAGGTTCTG-3' NM_001198823.1 RP: 5'-GATGATGGCGCCTTTGTTCG-3'
<i>ACE</i>	FP: 5'-ACATACCCTAGCTGAGCC-3' NM_207624.6 RP: 5'-GTCGGTGTTAGACACTGG-3'
<i>APH-1B</i>	FP: 5'-AAATGCCCAGATGCCCATGA-3' NM_177583.4 RP: 5'-GTGGTGTCCACCCAGACTTTT-3'
<i>AT₁</i>	FP: 5'-GTTGGGAGGGACTGGATGATG-3' NM_177322.3 RP: 5'-CACTTGATCTGGTGATGGGCTTT-3'
<i>BACE1</i>	FP: 5'-CTGCACTGGCTCCTGCTATG-3' NM_011792.7 RP: 5'-AGGATGTTGAGCGTCTGTGG-3'
<i>GAPDH</i>	FP: 5'-CATGGCCTTCCGTGTTCCCTA-3' NM_001289726.2 RP: 5'-GCGGCACGTCAGATCCA-3'
<i>gp91^{phox}</i>	FP: 5'-CACCCATTACACTGACCTCTG-3' NM_007807.5 RP: 5'-CTTATCACAGCCACAAGCATTGAA-3'
<i>p47^{phox}</i>	FP: 5'-CTGCTGTTGAAGAGGACGAGATG-3' NM_198326.3 RP: 5'-AGCCGGTGATATCCCCTTTCC-3'

APP, amyloid- β precursor protein; *ACE*, angiotensin converting enzyme; *APH-1B*, Aph-1 homolog B (gamma-secretase subunit); *AT₁*, angiotensin II receptor type 1; *BACE1*, beta-secretase 1; FP, forward primer; *GAPDH*, Glyceraldehyde 3-phosphate dehydrogenase; *gp91^{phox}*, glycosylated 91 Kda protein (membrane subunit); *p47^{phox}*, p47 phagocyte oxidase (cytosolic subunit); RP, reverse primer.

2.5. Angiotensin II ELISA

Angiotensin II was measured in plasma using an ELISA kit (CUSABIO Life Sciences, Houston, TX, USA, murine Ang II kit, ABIN366511) following the manufacturer's protocol. Sample values were derived from a standard curve, and the results were expressed as pg/mL/mg tissue.

2.6. Statistical Analysis

Data are shown as mean $\pm$ SEM. A 2-way ANOVA with post hoc Holm–Sidak test was used to assess statistical significance between treatment, exposure, and treatment $\times$ exposure interactions for each endpoint. Statistical analyses were conducted using GraphPad Prism v10.0. A *p*-value of <0.050 was considered statistically significant for all endpoints.

3. Results

3.1. MVE Exposure Promotes Oxidative Stress in the Hippocampus CA1 Area of *ApoE*^{−/−} Male Mice, Which Is Reduced with ACEi Treatment

As oxidative stress is known to contribute to both neuroinflammation and neurodegeneration, we quantified 8-hydroxy-2'-deoxyguanosine (8-OHdG) expression in our study animals. Compared to FA control (Figure 1A) or FA + ACEi-treated (Figure 1B) animals, we observed a significant increase in 8-OHdG expression in the CA1 region of mice exposed to MVE (Figure 1C), which was significantly reduced with ACEi treatment (Figure 1D), as quantified in Figure 1E ($p = 0.0305$). The two-way ANOVA showed that both ACEi treatment ($F = 17.32$, $p = 0.0032$) and MVE exposure ($F = 12.97$, $p = 0.0070$) mediated the increase in oxidative stress observed in the CA1 region of the hippocampus. There was no significant interaction between treatment $\times$ exposure ($F = 1.106$, $p = 0.3237$).

