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Acute exposure to diesel exhaust impairs adult neurogenesis in mice: prominence in males and protective effect of pioglitazone

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Abstract

Adult neurogenesis is the process by which neural stem cells give rise to new functional neurons in specific regions of the adult brain, a process that occurs throughout life. Significantly, neurodegenerative and psychiatric disorders present suppressed neurogenesis, activated microglia, and neuroinflammation. Traffic-related air pollution has been shown to adversely affect the central nervous system. As the cardinal effects of air pollution exposure are microglial activation, and ensuing oxidative stress and neuroinflammation, we investigated whether acute exposures to diesel exhaust (DE) would inhibit adult neurogenesis in mice. Mice were exposed for 6 h to DE at a PM_{2.5} concentration of 250–300 µg/m³, followed by assessment of adult neurogenesis in the hippocampal subgranular zone (SGZ), the subventricular zone (SVZ), and olfactory bulb (OB). DE impaired cellular proliferation in the SGZ and SVZ in males, but not females. DE reduced adult neurogenesis, with male mice showing fewer new neurons in the SGZ, SVZ, and OB, and females showing fewer new neurons only in the OB. To assess whether blocking microglial activation protected against DE-induced suppression of adult hippocampal neurogenesis, male mice were pre-treated with pioglitazone (PGZ) prior to DE exposure. The effects of DE exposure on microglia, as well as neuroinflammation and oxidative stress, were reduced by PGZ. PGZ also antagonized DE-induced suppression of neurogenesis in the SGZ. These results suggest that DE exposure impairs adult neurogenesis in a sex-dependent manner, by a mechanism likely to involve microglia activation and neuroinflammation.

Keywords

Diesel exhaust; Adult neurogenesis; Microglia; Pioglitazone; Neuroinflammation

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Compliance with ethical standards

Ethical statement C57BL/6J mice of both sexes were used in these studies. All animal procedures were pre-approved by the University of Washington Institutional Animal Care and Use Committee (IACUC), Protocol 2077-14. Experiments were carried out in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals, as adopted by the National Institutes of Health.

Conflict of interest The authors declare that they do not have any conflict of interest.

Introduction

Air pollution derives from a variety of sources, including industrial and vehicular emissions and biomass burning, and contains several components such as organic and inorganic particulates, metals, volatile organic compounds, and gases (Monks et al. 2009). Long associated with the development of chronic respiratory conditions and later with cardiovascular disorders and metabolic dysfunction, elevated air pollution has been associated recently with increased risk of adverse effects in the central nervous system (CNS) (Calderón-Garcidueñas et al. 2008, 2012; Costa et al. 2014a, 2017; Costa 2017; Genc et al. 2012). For example, epidemiological studies have shown an association between air pollution and cognitive impairment, dementia, and other neurodegenerative diseases (Chen et al. 2017; Weuve et al. 2012). One of the air pollution components of most concern is PM_{2.5}, i.e., particulate matter having an aerodynamic diameter of 2.5 micrometers or less. PM_{2.5} is capable entering the circulatory system through the pulmonary or olfactory mucosa, and can also enter the brain through the olfactory nerve (Oberdörster et al. 2004). Concentration of PM_{2.5} routinely exceeds 100 µg/m³ for extended periods of time in some parts of the world, especially in certain areas of China and India (Kandlikar and Ramachandran 2000; Sun et al. 2004). Traffic-related air pollution is a major contributor to global air pollution, and diesel exhaust (DE) is one of the predominant components (Ghio et al. 2012).

Oxidative stress and inflammation are the two important processes by which air pollution exerts its systemic and central nervous system toxicity (Genc et al. 2012; Costa et al. 2017). Experimental exposure of mice to DE causes priming and activation of microglia and subsequent neuroinflammation and oxidative stress (Levesque et al. 2011; Cole et al. 2016). In vitro experiments have also shown that the toxicity of DE particulates (DEP) is dependent upon the activity of microglia, with monocultured neurons showing none of the neurotoxic response that the neurons co-cultured with microglia displayed. In addition, blocking microglial activation with the PPAR-γ agonist pioglitazone (PGZ) attenuated DEP neurotoxicity in vitro (Roqué et al. 2016).

Neuroinflammation and microglial activation can adversely influence regeneration of neurons in the brain (Carpentier and Palmer 2009; Ekdahl et al. 2003). The birth of new neurons in the adult brain, and their survival and functional integration into existing neural circuitries, known as adult neurogenesis, is restricted in rodents to two regions: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the area adjacent to the lateral ventricles and striatum, known as the subventricular zone (SVZ) (Alvarez-Buylla et al. 2001). The SVZ is also the point of origin for immature neuroblasts that migrate along the rostral migratory stream to the olfactory bulb (OB), where they develop into local interneurons (Pignatelli and Belluzzi 2010). Neurogenesis occurs in the adult brain constantly, though at a rate that decreases with age (Ming and Song 2011), possibly because of increased neuroinflammation and microglial activation (Schuitemaker et al. 2012). Microglial activation resulting from lipopolysaccharide administration, or increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), or tumor necrosis factor-α (TNF-α) can impair hippocampal neurogenesis (Ekdahl et al. 2003; Iosif et al. 2006; Vallières et al. 2002). Even chronic peripheral inflammation may have an adverse effect on the CNS, as

increased permeability of the endothelium of the blood–brain barrier allows activated macrophages to migrate into the relatively restricted cerebrospinal compartment, triggering microglial activation and neuroinflammation (Raghavendra et al. 2004; Takeshita and Ransohoff 2012).

