Published in final edited form as:

Toxicology. 2016 December 30; 374: 1–9. doi:10.1016/j.tox.2016.11.010.

Sex and genetic differences in the effects of acute diesel exhaust exposure on inflammation and oxidative stress in mouse brain

Toby B. Cole^{1,2}, Jacki Coburn¹, Khoi Dao¹, Pam Roqué¹, Yu-Chi Chang¹, Vrinda Kalia³, Tomas R. Guilarte^{3,*}, Jennifer Dziedzic^{3,*}, and Lucio G. Costa^{1,4}

¹Dept. of Environmental and Occupational Health Sciences, School of Public Health, University of Washington, Seattle, WA, USA

²Center on Human Development and Disability, University of Washington, Seattle, WA, USA

³Dept. of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, NY, USA

⁴Dept. of Neuroscience, University of Parma, Italy

Abstract

In addition to increased morbidity and mortality caused by respiratory and cardiovascular diseases, air pollution may also contribute to central nervous system (CNS) diseases. Traffic-related air pollution is a major contributor to global air pollution, and diesel exhaust (DE) is its most important component. DE contains more than 40 toxic air pollutants and is a major constituent of ambient particulate matter (PM), particularly of ultrafine-PM. Limited information suggest that exposure to DE may cause oxidative stress and neuroinflammation in the CNS. We hypothesized that males may be more susceptible than females to DE neurotoxicity, because of a lower level of expression of paraoxonase 2 (PON2), an intracellular anti-oxidant and anti-inflammatory enzyme. Acute exposure of C57BL/6 mice to DE (250–300 µg/m³ for 6h) caused significant increases in lipid peroxidation and of pro-inflammatory cytokines (IL-1α, IL-1β, IL-3, IL-6, TNF-α) in various brain regions (particularly olfactory bulb and hippocampus). In a number of cases the observed effects were more pronounced in male than in female mice. DE exposure also caused microglia activation, as measured by increased Iba1 (ionized calcium-binding adapter molecule 1) expression, and of TSPO (translocator protein) binding. Mice heterozygotes for the modifier subunit of glutamate cysteine ligase (the limiting enzyme in glutathione biosynthesis; *Gclm*^{+/-} mice) appeared to be significantly more susceptible to DE-induced neuroinflammation than wild type mice. These findings indicate that acute exposure to DE causes neuroinflammation and

Correspondence: Dr. Lucio G. Costa, Dept. of Environmental and Occupational Health Sciences, University of Washington, 4225 Roosevelt, Suite No. 100, Seattle, WA 98105, Tel. 206 543-2831, Fax 206 685-4696, lgcosta@u.washington.edu.

*Present address: Dept. of Environmental and Occupational Health, Robert Stempel College of Public Health and Social Work, Florida International University, Miami, FL, USA.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

oxidative stress in brain, and suggests that sex and genetic background may play important roles in modulating susceptibility to DE neurotoxicity.

Keywords

Air pollution; Diesel exhaust; Neurotoxicity; Neuro-inflammation; Oxidative stress; Microglia activation; Sex differences; Genetic susceptibility

Introduction

Air pollution is a mixture comprised of several components, including ambient particulate matter (PM), gases, organic compounds, and metals. While the association between air pollution and morbidity and mortality caused by respiratory and cardiovascular diseases is well established (Brook and Rajagopalan, 2007; Gill et al., 2011), emerging evidence suggests that air pollution may also negatively affect the central nervous system (CNS) and contribute to CNS diseases (Calderon-Garciduenas et al., 2002; Genc et al., 2012; Costa et al., 2014a; 2016). Human epidemiological studies have shown that elevated air pollution is associated with decreased cognitive functions, olfactory and auditory deficits, and depressive symptoms, as well as increased incidence of neurodegenerative disease pathologies, i.e. increased beta-amyloid 42, phosphorylated tau, and alpha-synuclein (Calderon-Garciduenas et al., 2004; 2010; 2011; 2012; Ranft et al., 2009; Fonken et al., 2011; Power et al., 2011; Weuve et al., 2012; Guxens and Sunyer, 2012; Levesque et al., 2011). Among air pollution components, PM is believed to be the most widespread threat, and has been heavily implicated in disease (Brook et al., 2010; Moller et al., 2010; Costa et al. 2014a). PM is broadly characterized by aerodynamic diameter (e.g. PM₁₀ and PM_{2.5}, equivalent to <10 μ m and 2.5 μ m in diameter, respectively). Ultrafine particulate matter (UFP; <100 nm) is of much concern, as these particles can more easily enter the circulation and distribute to various organs, including the brain (Oberdoerster et al., 2002; 2004; Genc et al., 2012). Of most relevance is also the fact that UFP can access the brain through the nasal olfactory mucosa, reaching first the olfactory bulb (Oberdoerster et al., 2004; Peters et al., 2006; Genc et al., 2012; Lucchini et al., 2012). The populations of many countries (e.g. China, India, Middle East, Central America) are commonly exposed for extended periods to relatively high levels of PM (100 μ g/m³), and such concentration can be easily reached near roads with heavy traffic, and exceeded in certain occupational settings (van Donkelaar et al., 2015).