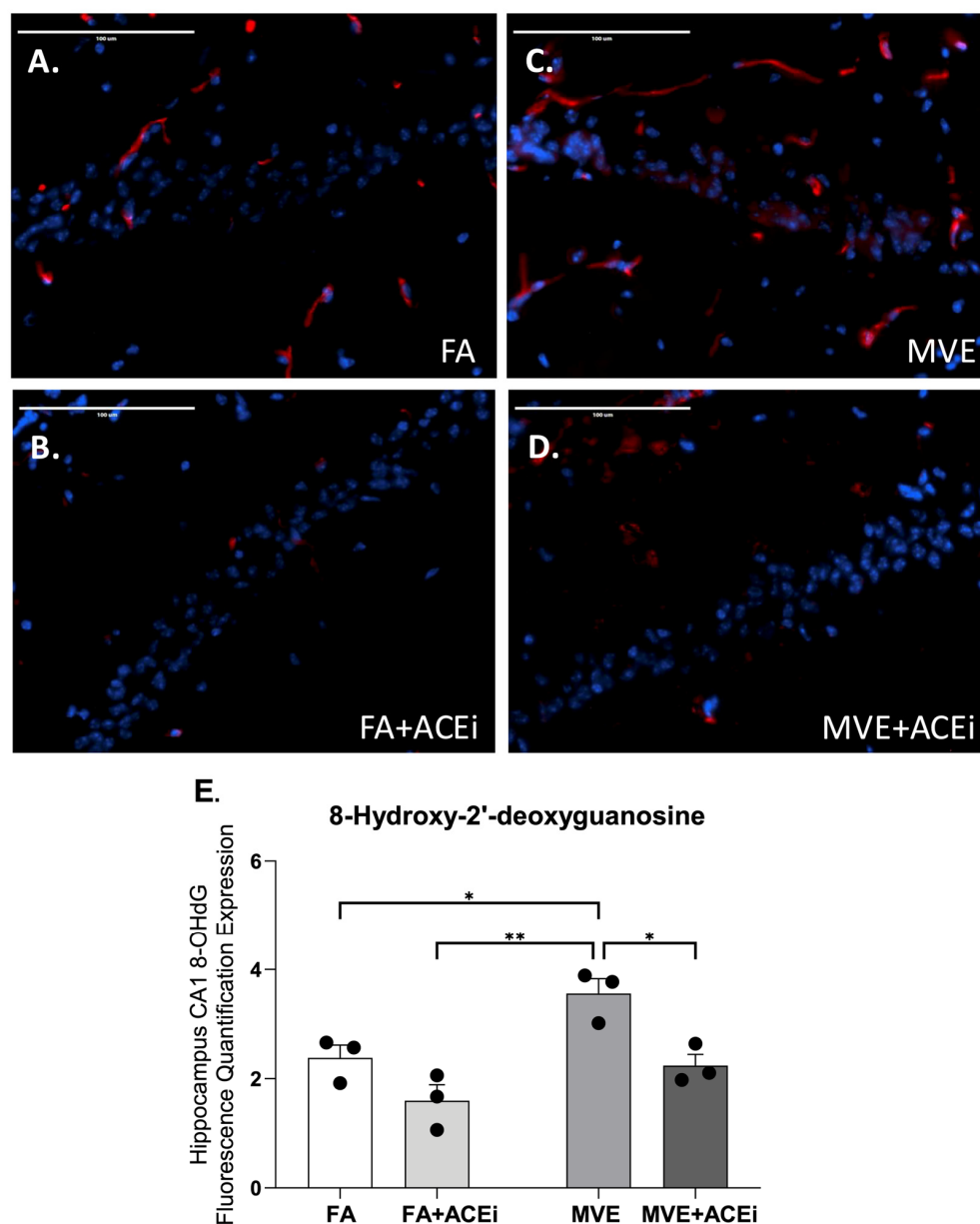


Figure 1. Representative images of 8-hydroxy-2'-deoxyguanosine (8-OHdG, red) in the hippocampus CA1 region in either (A) apolipoprotein E-null ($ApoE^{-/-}$) mice exposed to filtered air (FA); (B) $ApoE^{-/-}$ mice exposed to FA+captopril (FA+ACEi); (C) $ApoE^{-/-}$ mice exposed to mixed vehicle exhaust (MVE: 200 $\mu\text{g PM}/\text{m}^3$ mixed gasoline and diesel exhaust, 6 h/day, 7 days/week, 30 days); or (D) $ApoE^{-/-}$ mice exposed to MVE+ACEi. (E) Fluorescence was quantified and represented as mean fluorescence for the CA1 region in each animal. $n = 3$ animals per group, four sections per animal. Blue fluorescence = nuclear Hoechst stain. Scale bar = 100 μm , 40 $\times$ magnification (CA1). Statistical analyses were conducted using a two-way pairwise ANOVA. * = $p \leq 0.05$, ** = $p \leq 0.01$.

3.2. $ApoE^{-/-}$ Mice Exhibit Altered NADPH Oxidase-2 in the Hippocampus CA1 Region and Upregulation of Cerebral Catalytic Subunit Transcript Levels in Response to MVE Exposure

NADPH oxidase-2 (NOX2) is a prominent source of reactive oxygen species (ROS). NADPH subunits, cytosolic protein $p47^{\text{phox}}$ and transmembrane protein $gp91^{\text{phox}}$, act as markers for its activation. We quantified NOX2 in the hippocampus CA1 region via immunofluorescence. The two-way ANOVA revealed a significant main effect of ACEi treatment ($F = 42.09$, $p = 0.0002$), as well as a significant interaction between treatment and exposure ($F = 0.1893$, $p = 0.0134$). In contrast, the effect of exposure alone did not significantly affect NOX2 expression ($p = 0.6750$). Post hoc comparisons showed that relative

to both FA (Figure 2A) and MVE (Figure 2B), there was a decrease in NOX2 expression in the MVE+ACEi brains (Figure 2D); however, there was no statistical difference in NOX2 expression observed in the FA+ACEi brains (Figure 2B), as summarized in Figure 2E. At the transcript level, MVE exposure promoted an increase in cerebral gp91^{phox} mRNA expression compared to FA animals, which was attenuated with ACEi treatment (Figure 2F). The two-way ANOVA revealed significant differences for treatment ($F = 9.978$, $p = 0.006$), exposure ($F = 7.848$, $p = 0.013$), and interactions between treatment $\times$ exposure ($F = 16.921$, $p < 0.001$). We observed no statistical differences in cerebral p47^{phox} mRNA expression across our study groups (Supplemental Figure S2).