Disruption of adult neurogenesis may affect cognitive and olfactory function (Frankland and Miller 2008), and may lead to severe cognitive impairment in neurodegenerative diseases. Indeed, even in the presence of characteristic Alzheimer's disease (AD) neuropathology, cognitive function is equal to that of healthy individuals when neurogenesis is preserved (Briley et al. 2016). The gradual loss of neurons, coupled with a reduction in young adult-born neurons, plays a role in normal diminution of cognitive function due to the unique role of young neurons in memory (Bishop et al. 2010). Young neurons are very excitable, having a low threshold of depolarization, and they appear to be preferentially activated during the formation of new memories (Bischofberger 2007). Short-term memory also appears to be dependent on the activities of young neurons, and impairment of short-term memory is one of the first symptoms to emerge in the earliest stages of AD (Baudic et al. 2006). The ability to remember and forget is related to synaptic plasticity, or the weakening and strengthening of neural pathways over time, which also appears to be affected by neurogenesis (Saxe et al. 2006). Decreased neurogenesis, and the resulting impaired synaptic plasticity, may also be involved in depression and anxiety, and explain the therapeutic effectiveness of anti-depressants that increase neurogenesis (Hayley and Litteljohn 2013).

Based on our studies indicating that acute DE exposure induces neuroinflammation, microglial activation, and oxidative stress (Cole et al. 2016), we hypothesized that (1) mice acutely exposed to DE would show compromised neurogenesis relative to control animals and (2) given that neuroinflammation induced by DE was more pronounced in male mice (Cole et al. 2016), neurogenesis would be impaired to a greater extent in male animals. We further hypothesized that a pre-treatment with PGZ would protect against DE-induced inhibition of adult neurogenesis by attenuating microglial activation, neuroinflammation, and oxidative stress.

Materials and methods

Animals

Male and female 8-week-old C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in specific pathogen-free facilities with a 12-h dark–light cycle, with feed and water available ad libitum. The mice were assigned randomly to either filtered air (FA) or DE exposures.

Bromodeoxyuridine treatment

A stock solution of bromodeoxyuridine (BrdU) was prepared using 20-mg/mL BrdU (Sigma-Aldrich, St. Louis, Missouri) and 0.007-N sodium hydroxide to facilitate dissolution in sterile normal saline (0.9% sodium chloride). The solution was buffered to pH 7.4 and sterile-filtered prior to use. Each animal was weighed and given an initial dose of 100-mg/kg BrdU by intraperitoneal injection. Mice received four more doses of 100-mg/kg BrdU given

at 2-h intervals, resulting in a cumulative dose of 500 mg/kg/day (Pan et al. 2013a; Taupin 2007).

Exposure to diesel exhaust

Individually housed mice were exposed for 6 h to FA or DE (at a PM_{2.5} concentration of 250–300 µg/m³). Exposures to either FA or DE were conducted simultaneously in the University of Washington Controlled Exposure Laboratory's Northlake Diesel Facility, which includes an SPF mouse housing room with Allentown caging systems (Allentown, NJ), with the housing racks modified to ventilate cages with either diluted DE or FA. DE was derived from a Yanmar YDG5500 diesel generator, with a load bank maintaining 75% of rated capacity, using No. 2 undyed, ultra-low sulfur on-highway fuel and Royal Purple Duralec 15W-40 Synthetic crankcase oil to lubricate moving parts, as previously described (Fox et al. 2015; Gould et al. 2008). During exposures, DE concentrations were continuously measured and maintained at steady levels using a feedback controller monitoring fine particulate levels (Fox et al. 2015; Gould et al. 2008). DE was limited to PM_{2.5} or smaller, having a mean aerodynamic diameter of 100 nm. Characterization of DE is described in detail in a previous publication (Fox et al. 2015). For assays of lipid peroxidation and of TNFα mRNA levels, mice were euthanized by CO₂ asphyxiation within 2 h after the end of the exposure, and brain regions were dissected immediately, flash-frozen in liquid nitrogen, and stored at – 80 °C. For Iba1 and Ki67 immunohistochemistry, mice were euthanized 18 h after the end of exposure, and their brains fixed by transcardial perfusion and then embedded, sectioned, and stored at – 80 °C in cryoprotectant medium. For NeuN/BrdU immunohistochemistry, animals were pre-treated with 500-mg/kg BrdU the day before exposure, exposed for 6 h, then euthanized 21 days following exposure, to label adult-born cells that are still alive. Their brains were fixed, frozen, and sectioned as described above, and stored at – 80 °C in cryoprotectant medium (Pan et al. 2013a).

Lipid peroxidation assay

Lysates were prepared from frozen brain region samples homogenized in CLB lysis buffer (10-mM HEPES; 150mM NaCl; 1-mM CaCl₂; 0.5-mM MgCl₂; 10-µg/ml leupeptin; 10-µg/ml aprotinin; 1-mM PMSF; 50-mM NaF). The homogenate was incubated on ice for 10-min, centrifuged at 4 °C and 2000 × g for 5 min, and the resulting supernatant samples were stored at – 80 °C until ready for analysis. The protein content of each sample was determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA), with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol. Lipid peroxidation was measured by quantifying levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, using the Thiobarbituric Acid Reactive Substances (TBARS) assay (Cayman Chemical, Ann Arbor, MI) according to the manufacturer's instructions, as previously described (Giordano et al. 2013). MDA content was normalized to the amount of protein loaded per well.