Oxidative stress and inflammation are the two cardinal processes by which air pollution is believed to exert its peripheral toxicity (Brook et al., 2010; Lodovici and Bigagli, 2011; Anderson et al., 2012), and the same seems to be true with regard to the CNS, as markers of oxidative stress and neuroinflammation are increased as a result of exposure to air pollution (Calderon-Garciduenas et al., 2008; Genc et al., 2012; Costa et al., 2016).

Traffic-related air pollution is a major contributor to global air pollution, and diesel exhaust (DE) is its most important component (Ghio et al., 2012). DE contains more than 40 toxic air pollutants and is a major constituent of ambient PM, particularly of UFP; DE exposure is often utilized as a measure of traffic-related air pollution. Few studies have examined controlled acute exposure of humans to DE; for example, acute exposure of humans to DE

(300 $\mu\text{g}/\text{m}^3$) has been shown to induce EEG changes (Cruts et al., 2008). Exposure of mice to DE has been reported to alter locomotor activity and spatial learning and memory (Yokota et al., 2009; Hougaard et al., 2009; Suzuki et al., 2010; Win-Shwe et al., 2008; 2014). Biochemical and molecular studies have evidenced that the most prominent effects of DE exposure on the CNS are oxidative stress and neuroinflammation (MohanKumar et al., 2008; Kraft and Harry, 2011; Win-Shwe and Fujimaki, 2011). Indeed, alterations in some oxidative stress-related genes and other markers of oxidative stress (Hartz et al., 2008; Tsukue et al., 2009; van Berlo et al., 2010) and increased markers of neuroinflammation (Levesque et al., 2011; Gerlofs-Nijland et al., 2010) have been found in rodents following DE exposure. *In vitro*, DE particles can activate microglia, and microglia-derived oxidant and/or inflammatory agents cause the demise of neurons (Block et al., 2004; Roqué et al. in preparation). Altogether, the available evidence suggests that exposure to traffic-related air pollution (and to DE as its major contributor) is associated with adverse CNS effects, with primary mechanisms related to induction of oxidative stress and to neuroinflammation.

Among the factors that can affect neurotoxic outcomes, sex, genetic background, and age are considered the most relevant (Costa et al., 2004; Tiffany-Castiglioni et al., 2005; Weiss, 2011). An aim of the present study was to investigate whether two of these variables (sex and genetic background) would modify susceptibility to DE neurotoxicity. The hypothesis of sex differences was primarily based on recent findings in our laboratory on the differential expression of the enzyme paraoxonase 2 (PON2) between males and females (Giordano et al. 2011; 2013; Costa et al. 2014b). In brain and in other tissues, PON2 was shown to exert antioxidant and anti-inflammatory effects (Ng et al., 2001; Horke et al., 2007; Giordano et al., 2011; Bourquard et al., 2011; Levy et al., 2007), and higher levels of PON2 are associated with decreased susceptibility to oxidative stress and neuroinflammation (Giordano et al. 2013; Costa et al. 2013). We found that females express higher levels of PON2 than males, possibly because this enzyme is modulated by estrogens (Giordano et al. 2013; Costa et al. 2014b). Thus the overall hypothesis was that males would be more susceptible than females to DE neurotoxicity because of a significantly lower level of PON2 expression in brain tissue.