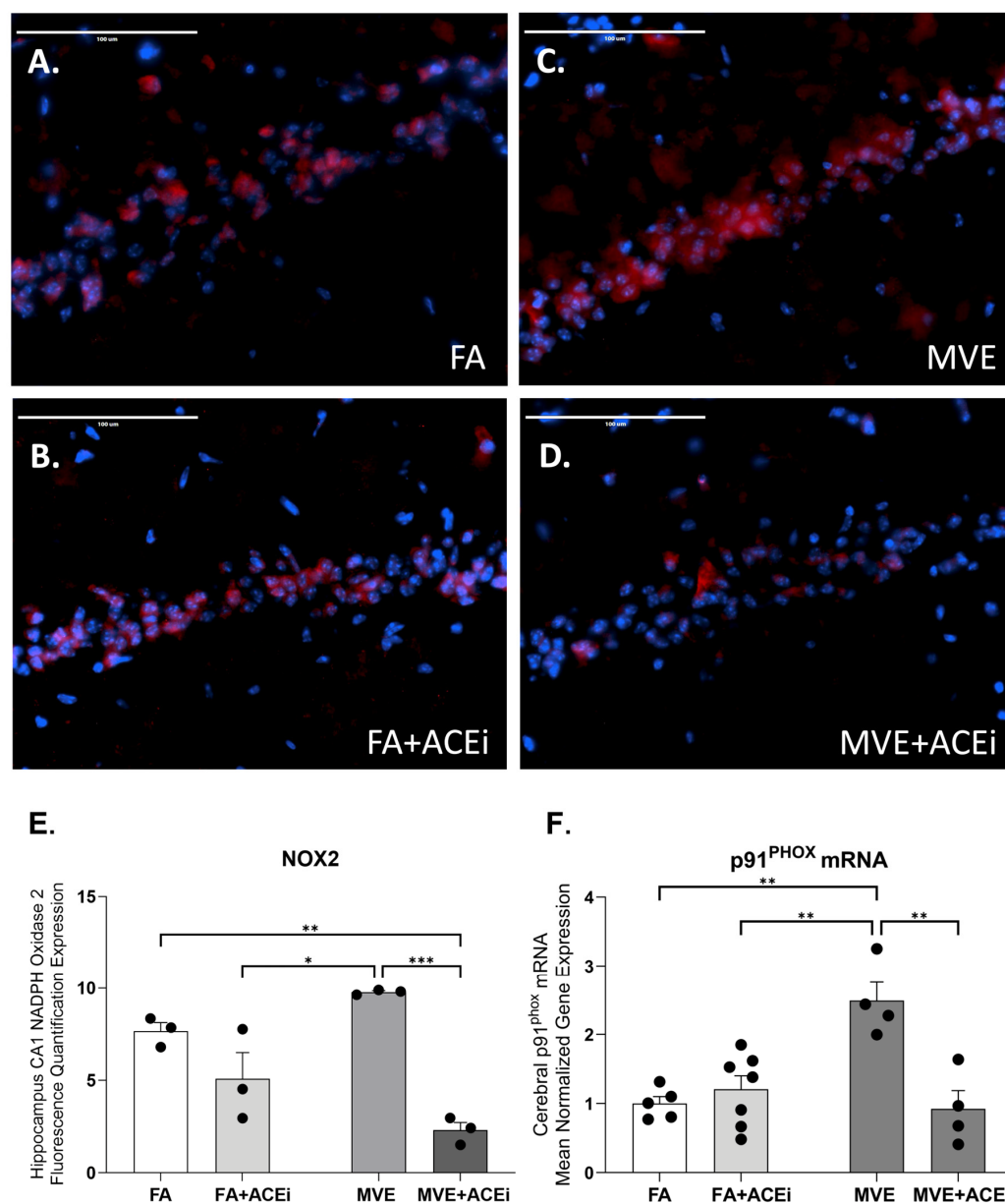


Figure 2. Representative images of NADPH oxidase (NOX2, red) in the CA1 region of the hippocampus in either (A) ApoE^{-/-} mice exposed to filtered air (FA); (B) ApoE^{-/-} mice exposed to FA + captopril (FA+ACEi); (C) ApoE^{-/-} mice exposed to mixed vehicle exhaust (MVE: 200 μg PM/m³ mixed gasoline and diesel exhaust, 6 h/day, 7 days/week, 30 days); or (D) ApoE^{-/-} mice exposed to MVE+ACEi. (E) Fluorescence was quantified and represented as mean fluorescence for the CA1 region in each animal. $n = 3$ animals per group, four sections per animal. Blue fluorescence = nuclear Hoechststain. Scale bar = 100 μm, 40 $\times$ magnification (CA1). (F) Real-time RT-qPCR analysis of cerebral mRNA expression of NADPH oxidase subunit gp91^{phox}, $n = 4$ –7 animals per group. Statistical analyses were

conducted using a two-way pairwise ANOVA. Data is expressed as mean normalized gene expression using $\Delta\Delta CT$ values, with GAPDH used as the housekeeping gene. * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

3.3. MVE Exposure Promotes Inflammation in the Cerebral Parenchyma of $ApoE^{-/-}$ Mice, Which Is Mitigated with ACEi

Increased Ang II is associated with inflammation at and around the cerebral microvasculature, via AT_1 receptor stimulation and activation of microglia [51]. Thus, we analyzed the cerebral expression of a key inflammatory mediator, IL-1 β , and characterized its expression in proximity to the cerebral microvasculature. Compared to FA controls (Figure 3A), MVE (Figure 3C) exposure resulted in a significant increase in inflammatory IL-1 β cerebral expression ($p = 0.0483$). ACEi treatment mitigated IL-1 β expression in the cerebrum of both FA (Figure 3B) and MVE animals (Figure 3D), as quantified in Figure 3E ($p < 0.05$). The two-way ANOVA showed that both treatment ($F = 16.97$, $p = 0.0033$) and exposure ($F = 11.08$, $p = 0.0104$) mediated IL-1 β expression. Conversely, when quantifying IL-1 β mRNA expression in cerebral homogenate, we did not observe any statistical differences among our study groups (Figure 3F).

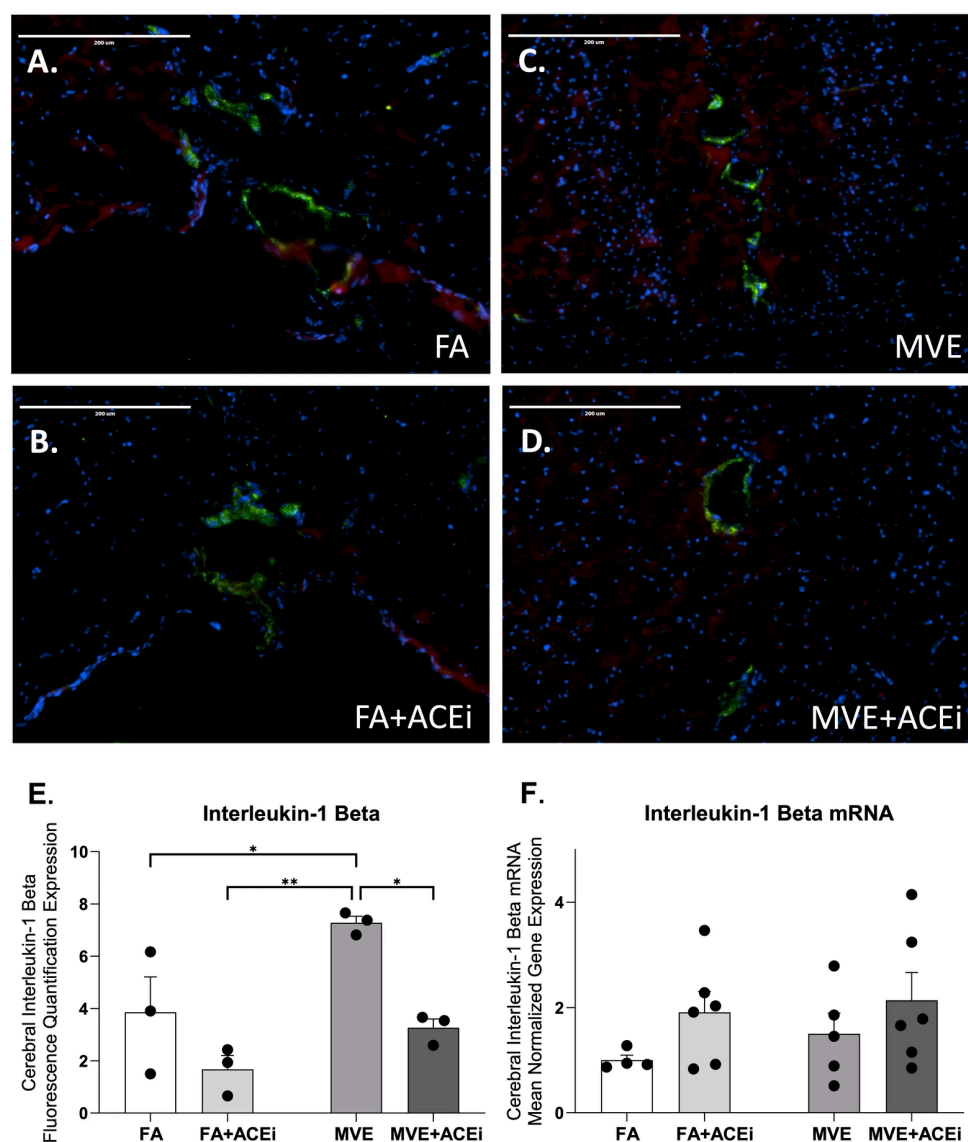


Figure 3. Representative images of interleukin-1 beta (IL-1 β , red) and Von Willebrand Factor (vWF, green) in the parenchyma surrounding the cerebral vasculature in either (A) $ApoE^{-/-}$ mice exposed

to filtered air (FA); (B) ApoE^{-/-} mice exposed to FA + captopril (FA+ACEi); (C) ApoE^{-/-} mice exposed to mixed vehicle exhaust (MVE: 200 µg PM/m³ mixed gasoline and diesel exhaust, 6 h/day, 7 days/week, 30 days); or (D) ApoE^{-/-} mice exposed to MVE + ACEi. (E) Fluorescence was quantified and represented as mean fluorescence for standardized areas in surrounding cerebral vessels (vWF positive) in each animal. *n* = 3 animals per group, two slides per animal, two sections per slide, 8–10 vessels on each section. Blue fluorescence = nuclear Hoechst stain; 20× magnification (scale bar = 200 µm). (F) Mean normalized gene expression of cerebral IL-1β was quantified using RT-qPCR, *n* = 4–6 animals per group. Statistical analyses were conducted using a two-way pairwise ANOVA. * = *p* ≤ 0.05, ** = *p* ≤ 0.01.