RNA extraction and real time PCR analysis

Prior to extraction, all working surfaces and instruments were cleaned with an RNase inhibitor. Tissue homogenates were prepared using TRIzol RNA extraction reagent (Invitrogen, Carlsbad, California) and purified according to the manufacturer's protocol.

Concentration and quality of RNA were determined using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). RNA samples (1 µg) were reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) according to the manufacturer's protocol. The resulting cDNAs were used for PCR amplification in the presence of primers specific to tumor necrosis factor (TNFα) (forward: GTCGTA GCAAACCACCAAGTG; reverse: CTTTGAGATCCATGC CGTTGG; 21 bp) and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT1) (forward: GAGGAG TCCTGTTGATGTTGCCAAG; reverse: GGCTGGCCT ATAGGCTCATAGTGC; 25 bp). Amplification was carried out in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, California) and quantification was carried out in a Bio-Rad CF384 Real-time System thermal cycler, using iTaq Universal SYBR Green Supermix.

Pioglitazone treatment

A stock suspension of 1.25-mg/mL pioglitazone hydrochloride (PGZ; 98% pure; Sigma-Aldrich, St. Louis, Missouri) was prepared in a vehicle (VEH) of 0.5% carboxymethylcellulose sodium dissolved in PBS. For 4 days, animals were treated with 10 µL/g of PGZ (12.5 mg/kg) or 10-µL/g VEH by oral gavage, using a 20G stainless-steel curved feeding needle, each morning, up to and including the day of DE exposure (Drew et al. 2015; Maeda et al. 2008).

Transcardial perfusion and post-fixation

Mice were euthanized by CO₂ asphyxiation, and the thoracic cavity was opened to expose the heart. A small incision was cut into the left ventricle of the heart, into which a blunted 20-gauge needle attached to a line containing saline was inserted and secured with a bulldog clamp. A small incision was cut into the right atrium to provide an outlet for the blood and perfusion fluids. First, 15 ml of 0.9% normal saline were pumped through the vasculature using a Minipuls 2 peristaltic pump (Gilson, Middleton, Wisconsin) to clear out the blood, followed by the same volume of 4% paraformaldehyde (PFA) in PBS (Santa Cruz Biotech, Santa Cruz, California) to achieve thorough fixation of tissues. Brains were resected intact from the perfused animals, placed in 50-mL conical tubes containing ice-cold 4% PFA in PBS, and post-fixed overnight at 4 °C. They were then removed from the PFA and placed in a solution of 30% sucrose in PBS at 4 °C until negative buoyancy was reached. The brains were rinsed in PBS and then embedded individually in O.C.T. Compound Embedding Medium (Sakura Finetek, Torrance, California), frozen, and stored at – 80 °C prior to sectioning (Pan et al. 2013a).

Olfactory bulb immunohistochemistry

Frozen, OCT-embedded, OB tissue was cut at a thickness of 14 µm using a Reichert-Jung Cryocut-1800 cryostat (Leica, Wetzlar, Germany) and every eighth section was placed directly on VWR SuperFrost-Plus Microslides. Slides were stored at – 20° until ready for processing. Slides destined for processing were removed from storage and allowed to sit at RT for 20 min to allow for adhesion of tissue to the slide. A Pap-Pen (Electron Microscopy) was used to draw around the sections to confine reagents to the surface of the slide. Slides were placed in a humidification chamber and washed with PBS to rehydrate the tissues.

Sections were rinsed in water briefly, and then treated with 2-N hydrochloric acid for 30 min at 38 °C. Acid was neutralized with 0.1-M borate buffer (pH 8.5); sections were then permeabilized, first with 1% sodium dodecyl sulfate, then with PBS containing 0.25% Triton X-100 (PBST). Sections were blocked overnight using a blocking solution containing 10% goat serum and 1% bovine serum albumin (BSA) in PBST. Following blocking, sections were probed for BrdU and NeuN using monoclonal antibodies specific to the markers (BrdU (1:1000): MCA2060, AbD Serotec/Bio-Rad, Hercules, California; NeuN (1:1000): MAB377, EMD Millipore, Temecula, California). Following 48-h incubation in primary antibodies at 4 °C, the slides were rinsed in PBST and then probed with AlexaFluor 594 goat anti-rat and AlexaFluor 488 anti-mouse antibodies [(1:1000 dilutions) Invitrogen/ThermoFisher, Waltham, Massachusetts] for 48 h. Following nuclear staining with Hoechst 33,342, sections were rinsed in PBS, mounted using VectaShield Fluorescence Mounting Medium (H-1000, VectaShield, Burlingame), and cover slips were secured using nail polish (Pan et al. 2013a).