As gene-environment interactions play an important role in toxicology (Costa and Eaton, 2006), we also investigated the possibility that genetic polymorphisms may affect susceptibility to air pollution-induced neurotoxicity. We hypothesized that genetically-based deficiencies in antioxidant defense mechanisms may exacerbate DE neurotoxicity. To test this hypothesis we utilized the *Gclm* mouse, which lacks the modifier subunit of glutamate-cysteine ligase, the first and rate-limiting enzyme in the synthesis of glutathione (GSH), a main player in cellular defense against oxidative stress. *Gclm*^{-/-} mice have very low levels of GSH in all tissues including the brain (Giordano et al. 2006), though they may up-regulate other antioxidant pathways; in contrast, *Gclm*^{+/-} mice have only moderate reductions in GSH but may more closely resemble an human polymorphism of *Gclm* (Nakamura et al. 2002). Interestingly, an enhanced lung inflammation has been observed in *Gclm*^{+/-} mice compared to wild-type mice upon exposure to DE (Weldy et al. 2012).

Materials and methods

Animals

Adult (3 month-old) male and female mice of C57Bl/6 strain background, purchased from Charles River Laboratories (Wilmington, MA) were used in most of these studies. Mice were housed in specific pathogen-free facilities with a 12-h dark–light cycle and unlimited access to food and water. Animals were randomly assigned to exposure to either filtered air (FA) or DE. In some experiments, *Gclm*-null (*Gclm*^{−/−}) mice of backcrossed C57Bl/6J (B6.129) strain background (Giordano et al. 2006; McConnachie et al. 2007) were utilized. Male and female mice hemizygous for the *Gclm* deletion (*Gclm*-Hz) were intercrossed, generating wild type, *Gclm*^{−/−}, and *Gclm*-Hz mice, in the expected Mendelian ratios. To genotype pups, genomic DNA was isolated from ear punch tissue using a Qiagen DNeasy kit, and mice were genotyped by PCR amplification of the wild type and disrupted *Gclm* alleles (i.e., amplification of β-geo), as previously described (Giordano et al. 2006; McConnachie et al. 2007). As seen previously, all pups developed normally and exhibited no differences in phenotypic landmarks compared to wild type littermates. The number of mice in each experimental group ranged from three to six. The animal use protocols used were approved by the Institutional Animal Care and Use Committee at the University of Washington. All animal 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.

In vivo exposure of mice 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 under SPF conditions in our diesel exposure facility (Gould et al., 2008; Fox et al., 2015), using an Allentown caging system (Allentown, NJ) with racks modified to receive either diluted DE or FA through their air intakes. DE was derived as described (Gould et al., 2008; Fox et al., 2015), 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. During exposures, DE concentrations were continuously measured and maintained at steady concentrations using a feedback controller monitoring fine particulate levels (Gould et al., 2008; Fox et al., 2015). DE was composed of PM_{2.5} or smaller, with a mean aerodynamic diameter of 100 nm. The DE characteristics have been described in detail previously (Fox et al., 2015). For assays of lipid peroxidation, inflammatory cytokine levels and Western blots, 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 immunohistochemistry and measurement of TSPO binding, mice were euthanized 18 h after the end of exposure.

Assessment of lipid peroxidation

Frozen brain-region samples were thawed and homogenized in CLB lysis buffer (10 mM HEPES; 150 mM 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 then incubated on ice for 10-min, centrifuged at 4°C and 2000×g for 5 min, and aliquots of the supernatant were stored

at -80°C until assay. 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., 2006).

Luminex quantification of cytokines

Levels of neuroinflammatory cytokines were measured using the MILLIPLEX MAP system (Merck-Millipore, Billerica MA), which uses Luminex xMAP technology for simultaneous quantification of the levels of multiple cytokines (Datta and Opp, 2008). Brain tissue was disrupted in lysis buffer (10 mM HEPES / 150 mM NaCl / 1 mM CaCl_2 / 0.5 mM MgCl_2 / 1 mM PMSF / 50 mM NaF). The homogenate was then shaken for 30–40 min on ice and centrifuged at 4°C and $6000 \times g$ for 5 min. The supernatant was removed and aliquoted. Aliquots were stored at -80°C until assay. The protein content of each sample was determined using the bicinchoninic acid (BCA) assay with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol. The assays were run according to instructions included with the Millipore cytokine kit (IL-1a, IL-1b, IL-3, IL-6, IL-9, and TNF α ; Cat # MYCOTMAG-70K-06). Each brain lysate sample was measured in triplicate. After completion of all steps in the assay, the plates (96 well plate format) were read in the MagPix xMAP system (Millipore) and the data was analyzed using xPONENT 4.2 software that is part of the MAGPIX System.