3.4. ApoE^{-/-} Mice Exposed to MVE Exhibit Elevated Levels of Circulating Plasma Ang II, Which Is Attenuated with ACEi

To determine if MVE exposure alters systemic RAS signaling, we quantified plasma Ang II levels. ApoE^{-/-} mice treated with ACEi had a significant decrease in circulating plasma Ang II versus treatment controls (Figure 4). In agreement with our previous findings [43], MVE exposure increased plasma Ang II in ApoE^{-/-} mice, compared to FA; however, ACEi-treatment decreased plasma Ang II levels (Figure 4). The two-way ANOVA showed treatment (*F* = 39.760, *p* < 0.001), exposure (*F* = 56.921, *p* < 0.001), and the interaction between treatment × exposure (*F* = 11.134, *p* = 0.003) significantly altered plasma Ang II in our study.

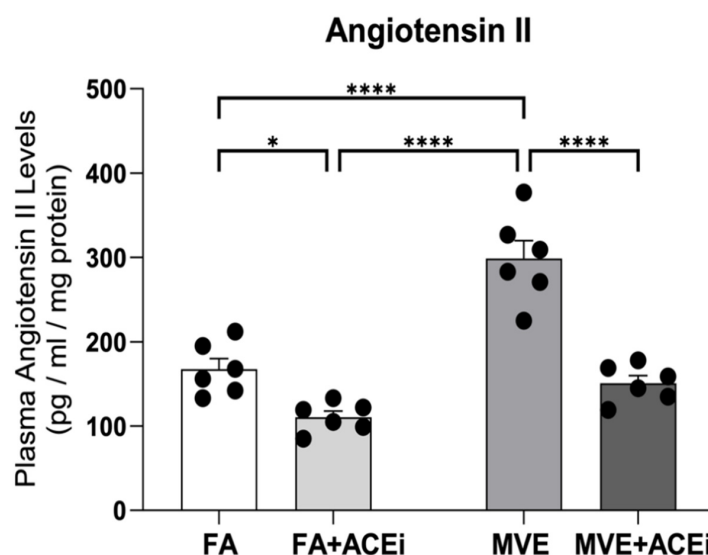


Figure 4. Plasma angiotensin (Ang) II levels, as determined by ELISA, in ApoE^{-/-} mice exposed to either filtered air (FA) or mixed gasoline and diesel vehicle emissions (MVE: 200 µg/m³ PM for 6 h/day, 5 days/week, 30 days) with or without captopril treatment (ACEi; 4 mg/kg/day) in the drinking water. *n* = 6 per group. Statistical analyses were conducted using a two-way pairwise ANOVA. * = *p* ≤ 0.05, **** = *p* ≤ 0.0001.

3.5. ACEi Treatment Reduces AT1 Expression in MVE-Exposed ApoE^{-/-} Mice

To investigate whether Ang II receptor expression is altered in the hippocampus with MVE exposures, we quantified AT₁ expression in ApoE^{-/-} mice using immunofluorescence (Figure 5A–D). A two-way ANOVA revealed a significant decrease with ACEi treatment in the CA1 region of MVE-exposed mice (Figure 5E, *F* = 11.687, *p* = 0.009); however, there were no significant effects for MVE exposure (*F* = 0.76, *p* = 0.409) or ACEi treatment (*F* = 4.144, *p* = 0.076) in the FA group (Figure 5E). At the transcript level, we did not observe any statistical changes in cerebral AT₁ mRNA expression in response to either MVE or ACEi treatment (Figure 5F). Additionally, we examined the expression of ACE in

the hippocampus CA1 (Supplemental Figure S3A–D) and at the cerebral transcript level (Supplemental Figure S3F) but did not observe any statistical differences in ACE expression at either the protein or transcript levels, respectively (Supplemental Figure S3E,F).