SGZ and SVZ immunohistochemistry

Every eighth coronal section of the subventricular and hippocampal region of each brain was cut at a thickness of 30 µm using a Reichert-Jung Cryocut-1800 cryostat (Leica) with temperature set at – 25 °C. Sections were stored at – 20 °C in 24-well plates containing cryoprotectant medium until processed. Prior to staining, sections were rinsed in PBS to remove cryoprotectant and embedding medium. For Ki67 and Iba1 immunohistochemistry, no antigen retrieval method was used prior to permeabilization. For NeuN/BrdU double-staining, a 30-min DNA-denaturing step with 2N hydrochloric acid and subsequent neutralization with 0.1-M borate buffer was required to improve immunolabeling of BrdU. The sections were then permeabilized with PBST and blocked overnight in a buffer containing 1% BSA (w/v) and 10% normal goat serum (v/v) in PBST. Sections were then incubated for 48 h with the appropriate primary antibodies [BrdU (1:1000): MCA2060, AbD Serotec/Bio-Rad; NeuN (1:1000): MAB377, EMD Millipore; Iba1 (1:1000): ab107159, and Ki67 (1:1000): ab15580, Abcam, Cambridge, Massachusetts]. Sections were rinsed in PBST then incubated with AlexaFluor 594 goat anti-rat and AlexaFluor 488 goat anti-mouse antibodies (1:1000), AlexaFluor 568 donkey anti-goat (1:1000), or AlexaFluor 555 donkey anti-rabbit antibody (1:1000) for 48 h. The sections were rinsed in PBST and incubated in Hoechst 33,342 to identify nuclei. Following nuclear staining, sections were washed in PBS, and then mounted on gelatin-coated slides. VectaShield Fluorescence Mounting Medium (H-1000, VectaShield, Burlingame, California) was used to mount the sections and prevent photobleaching. No. 1 coverslips (VWR, South San Francisco, California) were placed on top of the sections and secured using nail polish. The slides were then placed in slide boxes and kept in the dark at 4 °C until ready for imaging (Jongbloets et al. 2017; Pan et al. 2012, 2013a).

Image acquisition and analysis

For assessment of adult neurogenesis, images were captured in three channels using a Marianas Imaging System, which included a Zeiss 200M Axiovert microscope with a motorized stage, a 175-W xenon lamp, and a Roper HQ Cool Snap digital camera. The software SlideBook 6.0 (3i Intelligent Imaging Innovations, Denver, Colorado) was used to

set parameters of image capture. In the SGZ and SVZ, 40× 3D images of all BrdU-positive cells were collected. Images were uniformly adjusted for brightness and contrast. All BrdU-positive cells that contained a well-defined nucleus were counted, and BrdU-positive nuclei immunoreactive to NeuN were counted as double-labeled cells. The ratio of BrdU+/NeuN+ cells to all BrdU+ cells was calculated for each specimen and expressed as a percentage (Pan et al. 2012, 2013a, b).

For adult neurogenesis in the OB, a 20× montage of each section was taken. The granular cell layer of each section was then defined and stereologically sampled at 12.5% of the cross-sectional area. As with the SGZ and SVZ, three-channel 40× images were uniformly adjusted for color and brightness. The number of BrdU+ cells and the number of newborn neurons (NeuN+/BrdU+ cells) were expressed as density (number per cubic mm of region of interest) rather than a total number per region. Newborn neurons were also expressed as a percentage of all BrdU+ cells.

To assess proliferation in the SGZ and SVZ, two-channel images of all Ki67+ cells were taken in the SGZ and SVZ. Images were uniformly adjusted for contrast and brightness, and all Ki67+ cells with a well-defined nucleus were counted and expressed as a total number per region.

To assess microglial morphology, two-channel 40× images of the hippocampal dentate gyrus were captured using the Marianas Imaging System for imaging and Slide-Book 6.0 to set parameters of image capture. Two-channel 40× images were adjusted uniformly for contrast and brightness, and perimeter of microglial somata on the plane with the sharpest resolution of the nucleus were traced using the irregular drawing tool and analyzed to evaluate shape descriptor parameters using the open-source software FIJI. In addition to the microglial soma area, three other parameters were assessed: circularity ($C = 4\pi \cdot \text{area} / \text{perimeter}^2$), roundness ($R = 4 \cdot \text{area} / \pi (\text{major axis})^2$), and aspect ratio (AR = major axis/minor axis) (Jonas et al. 2012; Morrison and Filosa 2013; Torres-Platas et al. 2014).

Statistical analysis

Statistical analysis of all data was by one-way ANOVA with Bonferroni's post-test. All graphs show the mean and SEM. Prism 5.02 (GraphPad, San Diego, California) was used for the preparation of graphs as well as statistical analysis of data.

Results

As observed previously with this paradigm of DE exposure (Cole et al. 2016), no morbidity was observed in animals exposed to 250–300- $\mu\text{g}/\text{m}^3$ DE for 6 h, and there were no salient behavioral or physical differences between DE- and FA-exposed animals of either sex.

Fluorescence immunohistochemistry was used to analyze the neurogenic regions of the brain (OB, SVZ, and SGZ). Cellular proliferation was assessed by immunohistochemistry using Ki67 in the SGZ and SVZ. In male mice, DE exposure was associated with a significant reduction in the number of Ki67-immunopositive cells in both the SGZ (Fig. 1a) and the SVZ (Fig. 1b). In contrast, no significant differences were seen between DE- and FA-

exposed female mice in the number of Ki67-immunopositive cells in either SGZ (Fig. 1a) or SVZ (Fig. 1b).