Measurement of Iba1 by Western blot and Iba1 immunocytochemistry

Frozen brain tissue was homogenized in Tris-Triton buffer (10 mM Tris, pH 7.4; 100 mM NaCl; 1 mM EDTA; 1 mM EGTA; 1% Triton X-100; 10% glycerol; 0.1% SDS; 0.5% deoxycholate) using 12 strokes in a glass Potter-Elvehjem homogenizer, incubated on ice 10-min, and centrifuged for 10 min at $2,000 \times g$ at 4°C . The protein content of each sample was determined by the BCA assay, with BSA as a standard. After SDS-PAGE (using 30 μg of protein per well), proteins were electro-transferred (1 h at 100 V) onto a polyvinylidene difluoride immunoblot membrane (PVDF, 0.45 μm) using a transblot apparatus in Tris-Triton buffer. The membranes were blocked by incubation with 5% (w/v) nonfat dried milk powder dissolved in $1 \times$ Tris-buffered saline, pH 7.5 (Bio-Rad Laboratories, Hercules, CA) containing 0.05% v/v Tween 20 for 1 h and incubated overnight with a 1:250 dilution of polyclonal rabbit anti-Iba1 antibody (Catalog No. 016-20001; Wako Chemicals, Richmond, VA) or 1:1000 dilution of mouse monoclonal anti- β -actin antibody (Catalog No. 554022; BD Pharmingen, San Jose, CA) for 1 h. Blots were rinsed in Tris-buffered saline, blocked for 1 h, and incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (Catalog No. 7074; Cell Signaling Technology, Danvers, MA) at a dilution of 1:750. The membranes were developed with a chemiluminescent substrate (ECL kit from Thermo Scientific, Waltham, MA). Band intensity was measured by densitometry using ImageJ (provided by the National Institutes of Health), and the intensity of the bands was normalized to β -actin content.

For Iba1 immunohistochemistry mice were perfused transcardially with saline and 4% paraformaldehyde (PFA) in PBS, using a Minipuls 2 peristaltic pump (Gilson, Middleton, Wisconsin) to achieve thorough fixation of tissues. Brains were placed intact in ice-cold 4% PFA in PBS, post-fixed overnight at 4°C, and embedded individually using O.C.T. Compound Embedding Medium (Sakura Finetek, Torrance, California). Coronal sections of the hippocampal region were taken at a thickness of 30 µm using a Reichert-Jung Cryocut-1800 cryostat (Leica, Wetzlar, Germany). Sections were placed in 24-well plates in a cryoprotectant medium composed of 30% ethylene glycol, 30% glycerol, and 40% PBS and stored at -20°C. After further washings, the sections were permeabilized with PBS containing 0.25% Triton X-100 (PBST), blocked in a buffer containing 1% BSA (w/v) and 10% normal serum (v/v) in PBS, then incubated with a polyclonal goat anti-Iba1 antibody (ab107159, Abcam, Cambridge, Massachusetts). Sections were then washed four times in PBST, and then incubated with AlexaFluor 568 donkey anti-goat IgG secondary antibody in a buffer containing 2% donkey serum and 1% BSA. Nuclei were stained with 30 min incubation with 2.5 µg/mL Hoechst 33342 (Molecular Probes, Eugene, Oregon). VectaShield Fluorescence Mounting Medium (VectaShield, Burlingame, California) was used to mount the sections and prevent photobleaching. Images were captured 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. Images were analyzed using Fiji (ImageJ) open source software. Cross-sectional area of microglia was measured on the plane at which resolution of the nucleus was sharpest. The irregular drawing tool was used to determine the borders of each microglial soma. Area (µm²) and circularity (a measure of inverse aspect ratio) of each soma were measured and recorded. Circularity was calculated using Fiji software as $C=4\pi \cdot \text{Area}/(\text{Perimeter})^2$.