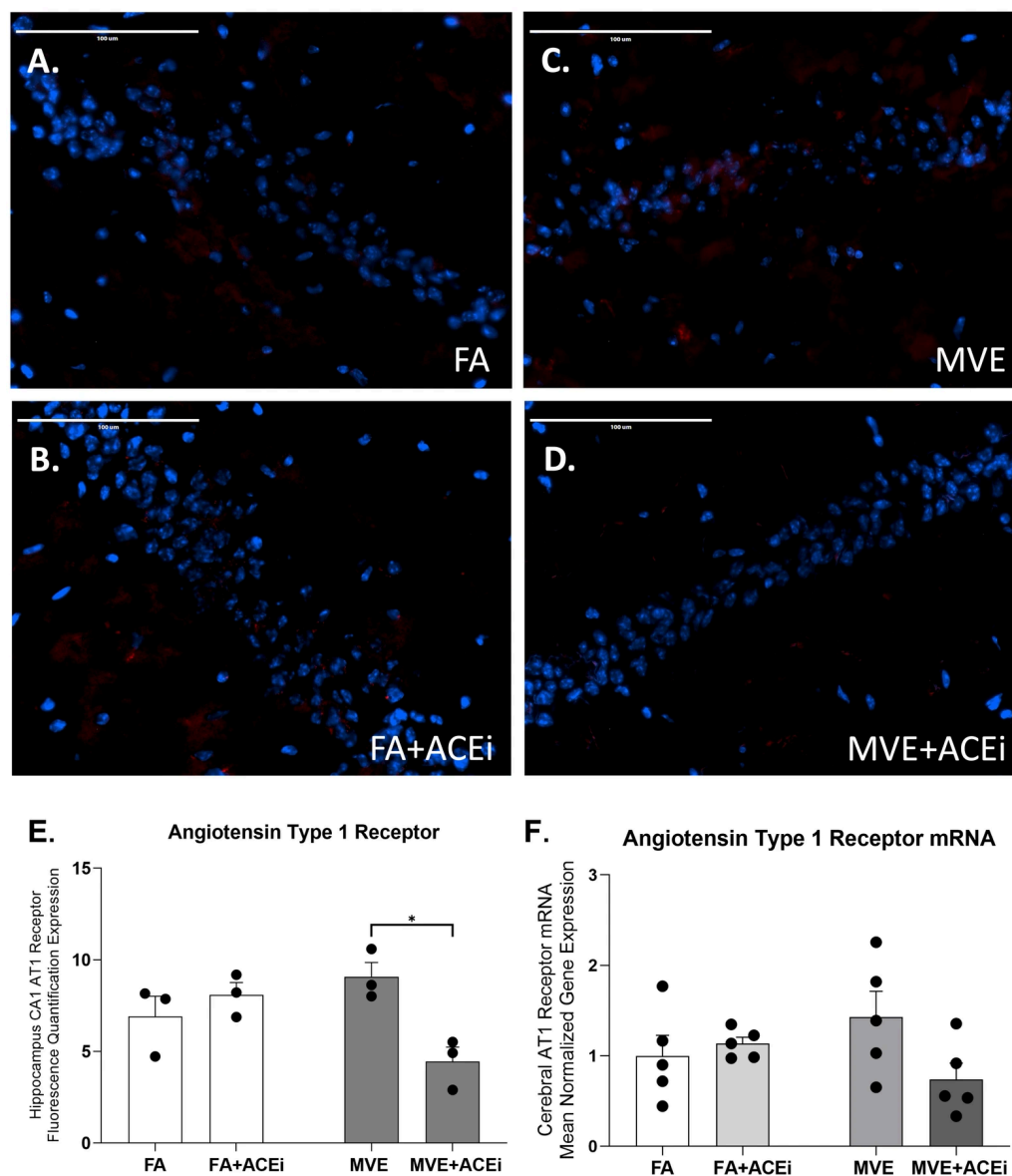


Figure 5. Representative images of angiotensin type 1 receptor (AT1, red) in the CA1 region of the hippocampus in (A) ApoE^{−/−} mice exposed to water + filtered air (FA); (B) ApoE^{−/−} mice exposed to captopril + FA (FA+ACEi); (C) ApoE^{−/−} mice exposed to water + mixed vehicle exhaust (MVE: 200 µg PM/m³ mixed gasoline and diesel exhaust, 6 h/day, 7 days/week, 30 days); or (D) ApoE^{−/−} mice exposed to MVE + captopril treatment (MVE+ACEi). (E) Fluorescence was quantified and represented as mean fluorescence for the CA1 region in each animal. *n* = 3 animals per group, four sections per animal. Blue fluorescence = nuclear Hoechst stain. Scale bar = 100 µm, 40x magnification (CA1 region). (F) Mean normalized gene expression of the AT₁ receptor was quantified in the cerebrum using RT-qPCR, *n* = 5 animals per group. Statistical analyses were conducted using a two-way pairwise ANOVA. * = *p* ≤ 0.05.

3.6. ApoE^{−/−} Mice Exhibit Altered Expression of Aβ Signaling Pathway in Response to MVE and ACEi Treatment

Cleavage of amyloid beta precursor proteins by secretase enzymes is necessary for Aβ production and deposition in the brain. To determine whether MVE exposure or treatment with an ACEi affects amyloid processing in the brains of ApoE^{−/−} mice, we analyzed

the expression of key A β signaling pathway members using real-time qPCR. We did not observe any statistical differences in APP mRNA (Figure 6A) in animals exposed to MVE or treated with ACEi. BACE1 mRNA was increased in MVE+ACEi animals ($p = 0.001$) (Figure 6B). A two-way ANOVA analysis revealed that exposure was significantly associated with a difference in cerebral BACE1 mRNA expression ($F = 14.355$, $p = 0.001$); however, neither ACEi treatment ($F = 4.183$, $p = 0.053$) nor interactions between treatment and exposure ($F = 1.601$, $p = 0.219$) were associated with BACE1 expression. Gamma-secretase is responsible for the final proteolytic cleavage and release of A β into the extracellular space. We found that exposure to MVE increased the mRNA expression of APH1B, a gamma secretase subunit, regardless of ACEi treatment (Figure 6C). The two-way ANOVA analysis showed that MVE exposure was strongly associated with increased APH1B ($F = 19.369$, $p < 0.001$), while ACEi treatment ($F = 0.0863$, $p = 0.362$) and interactions between ACEi treatment $\times$ exposure ($F = 0.758$, $p = 0.392$) were not.