For assessment of adult neurogenesis, tissue sections containing the OB, SVZ, and SGZ were probed with antibodies specific to the cellular proliferation marker BrdU and the neuronal nuclear marker NeuN. BrdU+ and double-labeled cells (NeuN+/BrdU+) were counted and expressed as a total number and as a percentage of all BrdU+ cells. In male mice, the total number of BrdU+ cells was significantly reduced following DE exposure in both the SGZ (Fig. 2a) and the OB (Fig. 4a), but not in the SVZ (Fig. 3a). The number of double-positive NeuN+/BrdU+ cells was reduced in DE-exposed males in all three regions (Figs. 2b, 3b, 4b) compared to FA-exposed males. NeuN+/BrdU+ double-labeled cells expressed as a percentage of all BrdU+ cells were also significantly reduced in the SGZ (Fig. 2c), SVZ (Fig. 3c), and OB (Fig. 4c) of DE-exposed males compared to FA controls. No significant differences were seen between DE- and FA-exposed female mice in the SGZ (Fig. 2) or SVZ (Fig. 3) in either the number of BrdU+ cells (Figs. 2a, 3a) or the number or percentage of double-labeled NeuN+/BrdU+ cells (Figs. 2b, c, 3b, c). However, in the OB (Fig. 4), the total number of double-labeled NeuN+/BrdU+ cells was significantly reduced in DE-exposed females compared to FA-exposed controls (Fig. 4b).

Pioglitazone (PGZ) has been shown to suppress DE-induced microglial activation in vitro (Roque et al. 2016). Since microglial activation and ensuing neuroinflammation has been shown to affect adult neurogenesis, we assessed the effect of pre-treatment with PGZ on DE-induced inhibition of adult neurogenesis in the SGZ. Male mice were used in this experiment, because they were more sensitive than females to the effects of DE exposure. Mice were pre-treated with PGZ or VEH for 4 days, then exposed to DE or FA for 6 h, and sacrificed 18 h later for assessment of microglial activation, or 3 weeks later for assessment of adult neurogenesis.

Iba1 immunohistochemistry was used to define the microglial somata in the dentate gyrus of the hippocampus, and shape descriptors associated with reactive microglial phenotypes were used to assess the degree of microglial activation associated with DE exposure. Acute DE exposure resulted in changes in microglial morphology typically associated with microglial activation, and these changes were antagonized by PGZ pre-treatment (Fig. 5). Specifically, microglial soma area was increased significantly with DE exposure (Fig. 5a), while circularity of microglial soma defined as $4\pi(\text{area}/\text{perimeter}^2)$ and roundness of the soma defined as $4*\text{area}/\pi(\text{major axis})^2$ were significantly reduced by DE exposure (Fig. 5b, c). Finally, aspect ratio (Fig. 5d), the quotient of the major and the minor axis of the best-fit ellipse, was significantly increased in microglia of the DE-exposed mice compared to FA controls. PGZ decreased the effects of DE exposure on all parameters of microglial morphology (Fig. 5a–d), indicating that the treatment was effective at inhibiting microglial activation. This was further confirmed by the findings that the increases in the levels of TNF- α mRNA and of lipid peroxidation in cortex and hippocampus caused by DE exposure were antagonized by pre-treatment with PGZ (Fig. 6a, b).

As expected, DE exposure significantly reduced cellular proliferation in the SGZ of the VEH-treated mice (Fig. 7). PGZ pre-treatment antagonized this effect of DE. In animals pre-

treated with PGZ DE exposure did not affect proliferation (Fig. 7). With regard to neurogenesis, in VEH-treated control animals, the total number of BrdU+ cells in the SGZ was reduced by DE exposure, and this effect was antagonized by PGZ (Fig. 8a). Similarly, NeuN+/BrdU+ cells expressed both as a total number and as percentage of all BrdU+ cells were reduced by DE exposure in VEH-treated controls, and this was also antagonized by pre-treatment with PGZ (Fig. 8b, c).

Discussion

The main findings of this study are that acute exposure to DE causes an impairment of adult neurogenesis in mice, which is likely secondary to neuroinflammation and oxidative stress that follow microglial activation, and that this effect is more pronounced in male animals. Young adult mice (8 weeks) were used in this study, and the exposure was for 6 h to a moderate/high concentration of DE (250–300- $\mu\text{g}/\text{m}^3$ PM_{2.5}). Such levels of PM_{2.5} can often be reached and even exceeded in several cities worldwide, particularly in India and China (Allen et al. 2013; Kandlikar and Ramachandran 2000; Sun et al. 2004; Costa et al. 2017). Traffic-related air pollution is a major contributor of PM_{2.5} levels and DE is the major source of PM_{2.5} and ultrafine particles (Ghio et al. 2012; Calderón-Garcidueñas et al. 2015). In some megacities such as New Delhi, where diesel fuel powers many passenger vehicles in addition to heavy machinery and public transportation conveyances, the contribution of traffic to air pollution is estimated to be as high as 72% (Goyal et al. 2006). The use of diesel fuel to power heavy machinery and vehicles of mass transit results in high daily exposures for those who work in certain occupations; miners and mechanics in bus garages regularly experience some of the very highest levels of particulate air pollution, where PM_{2.5} may range from 300 to 1000 $\mu\text{g}/\text{m}^3$ (Pronk et al. 2009). It should also be noted that DE contains several other components that were not measured in this study, and their potential contribution to neurotoxicity cannot be discounted. In future studies, it would be of much interest to also measure PM deposition in brain over time.