Measurement of TSPO binding

TSPO binding was measured as described by Loth et al. (2016). Briefly, fresh-frozen brains were sectioned (20 µm) on a freezing cryostat in the horizontal plane. Brain sections were thaw-mounted onto poly-L-lysine-coated slides (Sigma) and stored at -80°C until used. [3H]-DPA-713 autoradiography to measure TSPO levels was performed on adjacent brain sections using the following procedures. Slides were thawed and dried at 37°C for 30 min and prewashed in 50 mM Tris-HCl buffer (pH 7.4) for 5 min at room temperature. Sections were then incubated in 1 nM [3H]-DPA-713 in 50 mM Tris-HCl buffer for 30 min at room temperature. For non-specific binding, adjacent sections were incubated in the presence of 10 µM racemic PK11195. The reaction was terminated by two 3-min washes in cold buffer (4°C) and two dips in cold deionized water (4°C). Sections were apposed to Kodak Bio-Max MR films with tritium microscopes for 4 weeks (GE Healthcare, Piscataway, NJ). Images were acquired and quantified using the MCID software (InterFocus Imaging Ltd., Cambridge, England).

Statistical analysis

All data are presented as means ± standard error. Results were analyzed by one-way ANOVA with Bonferroni correction for multiple comparisons.

Results

Adult mice exposed to 250–300 $\mu\text{g}/\text{m}^3$ DE for 6-h did not exhibit any overt signs of toxicity, and were similar to the mice exposed concurrently to FA with respect to activity, alertness and general health. Body weights were unaffected by the exposure. At the end of the exposure, a number of pro-inflammatory cytokines (IL-1 α , IL-1 β , IL-3, IL-6, TNF- α) were measured in the olfactory bulb and the hippocampus. Microglia activation was also assessed by measuring Iba1 (by Western blot and immunocytochemistry) and by TSPO binding. Furthermore, oxidative stress was assessed in brain regions by measuring lipid peroxidation.

Levels of pro-inflammatory cytokines, as well as the anti-apoptotic cytokine IL-9, were measured in two brain regions (olfactory bulb and hippocampus) following exposure to DE or FA, using a multiplexed Luminex assay. Significant increases in the levels of all of the pro-inflammatory cytokines tested (IL-1 α , IL-1 β , IL-3, IL-6, and TNF α) were found (Table 1). In a number of cases the effects were greater in males than in females. For example, the effect of DE exposure on IL-6 levels was particularly striking, with a 17-fold increase in both areas of male mice within 2 h after the end of the exposure. In contrast, in female mice, the increases of IL-6 levels were 12-fold and 8-fold in olfactory bulb and hippocampus, respectively. Similarly, in males IL-3 levels were increased by 11-fold in both brain regions, compared to 3-fold in females (Table 1). Levels of the anti-apoptotic cytokine IL-9 were decreased by about 40–50% in both brain regions, with no significant sex differences (Table 1).

Because the production of neuroinflammatory cytokines is one of the hallmarks of microglial activation, and ongoing *in vitro* studies in our laboratory indicate an important role for microglia in the mechanisms of toxicity of DE particles (Roqué et al., 2016), microglia activation upon DE exposure was also assessed. Figures 1 and 2 show that the expression of Iba1, a marker of microglia activation, is increased in both the olfactory bulb and the hippocampus. Iba1 levels, quantified by Western blot and expressed relative to β -actin, were increased by 40- to 50% in both brain regions of male and female mice exposed acutely to DE, as compared to the FA-exposed controls (Fig. 1). The increases in Iba1 observed by Western blot were associated with phenotypic changes in microglia measured by immunohistochemistry in the dentate gyrus region of the hippocampus (Fig. 2). In the dentate gyrus of DE-exposed mice, microglia showed morphological changes associated with activation, with apparent decreases in branching accompanied by increased area of the soma and decreased circularity. Quantification of soma area and circularity of microglia within the dentate gyrus revealed an increase (by 25%) in soma area, and a decrease (by 10%) in circularity (Fig. 2).