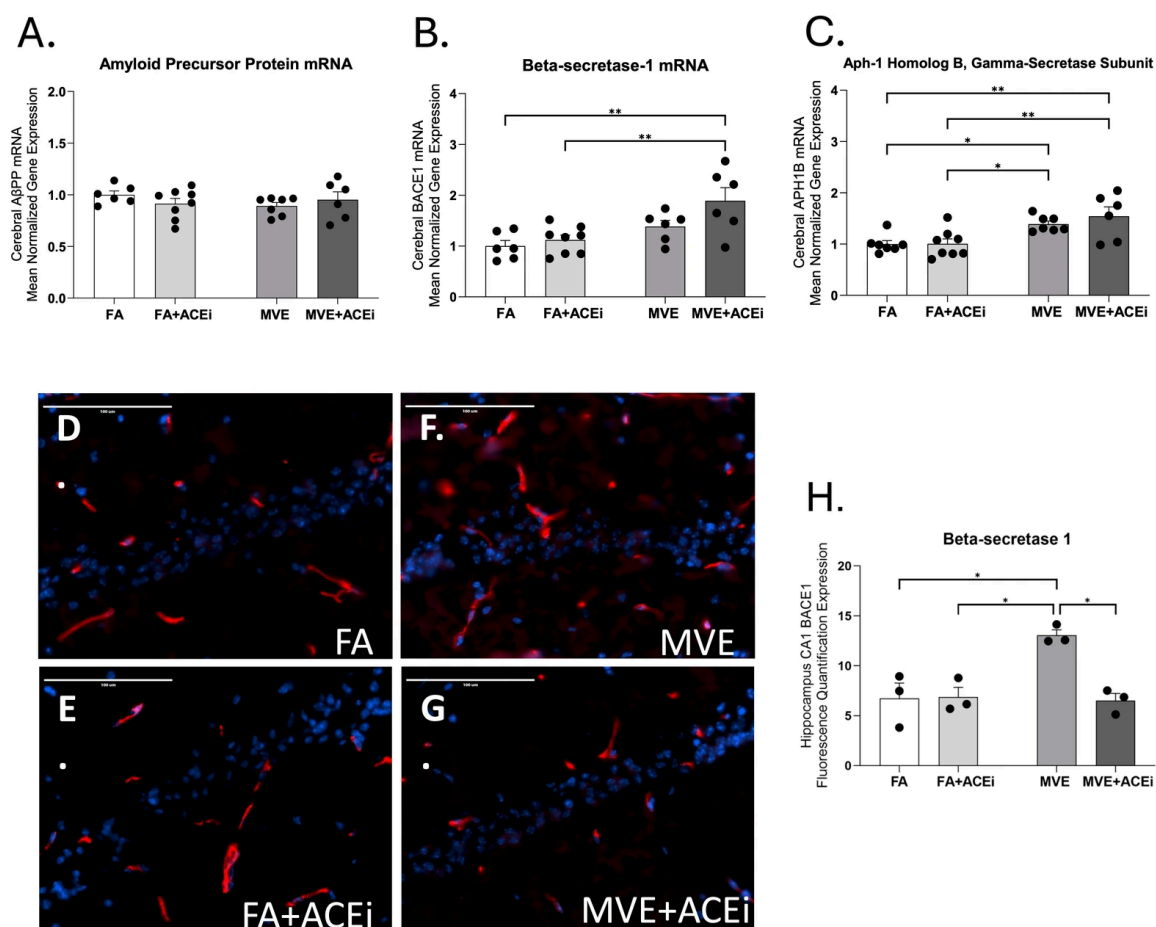


Figure 6. Real time RT-qPCR analysis of cerebral mRNA expression of (A) amyloid beta precursor protein (APP), (B) beta-secretase 1 (BACE1), (C) Aph-1 homolog B gamma-secretase subunit (APH1B) mRNA expression in cerebral tissue from ApoE^{-/-} mice exposed to either filtered air (FA), FA with concurrent treatment of captopril (FA+ACEi) or mixed vehicle exhaust (MVE) (200 μ g PM/ m^3 mixed gasoline and diesel exhaust, 6 h/day, 7 days/week, 30 days), or MVE with concurrent treatment of captopril (MVE+ACEi). Data is expressed as mean normalized gene expression using $\Delta\Delta$ CT values, with GAPDH used as the housekeeping gene, $n = 6-8$ animals per group. (D–G) Representative images of β -secretase (BACE1, red) in the hippocampus CA1 region of ApoE^{-/-} mice exposed to (D) FA, (E) FA+ACEi, (F) MVE, or (G) MVE + ACEi. (H) Fluorescence was quantified and represented as mean fluorescence for the CA1 region in each animal. $n = 3$ animals per group, four sections per animal. Blue fluorescence = nuclear Hoechst stain. Scale bar = 100 μ m, 40 $\times$ magnification (CA1). Statistical analyses were conducted using a two-way pairwise ANOVA * = $p \leq 0.05$, ** = $p \leq 0.01$.

BACE1 is one of the key enzymes involved in the proteolytic cleavage of APP to A β and is thought to be the pathway by which pathogenic A β_{42} , a hallmark of AD, is deposited in the brain. As such, we examined BACE1 expression in the hippocampus CA1 region. Compared to FA controls (Figure 6D), MVE-exposure led to significantly higher levels of BACE1 (Figure 6F; $p = 0.0108$) in the hippocampal CA1 region of ApoE $^{-/-}$ mice. While treatment with ACEi did not alter BACE expression in FA-exposed animals (Figure 6E), it did lead to a significant reduction in BACE1 expression in the hippocampus of MVE-exposed mice (Figure 6G, $p = 0.0104$). The two-way ANOVA results revealed significant effects for treatment ($F = 0.1018$, $p = 0.0128$) and exposure ($F = 8.717$, $p = 0.0184$), and an interaction between ACEi treatment and exposure ($F = 11.05$, $p = 0.0105$), as shown in Figure 6H. We also quantified A β expression in the CA1 regions of the hippocampus (Supplemental Figure S4), which was not significantly affected by MVE exposure (Figure S4C), compared to FA controls (Figure S4A; $F = 0.0076$, $p = 0.933$), ACEi treatment (Figure S4B,D; $F = 1.829$, $p = 0.213$) or interactions between the two ($F = 0.0103$, $p = 0.922$), as represented in Figure S4E.

4. Discussion

Increased RAS signaling in the CNS is associated with oxidative stress and neuroinflammation through NADPH oxidase (NOX) and IL-1 β expression, as well as other mechanisms [39,52,53]. Oxidative stress from ROS generation is thought to contribute to AD pathogenesis, prior to the accumulation of A β and the manifestation of clinical symptoms [10]. Additionally, exposure to TRAP has been shown to increase oxidative stress in the BBB microvasculature, the cerebral cortex, and hippocampus of animals exposed to environmental air pollutants [28,54–56]. These findings are consistent with post-mortem autopsy reports examining inflammation, amyloid plaque development, and oxidative stress in humans as young as adolescents in highly polluted Mexico City [57–59]. Our current study examined oxidative stress by measuring 8-OHdG in the hippocampus of six- to eight-week-old male ApoE $^{-/-}$ mice. 8-OHdG is formed from the oxidation of nucleic acids and is reported to be significantly increased in AD patients [60]. Other studies have found that lipid peroxidation induced by oxidative stress leads to increased A β levels through upregulation of BACE1 [61]. Previously, we have reported that MVE significantly increased 8-OHdG in the hippocampus of aged (18-month-old) male C57BL/6 (wild-type) mice fed a standard (low-fat) mouse diet [46]. In contrast, in our prior studies utilizing male 6–8-week-old C57BL/6 wild-type mice fed a standard mouse chow, MVE exposure did not produce a significant increase in oxidative stress in the hippocampal CA1 region [46]. Together, these findings suggest that MVE-induced oxidative stress in the hippocampus is likely modulated by multiple factors, such as age, comorbidities, diet, and genetics, and may require an underlying priming condition to manifest observable oxidative stress markers in the CNS.

In the current study, we observed that exposure to MVE significantly increased 8-OHdG expression, which was attenuated by ACEi treatment, indicating a protective effect through reduced Ang II signaling in the brain. Interestingly, 8-OHdG was also reduced in the hippocampus of FA control animals, reflecting a likely role for Ang II signaling in the underlying baseline CNS pathologies observed in the ApoE $^{-/-}$ mouse model. The reduction in oxidative stress in the ApoE $^{-/-}$ mice treated with ACEi is consistent with other vulnerable vascular disease models, as a decrease in oxidative stress was also observed with captopril treatment in a hypertensive rat model [62]. Furthermore, Gannon et al. show that captopril improved hippocampus and memory function, a variable that was not measured in our current study [62]. Since recent studies observed memory and behavioral alterations in AD mice after acute exposure to ultrafine PM [63], future studies are needed

to determine whether MVE exposure results in similar behavioral outcomes and whether RAS signaling contributes mechanistically to these outcomes.