A short-term (6 h) exposure to DE was sufficient to affect adult neurogenesis in different brain regions. We had previously shown that the same DE exposure caused activation of microglia, increased oxidative stress (elevated lipid peroxidation), and neuroinflammation (increased levels of pro-inflammatory cytokines; Cole et al. 2016). These findings were confirmed in the present study, as lipid peroxidation and TNF- α mRNA levels were increased in cerebral cortex and hippocampus of DE-exposed mice (Fig. 6) and hippocampal microglia were activated (Fig. 5). Our earlier findings (Cole et al. 2016) also indicated that male mice are more sensitive to DE-induced oxidative stress and neuroinflammation. We had formulated the hypothesis of a possible higher susceptibility of male mice on the basis of a series of findings related to the levels of expression of the intracellular enzyme paraoxonase-2 (PON2). This enzyme has antioxidant and anti-inflammatory properties (Giordano et al. 2011; Schweikert et al. 2012), and is believed to have a neuroprotective role (Costa et al. 2014b). In vitro and in vivo studies have shown that males express lower levels of PON2 in brain and other tissues (Giordano et al. 2011, 2013); as such, they are more susceptible to oxidative stress and neuroinflammation than female mice (Giordano et al. 2011, 2013; Costa et al. 2014b), though additional mechanisms may also be involved in the

differential susceptibility of male and female mice to DE-neurotoxic effects (Cole et al. 2016).

Since it has been shown that microglial activation and increased levels of pro-inflammatory cytokines such as IL-6 or TNF- α impair hippocampal neurogenesis (Ek Dahl et al. 2003; Iosif et al. 2006; Vallières et al. 2002), we hypothesized that DE exposure would inhibit adult neurogenesis. Furthermore, based on our previous findings, we also hypothesized that DE-induced inhibition of neurogenesis would be more pronounced in male mice. Results of this study confirm both hypotheses. DE exposure impaired proliferation in the SGZ and SVZ only in male mice (Fig. 1); in the same two regions, adult neurogenesis was also impaired only in males (Figs. 2, 3). In the OB, the total number of adult-born neurons was reduced in males (Fig. 4), and to a minor extent was reduced in females (Fig. 4).

Gender differences in adult neurogenesis have been observed in some studies. Male rodents show higher hippocampal neurogenesis (as we also observed, see Fig. 2), accompanied by better performance in tests of spatial memory (Perfilieva et al. 2001). Anxiety disorders, which are also associated with reduced neurogenesis, are more prevalent and disabling in women than in men (McLean et al. 2011). These differences may, in part, be due to the effect of the differential levels of androgens; indeed, androgens increase adult hippocampal neurogenesis by promoting survival of young neurons in the dentate gyrus (Hamson et al. 2013). Gender differences also appear to extend to the effect of environmental factors on adult neurogenesis; for example, hippocampal neurogenesis is impaired by high-fat diet and stress in male, but not female, rodents (Lindqvist et al. 2006).

To test the hypothesis that inhibition of adult neurogenesis was secondary to microglia activation and ensuing oxidative stress and neuroinflammation, we utilized the PPAR- γ agonist PGZ. PGZ and other thiazolidinediones are recognized for their antidiabetic and hypolipidemic properties (Smith 2001), effects that may be mediated by the modulation of different parameters of mitochondrial function, and the promotion of mitochondrial biogenesis (Smith 2001; Bogacka et al. 2005; Ghosh et al. 2007; Corona and Duchon 2016; Miglio et al. 2009). PGZ also has neuroprotective activities that are ascribed to attenuation of microglial activation (Ji et al. 2010). Indeed, PGZ can reduce inflammation and microglial activation by activating the PPAR- γ of microglia, inhibiting the release of a number of pro-inflammatory substances (Carta and Pisanu 2013; Ji et al. 2010; Drew et al. 2015). We had previously shown that PGZ inhibits the neurotoxicity of DE particles in a microglia-neuron co-culture (Roqué et al. 2016). In the present study, we found that administration of PGZ to male mice antagonizes DE-induced microglial activation, as well as increases in lipid peroxidation and neuroinflammation in the hippocampus and the cerebral cortex (Figs. 5, 6). Upon PGZ pre-treatment, DE-induced inhibition of proliferation and adult neurogenesis in the SGZ was also inhibited (Figs. 7, 8).

The precise mechanisms by which DE exposure may inhibit adult neurogenesis are still elusive, though activation of microglia appears to play a relevant role. Microglia undergoes extensive cytoskeleton remodeling to better carry out certain functions such as phagocytosis (Arcuri et al. 2017; Perry and Teeling 2013). While the default state of microglia is silent vigilance, in which they survey their territory with fine, highly branched processes (Ito et al.