To further assess microglial activation, TSPO autoradiography was also utilized (Fig. 3). TSPO (translocator protein 18 kD) is a biomarker of neuroinflammation produced in microglia and astrocytes. As in other experiments mice were exposed acutely (6 h) to DE or FA, and the sacrificed 18 h later; autoradiography was performed on horizontal sections, using [3H]-DPA 713 as the radiolabeled ligand to quantify TSPO levels. Regions analyzed included limbic cortex, orbital cortex, somatosensory cortex, entorhinal cortex, caudate putamen, thalamus, hippocampus, periaqueductal grey, and cerebellum. Each region was

analyzed on the right and left hemisphere. Figure 3 shows a representative image of a horizontal brain section illustrating the regions analyzed for TSPO autoradiography. There was a tendency towards increased TSPO in the cerebellum of males ($p=0.064$) and females ($p=0.126$), as well as increased TSPO in multiple cortical regions in males (limbic cortex, $p=0.278$; orbital cortex, $p=0.053$; somatosensory cortex, $p=0.059$; entorhinal cortex, $p=0.416$), the increases were not statistically significant (Fig. 3). Overall, female mice displayed a slightly lesser increase in TSPO binding compared to males.

In an additional experiment we compared the effect of acute DE exposure on cytokine levels in the olfactory bulb and the hippocampus of *Gclm*^{+/+}, *Gclm*^{+/-} and of *Gclm*^{-/-} mice, focusing on IL-3 and IL-6 which showed the greater increases upon DE exposure, and on male mice, which appeared to be more affected than females (Table 1). The underlying hypothesis was that *Gclm*-heterozygous and *Gclm*-null mice would be more susceptible to neuroinflammation caused by acute DE exposure. Results shown in Table 2 indicate that DE exerts a stronger effect on IL-3 and IL-6 in *Gclm*^{-/-} mice compared to wild-type mice, and the effects were even greater in *Gclm*^{+/-} mice.

As air pollution in general, and DE exposure specifically, have been reported to cause oxidative stress in brain, we also measured lipid peroxidation in several brain regions of male and female wild-type mice, following a 6 h exposure to DE (250–300 $\mu\text{g}/\text{m}^3$). As shown in Table 3, lipid peroxidation, measured by quantifying the levels of MDA, was increased in all brain regions analyzed (hippocampus, striatum, cerebral cortex, cerebellum, and olfactory bulb), and in some regions it was more pronounced in male than in female mice.

Discussion

The results obtained in this study provide confirmation that exposure to DE causes neuroinflammation and increase in oxidative stress, and suggests that sex and genetic determinants may influence these neurotoxic effects. In wild-type mice, even a short (6 h) exposure to moderate (250–300 $\mu\text{g}/\text{m}^3$) levels of DE caused significant microglia activation, neuroinflammation and oxidative stress. Such levels of $\text{PM}_{2.5}$ can often be reached, and even exceeded for extended periods, in several cities worldwide, particularly in India or China (Costa et al. 2016). In addition, as diesel engines power a wide range of vehicles, heavy equipment and other machinery utilized in numerous industries, occupational exposure are common and can often exceed 200–300 $\mu\text{g}/\text{m}^3$ in bus garage, construction and dock workers, with miners experiencing the highest exposures of up to 1000 $\mu\text{g}/\text{m}^3$ (Pronk et al. 2009; Costa et al. 2016). Acute exposure to DE caused increases in all measured pro-inflammatory cytokines in olfactory bulb and hippocampus (Table 1). Of interest is that in some cases, the increases were more pronounced in brains from male than female mice. Oxidative stress, measured as lipid peroxidation in several brain regions (olfactory bulb, hippocampus, cerebellum, cerebral cortex, and striatum) was also increased, and in some cases the increases were higher in male mice (Table 3).

We had formulated the hypothesis of a possible higher susceptibility of male mice on the basis of a series of recent findings related to the expression of the intracellular enzyme