One possible mechanism for the observed decrease in oxidative stress with ACEi treatment is the reduction in Ang II signaling. For example, Ang II-AT₁ signaling activates NOX via a G-protein-coupled signaling pathway, increasing the generation of cytoplasmic superoxide (O₂^{•−}) [39,52]. NOX is composed of several cytosolic (p47^{phox} and p67^{phox}) and transmembrane subunits (gp91^{phox}), of which the catalytic subunit gp91^{phox} is thought to be the driving force in ROS production [64]. We observed significant increases in gp91^{phox} mRNA in mice exposed to MVE, which were attenuated with ACEi treatment. Interestingly, we did not observe an increase in the cytosolic p47^{phox} mRNA expression, although there was significant variability within the exposed groups.

ApoE^{−/−} mice have classically been utilized as an important tool for studying CVD as they exhibit increased plasma cholesterol and atherogenesis when fed a high-fat diet [65]. Additionally, ApoE^{−/−} have been shown to have underlying cerebrovascular dysfunction [66], which we have previously shown is exacerbated by MVE exposure [31,67]. Due to the intricate relationship between CVD, cerebrovascular disease, and the progression of AD, we examined the effects of exposure to MVE on inflammation at or near the cerebral microvasculature. We observed a substantial increase in IL-1β expression in the parenchyma surrounding the brain's microvasculature following exposure to MVE, which was mitigated with treatment with ACEi. Studies have previously found links between acute inflammatory episodes in vascular smooth muscle cells and the promotion of cerebral amyloid angiopathy and the development of AD [68]. Importantly, our laboratory previously reported increased inflammation and oxidative stress in the cerebral microvasculature of wild-type C57BL/6 mice following MVE exposure [67].

Epidemiological studies have reported that ambient air pollution contributes to the initiation and progression of AD globally [5,69–72]. In regions where air pollutants regularly exceed the recommended World Health Organization (WHO) guidelines, studies show that the pathogenic onset of AD can occur in human populations as early as the first two decades of life [6,12,73,74]. TRAP is among the highest contributors to unsafe air pollution levels in most industrialized areas and is associated with numerous adverse health outcomes, including AD [75,76]. For example, Aβ accumulation, phosphorylated tau proteins, neurodegeneration, and increased cognitive decline are all correlated with TRAP exposure [5,69,77–79]. In addition to environmental factors, genetic risk factors such as the ε4 allele of the apolipoprotein E gene (ApoE4) are also associated with sporadic AD [25,80–83]. Furthermore, ApoE4 carriers exposed to TRAP have a higher risk of cerebrovascular dysfunction, neuroinflammation, oxidative stress, hyperphosphorylated tau protein, and Aβ plaques [84]. Laboratory studies further support these correlations, as exposure to TRAP increases Aβ, oxidative stress, and neuroinflammation, in both wild-type models and those genetically predisposed to AD [54,85–88].

Interestingly, data is lacking in the literature regarding the effects of TRAP exposure and AD pathology using the ApoE^{−/−} mouse model, despite ApoE4 being the most prominent genetic risk factor for sporadic AD. However, the literature is conflicting on whether the deletion of the ApoE gene in this animal model leads to AD pathology similar to that observed with the ApoE4 allele in humans. Shi et al. suggest that deletion of the ApoE gene confers protective effects against AD, while ApoE4 knock-in exhibits an increased propensity to AD [89]. Alternatively, other groups have reported that ApoE4 knock-in mice and ApoE^{−/−} exhibit comparable neuronal deficiencies in the development of AD [29,90,91]. As such, the results observed in the ApoE^{−/−} mouse model are likely not directly translatable to humans with the ApoE4 allele; however, this model remains a useful tool for investigating the contributions of ApoE to AD pathologies. In addition to a lack of

data on the effects of TRAP in ApoE^{-/-} mice, the current literature primarily focuses on exposure to single components of TRAP, such as PM_{2.5} or ozone exposure. While studies examining specific components of TRAP are crucial to understanding the mechanisms of toxicity associated with individual components, this is not representative of typical occupation or near-roadway exposure scenarios for most humans. As such, we analyzed the effects of combined gasoline and diesel engine emissions (MVE) exposure in ApoE^{-/-} mice in our current study.

We have previously investigated the effects of MVE in the brains of young and aged C57BL/6 mice and reported increased A β , BACE1, oxidative stress, and RAS signaling in the hippocampal CA1 region of aged mice exposed to MVE [46]. We observed similar trends of increased RAS signaling in other MVE exposures conducted by our laboratory in C57BL/6 mice [47]. The hippocampal CA1 region is responsible for consolidating short-term memories into long-term memories and is one of the key brain areas hypothesized to be involved in the initiation of AD pathology; thus, it is the primary area we examined histologically for this study. The RAS in the brain has been heavily implicated in mediating the initiation and progression of AD through Ang II signaling [40,42]. Recent studies also suggest that treatment with RAS-targeting drugs, such as ACEi and ARBs, may reduce classic neuropathologic features of AD, such as A β plaque deposition, in humans and animal models [62,92–95]. There remains a clear gap in the literature on the interactions of RAS, ApoE status, environmental exposure to TRAP, and AD pathology. Therefore, our current study utilized captopril, a popular ACEi inhibitor, in conjunction with our MVE exposure in ApoE^{-/-} mice.

Increased plasma Ang II is a classic hallmark of hypertension and CVD, and there is ample evidence of the association of midlife CVD with future cognitive impairment and diagnosis of AD [96]. In addition to the systemic RAS, there is a local CNS RAS, in which Agt secreted by astrocytes is converted to Ang II via renin and ACE, and signals via AT₁ and AT₂ receptors [34,92,97]. Ang II from the systemic circulation can promote BACE1 activity and A β accumulation via AT₁ receptor signaling [98]. Our laboratory previously demonstrated an increase in plasma Ang II following MVE exposure in C57BL/6 mice [47]. In agreement, in the current study with ApoE^{-/-} mice, MVE exposure increased plasma Ang II, which was attenuated through ACEi treatment. While we did not observe statistically significant increases in either AT₁ or A β in the CA1 region of the hippocampus in ApoE^{-/-} mice exposed to MVE, we did observe an increase in BACE1 expression, which was normalized through ACEi treatment. Furthermore, there was a significant decrease in AT₁ protein expression in MVE-exposed mice treated with captopril. These findings suggest a strong correlation between plasma Ang II levels and BACE expression in the hippocampus, which is elevated in AD pathologies. Thus, even though we did not observe an increase in A β deposition in the hippocampus of our study animals with 30-day MVE exposure, it is plausible that MVE exposure promotes alterations in the brain that may subsequently promote A β accumulation. Interestingly, we also did not observe an increase in ACE expression in the CA1 region of the hippocampus of ApoE^{-/-} mice exposed to MVE, despite observing an increase in MVE-exposed C57BL/6 mice in our previous studies [43]. The use of ApoE^{-/-} mice may contribute to this observation, as little is known about the expression of ACE in the brains of ApoE^{-/-} mice.