1998), certain chemical signals (e.g., extracellular ATP and subtle changes in concentration of potassium), that may indicate damaged or stressed neurons, can induce chemotaxis, and may activate microglia, triggering them to release TNF- α (Gehrmann et al. 1995; Hide et al. 2000). Reactive microglia have greater cell body area and thicker, shorter processes (Jonas et al. 2012; Morrison and Filosa 2013; Torres-Platas et al. 2014). It should be noted that microglia may either promote or inhibit adult neurogenesis (Sato 2015), depending on the activation state (Aarum et al. 2003; Xu et al. 2017). Notably, the M2 activation state, which is induced by anti-inflammatory cytokines such as IL-4, favors proliferation, and can direct neural progenitor cells (NPC) towards neurogenesis (Cherry et al. 2014; Choi et al. 2017; Zhao et al. 2017). In contrast, the inflammatory cytokines such as interferon- γ and TNF α , as well as oxidative stress, induce the M1 (classical activation) state, provoking killing behavior in microglia and other macrophages (Colton 2009). Thus, the two polarities of microglial activation have drastically different effects upon neurogenesis in the adult brain. Specific assessment of M1 (classical) and M2 (alternative) polarization upon exposure to DE would be useful to elucidate this issue. The mode of microglial activation may promote or inhibit NPC proliferation, cause them to embark upon a glial rather than a neuronal lineage, or favor either survival or apoptosis (Yuan et al. 2017). Another possibility is the phagocytosis of stressed but viable developing neurons by microglia, in a process called “phagoptosis” (Brown and Neher 2014). The ability of PGZ to inhibit both microglial activation and the effects of DE on neurogenesis suggests that this event is critical to DE-induced inhibition of neurogenesis. However, PGZ may also have other mechanisms of neuroprotection (e.g., improving the number and efficiency of mitochondria); hence, additional or alternative mechanisms for the effects of DE on neurogenesis are possible.

Another potential mechanism relates to the ability of traffic-related air pollution to suppress production of brain-derived neurotrophic factor (BDNF) (Bos et al. 2011, 2012). BDNF is a neurotrophin with stimulant effects on proliferation of NSCs, favoring differentiation of NSCs into neuro-blasts rather than glial cells, and promoting the survival of adult-born neurons (Binder and Scharfman 2004). Factors such exercise or dietary polyphenols may increase BDNF signaling, and thus enhance neurogenesis (Sleiman et al., 2016; Zhang et al. 2012). However, the increase in BDNF levels caused by exercise may be abrogated by exposure to particulate air pollution in both human and animal models (Bos et al. 2011, 2012). Increased lipid peroxidation and reduced BDNF resulting from a high-fat diet impair proliferation in the SGZ, and direct treatment of NPCs with malondialdehyde in vitro also significantly reduced proliferation, which was then restored by treatment with BDNF (Park et al. 2010).

Independent of the specific mechanism, suppression of neurogenesis by DE is of interest with regard to the reported reduced cognitive function and depression associated with traffic-related air pollution that has been observed in elderly adults (Chen et al. 2017; Lim et al. 2012; Weuve et al. 2012). Notably, impaired neurogenesis is an early event in the etiology of Alzheimer’s disease (AD) (Demars et al. 2010). In particular, older adults with high levels of exposure to particulate air pollution show poorer cognitive outcomes than non-exposed individuals (Ailshire and Clarke 2015). Aberrant neurogenesis is associated with poor cognitive functioning, and even individuals having characteristic AD neuropathology show no impairment of cognitive functions when neurogenesis is conserved (Briley et al. 2016).

These considerations should, however, be tempered by the fact that in the present study, the persistence over time of the effect of DE on neurogenesis was not assessed and that an assessment of cognitive behavior was not carried out. A follow-up of this study should also likely investigate neuroinflammation at timepoints beyond what done here, to determine whether persistent recurrent neuroinflammation remains, or waves of neuroinflammatory responses are seen.

In conclusion, results of this study show that short-term exposures to moderate/high concentrations of DE induce neuroinflammation, oxidative stress, and microglial activation, and suppress adult neurogenesis and cellular proliferation in male mice, as evidenced by a reduced number of Ki67+ cells in the SVZ and SGZ, and a reduced number of surviving NeuN/BrdU double-labeled cells in the OB, SVZ, and SGZ. The effects were significantly less pronounced in female mice. When microglial activation was blocked by pre-treatment with the PPAR- γ agonist PGZ, DE-induced suppression of adult neurogenesis was significantly antagonized. These findings underscore the importance of considering sex/gender when measuring neurotoxic effects, including alterations in neurogenesis, and reinforce the hypothesis that traffic-related air pollution may contribute to cognitive decline and perhaps also neurodegenerative diseases (Cacciottolo et al. 2017).

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Fig. 1.

Acute DE exposure decreases proliferation in the SGZ (**a**) and SVZ (**b**) of male mice. Eight-week-old male and female C57BL/6J mice were exposed to DE (250 μ g/m³) or FA for 6 h, and then sacrificed 18 h later. Images shown are representative micrographs of Ki67 immunohistochemistry in female and male mice. Results represent the mean ($\pm$ SE) with $n = 3$ per group. Significantly different from FA, ** $p < 0.01$, *** $p < 0.001$. SGZ subgranular zone of the hippocampus, SVZ subventricular zone

Fig. 2.

Effect of acute DE exposure on adult hippocampal neurogenesis in mice. Eight-week-old male and female C57BL/6J mice were given five 100-mg/kg doses of BrdU at 2-h intervals. On the following day they were exposed to DE (250 μg/m³) or FA for 6 h and then sacrificed 21 days later. The images shown are representative micrographs of NeuN/BrdU immunohistochemistry in the SGZ of FA- and DE-exposed mice. Scale bars in detail images represent 10 μm. The number of BrdU-stained cells (a) indicates surviving cells that been born since the beginning of the experiment. Double-stained NeuN/BrdU cells indicate surviving adult-born neurons, which are expressed both as a total number per brain region (b), and as a percentage of all BrdU-positive cells (c). Results represent the mean (± SE) with $n = 3$ per group. Significantly different from FA, ** $p < 0.01$, *** $p < 0.001$

Fig. 3.