Although not observed in the current study, multiple studies have reported increased accumulation of A β in the brain following exposure to air pollution [28,54,55,85–88]. A β accumulation in the brain occurs via proteolytic cleavage of APP by BACE and subsequent processing by gamma-secretase; therefore, increased BACE expression can result in accumulation of A β . While our findings show changes in the upstream amyloidogenic signaling via BACE, we did not quantify additional gamma-secretase components or directly

assess gamma-secretase activity; therefore, any observation related to gamma-secretase should be interpreted as suggestive of altered complex regulation rather than definitive evidence of enhanced gamma-secretase catalytic activity. At the transcript level, we observed an increase in BACE1 in ApoE^{−/−} mice exposed to MVE and treated with ACEi. This differs from what was observed at the protein level, where ACEi treatment prevented MVE-induced BACE1 expression in the hippocampal CA1 region. A plausible explanation for this observed difference may be related to the location of measured BACE1 expression. For transcript-level expression, mRNA was extracted from a homogenate of cerebral tissue, while immunofluorescent protein quantification was specific to the hippocampal CA1 region. Notably, the CA1 region of interest includes the pyramidal neuronal layer as well surrounding neuropil. Because this surrounding region contains a mixture of neuronal processes, glial cells, and microvessels, staining outside the pyramidal layer should be interpreted as regional CA1 signaling, and not as any cell-specific signaling in the absence of co-labeling.

Blockade of the RAS has previously shown to ameliorate inflammatory and apoptotic responses in cell culture and animal models through mechanisms that include protection of cerebral blood flow and maintenance of BBB function [99]. It should be noted that in human trials, treatment with ACEi did not significantly reduce inflammation. However, AT₁ receptor inhibitors are effective in lowering neuroinflammation [99]. There is increased interest in identifying drugs that can serve as adjunct therapies to treat and prevent the onset and progression of AD, and the current study suggests that decreased RAS signaling can reduce inflammation, oxidative stress, and signaling factors that mediate Aβ production in the CNS.

It is important to note the limitations of the current study. ApoE^{−/−} mice as an AD model have been scrutinized, as global ApoE knockout does not produce Aβ plaques [29]. In addition, we did not directly quantify Aβ peptide levels or measure gamma-secretase catalytic activity components; therefore, the present findings are best interpreted as changes in Aβ-production-related signaling markers rather than changes in Aβ peptide abundance. Additionally, while the rationale to utilize the ApoE^{−/−} mouse model on a high-fat diet in the current study was to investigate the contributions of MVE to mechanisms involved in AD pathology using the same study design for which we previously observed MVE-mediated BBB disruption and promotion of oxidative stress [32], further studies are needed to delineate the role of the genetic vs. dietary factors in promoting the observed outcomes. However, since we observe a statistical increase in the oxidative stress marker 8-OHdG in the hippocampus of the MVE-exposed ApoE^{−/−} mice, compared to FA control ApoE^{−/−} mice, we can conclude that the MVE exposure is driving a significant induction of ROS in the CNS through mechanisms that involve the RAS (at least in part). Furthermore, the concentration of MVE used for the current study (200 μg PM/m³) is considered an “unhealthy” Air Quality Index category and is higher than the average US Air Quality Index for major metropolitan areas. However, it is within the range of PM levels reported in highly polluted regions worldwide, thus representing realistic exposure levels in those regions [100,101]. The endpoints examined in the current study only represent one exposure time point (30-day, subchronic) and thus do not necessarily represent acute or chronic outcomes of MVE exposure. Finally, only male ApoE^{−/−} mice were used in the current study. Additional studies in ApoE^{−/−} females are necessary to fully determine the effects of MVE exposure in ApoE^{−/−} mice and potential sex-related differences.

While additional mechanistic studies are needed to fully elucidate the pathways involved, this study’s findings suggest that MVE exposure modulates oxidative stress and the expression of factors associated with AD pathogenesis. These outcomes appear to be in part regulated by the RAS, as captopril treatment reduced or normalized the expression of

factors associated with AD pathogenesis, including BACE and ROS production. Traffic-generated air pollution has been reported to contribute to detrimental outcomes in the CNS and promotion of AD pathology, especially in populations with additional risk factors such as ApoE allele status [23,50,84]. Understanding the connections among air pollution, the ApoE gene, ROS, and RAS signaling in the CNS is imperative for unraveling the gene–environment interactions in AD progression. This integrated perspective can potentially lead to more effective preventative and therapeutic approaches to treating AD.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/antiox15020161/s1>, Figure S1: Representative images of secondary antibodies negative controls; Figure S2: Cerebral expression of p47phox mRNA transcript in ApoE^{−/−} mice; Figure S3: Exposure to MVE does not alter the expression of ACE in the CNS of ApoE^{−/−} mice; Figure S4: Exposure to MVE does not promote increased beta amyloid deposition in the CA1 hippocampal region in ApoE^{−/−} mice.

Author Contributions: Conceptualization: T.D.A.; U.S. and A.K.L.; methodology: T.D.A., U.S. and A.K.L.; investigation: T.D.A., U.S., A.S., B.J., V.L.Y., I.S., S.M.G. and M.C.; formal analysis: T.D.A., U.S., V.L.Y., A.S., B.J., I.S., S.M.G., M.C. and A.K.L.; writing—original draft preparation: T.D.A. and U.S.; writing—review and editing: T.D.A., U.S., V.L.Y., A.S., B.J., I.S., S.M.G., M.C. and A.K.L.; supervision: T.D.A., U.S., V.L.Y. and A.K.L.; funding acquisition: A.K.L.; resources: A.K.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was supported by the National Institute of Health (NIH)/National Institute of Environmental Health Sciences (NIEHS), grants number R00ES016586 and 2R15ES026795 to A.K.L. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.

Institutional Review Board Statement: All procedures were approved by the Lovelace Biomedical Research Institute’s Animal Care and Use Committee (IACUC) Protocol #08-072; Amendment E (original protocol approved 29 April 2009). These procedures conform to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Informed Consent Statement: Not applicable.

Data Availability Statement: The original data presented in the study are openly available in the Mendeley Data repository at DOI:10.17632/cwb7tgbt9y.2.

Acknowledgments: We would like to thank Jacob D. McDonald and the Chemistry and Inhalation Exposure group, in the Environmental Respiratory Health Program, at Lovelace Biomedical Research Institute, for the characterization and monitoring of the original animal inhalation exposures, as well as fellow lab member Kayla Nguyen-Alley for her contributions to the work.

Conflicts of Interest: Funding from the National Institute of Health (NIH)—National Institute of Environmental Health Sciences (NIEHS) was used to conduct the exposures and experiments described in this manuscript; however, the authors declare no conflicts of interest or financial gains to the entities associated with this publication.

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