Effect of acute DE exposure on adult neurogenesis in the SVZ of mice. Eight-week-old male and female C57BL/6J mice were given five 100-mg/kg doses of BrdU at 2-h intervals. On the following day, they were exposed to DE (250 $\mu\text{g}/\text{m}^3$) or FA for 6 h, and then sacrificed 21 days later. Images shown are representative micrographs of NeuN/BrdU immunohistochemistry in the SVZ of FA- and DE-exposed mice. Scale bars in montage images represent 100 microns, while scale bars in detail images represent 10 μm . The number of BrdU-stained cells (**a**) indicates surviving cells that been born since the beginning of the experiment. Double-stained NeuN/BrdU cells indicate surviving adult-born neurons, which are expressed both as a total number per brain region (**b**) and as a percentage of all BrdU-positive cells (**c**). Data shown represent the mean ($\pm$ SE) with $n = 3$ per group. Significantly different from FA, $*p < 0.05$

Fig. 4.

Effect of acute DE exposure on adult neurogenesis in the OB of mice. Eight-week-old male and female C57BL/6J mice were given five 100-mg/kg doses of BrdU at 2-h intervals. On the following day, they were exposed to DE (250 $\mu\text{g}/\text{m}^3$) or FA for 6 h and then sacrificed 21 days later. The images shown are representative micrographs of NeuN/BrdU immunohistochemistry in the OB of FA- and DE-exposed mice. Scale bars in detail images represent 10 μm . **a** Number of BrdU-positive cells indicates surviving cells that been born since the beginning of the experiment. Double-stained NeuN/BrdU cells indicate surviving adult-born neurons, which are expressed both as a total number per brain region (**b**), and as a percentage of all BrdU-positive cells (**c**). Data shown represent the mean ($\pm$ SE) with $n = 3$ per group. Significantly different from FA, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. *OB* olfactory bulb

Fig. 5.

Pioglitazone (PGZ) pre-treatment suppresses microglial activation in the hippocampus of male mice. Eight-week-old male C57BL/6J mice were pre-treated with 12.5 mg/kg/day PGZ or vehicle (VEH) for 4 days up to and including the day of exposure, and were then exposed to DE (250 $\mu\text{g}/\text{m}^3$) or FA for 6 h and then sacrificed 18 h later. Representative micrographs (on the right) of Iba1 immunohistochemistry of FA- and DE-exposed mice show microglia from the hippocampus, both as originally captured, and then as defined using tracing tool (inset) to allow for shape descriptor analysis using FIJI. Increases in area of the microglial soma (a) and aspect ratio (d) and decreases in circularity (b) and roundness (c) were indicators of microglial activation. Microglial soma area (a) is measured in μm^2 , circularity (b) is calculated as $4\pi(\text{area}/\text{perimeter}^2)$, roundness (c), a measure of inverse aspect ratio, is calculated by $R = 4 \cdot \text{area} / \pi(\text{major axis})^2$. Aspect ratio (d) is defined as major axis/minor axis. Results represent the mean ($\pm$ SE) with $n = 3$ per group. VEH/FA significantly different from VEH/DE; VEH/DE significantly different from PGZ/DE; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 6. Effect of pioglitazone on oxidative stress and cytokine expression. Lipid peroxidation (levels of malondialdehyde) and levels of TNF- α mRNA were assessed in the cerebral cortex (**a**, **c**), and the hippocampus (**b**, **d**) of male mice following 4 days of pre-treatment with either 12.5-mg/kg/day PGZ or with VEH, followed by a 6-h exposure to DE (250 $\mu\text{g}/\text{m}^3$) or FA. Results represent the mean ($\pm$ SE) with $n = 6$ per group. Significantly different from FA control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

Fig. 7.

Effect of PGZ pre-treatment on DE-induced inhibition of proliferation in the SGZ of male mice. Eight-week-old male C57BL/6J mice were pre-treated with 12.5-mg/kg/day PGZ or VEH for 4 days up to and including the day of exposure, and were then exposed to DE (250 $\mu\text{g}/\text{m}^3$) or FA for 6 h and then sacrificed 18 h later. Images shown are representative micrographs of Ki67+ cells in the hippocampus of FA- and DE-exposed mice, without and with PGZ pre-treatment. Significantly different from FA control, * $p < 0.05$

Fig. 8.

Effect of PGZ pre-treatment on DE-induced inhibition of adult hippocampal neurogenesis in male mice. Eight-week-old male C57BL/6J mice were pre-treated with 12.5-mg/kg/day PGZ or VEH for 4 days. On day 3 of pre-treatment, they were given five 100-mg/kg doses of BrdU at 2-h intervals. On day 4, mice were exposed to DE (250 $\mu\text{g}/\text{m}^3$) or FA for 6 h, then sacrificed 21 days later. Images shown are representative micrographs of NeuN/BrdU immunohistochemistry in the SGZ of FA- and DE-exposed mice. **a** BrdU+nuclei indicate surviving cells that been born since the beginning of the experiment. Double-stained NeuN/BrdU cells indicate surviving adult-born neurons, which are expressed both as a total number per brain region (**b**) and as a percentage of all BrdU-positive cells (**c**). Results represent the mean ($\pm$ SE) with $n = 3$ per group. Significantly different from FA control or between VEH-pre-treatment and PGZ pre-treatment, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$