PON2. PON2 has antioxidant and anti-inflammatory properties (Ng et al. 2001; Levy et al. 2007; Altenhofer et al. 2010; Giordano et al. 2011; Schweikert et al. 2012) and for these reasons it has been suggested to play an important role in neuroprotection (Costa et al. 2014b). Indeed the absence of PON2, as in $PON2^{-/-}$ mice was found to be associated with higher susceptibility to neurotoxicity (Giordano et al. 2011; 2013; Costa et al. 2013). We also found that there is a sex differences in PON2 expression, with females (mice, rats, humans, non-human primates) displaying higher PON2 mRNA, protein and activity levels (Giordano et al. 2013; Costa et al. 2013; Garrick et al. 2016). This appears to be due by the ability of estrogens to stimulate PON2 expression, as seen *in vitro* and also *in vivo* in ovariectomized mice (Giordano et al. 2013). *In vitro* studies have shown that cells (neurons, astrocytes, and microglia) from male mice are more susceptible to neurotoxicity than cells from female mice (Giordano et al. 2011; 2013). Results from the present study would provide initial evidence to extend these finding to *in vivo* exposure to DE. Understandably, additional mechanisms may be involved in a differential susceptibility of male and female mice to DE neurotoxicity, and these should be further investigated. For example, one may hypothesize that DE exposure in $PON2^{-/-}$ mice would provide evidence of an overall increased susceptibility to oxidative stress and neuroinflammation in both sexes (compared to wild-type animals), and of an absence of sex differences. An important additional aspect to address would be that of determining the estrous cycle of mice at the time of exposure, as well as PON2 expression and estrogen levels in each animal. Of broader interest is also the fact that neuroinflammation and oxidative stress are involved in the etiopathology of various neurodevelopmental (El-Ansary and Al-Ayadi, 2012; Napoli et al. 2013; Hanamsagar and Bilbo, 2016) and neurodegenerative diseases (Teeling and Perry, 2009; Lee et al., 2010; Qian et al., 2010). Developmental exposure to traffic-related air pollution has been reported to be associated with autism spectrum disorders in humans and in experimental animals (Volk et al. 2013; Roberts et al. 2013; Thirtamara Rajamani et al. 2013; Allen et al. 2014a; 2014b; Chang et al. 2016). Furthermore, markers of neurodegenerative diseases appear to be increased upon air pollution exposure (Levesque et al. 2011; Calderon-Garciduenas et al., 2004; 2012; 2016; Wu et al. 2015). These findings thus suggest that age is a third important determinant which may modulate susceptibility to air pollution neurotoxicity. Of much interest is also the fact that both neurodevelopmental (e.g. autism spectrum disorders) and neurodegenerative (e.g. Parkinson's disease) diseases are more prevalent in males than in females, underscoring the importance of sex in modulating susceptibility.

To test the hypothesis that genetic background may modulate the effect of DE on neuroinflammation, we utilized *Gclm* transgenic mice. *Gclm*^{-/-} mice have very low levels of GSH in all tissues including the brain (Giordano et al. 2006), though they may up-regulate other antioxidant pathways; in contrast, *Gclm*^{+/-} mice have only moderate reductions in GSH but may more closely resemble an human polymorphism of *Gclm* (Nakamura et al. 2002). We had previously shown that cells derived from these mice were more susceptible to the toxicity of neurotoxicants causing oxidative stress (Giordano et al. 2006). Interestingly, an enhanced lung inflammation had also been observed in *Gclm*^{+/-} mice compared to wild-type mice, upon exposure to DE (Weldy et al. 2012). Findings of the present study in male *Gclm* transgenic mice indicate that levels of neuroinflammatory cytokines were increased by acute DE exposure in all three genotypes, and that the largest increases were seen in *Gclm*^{+/-}

mice, particularly for IL-6 (Table 2). For example, when comparing the most susceptible sex/genotype (male *Gclm*^{+/-} mice) to the least susceptible (female wild-type mice) the levels of IL-6 in hippocampus upon acute DE exposure were 314 and ~10 pg/ml, respectively, a 30-fold difference (Tables 1 and 2)

In summary, acute exposure to moderate levels of DE has been shown to induce neurotoxic effects in mice, as evidenced by increased oxidative stress and increased levels of pro-inflammatory cytokines in various brain regions. Of note are the following novel observations: 1) A single short-term (6 h) exposure to DE is sufficient to elicit significant increases in neuroinflammation and oxidative stress; 2) Males appear to be more susceptible to some of these effects, possibly because of lower expression of PON2; 3) Genetic polymorphisms which diminish the ability to cope with oxidative stress may also increase susceptibility to DE-induced neuroinflammation. These findings underscore the importance of considering both sexes when investigating neurotoxicity, and the fact that certain genetic polymorphisms may increase susceptibility to neurotoxic effects of traffic-related air pollution. Ongoing studies in our laboratory are examining a third variable that appears to modulate air pollution neurotoxicity, i.e. age. Further studies should also investigate the effects of prolonged exposure to DE, the reversibility of the observed effects upon cessation of exposure, and possible therapeutic interventions to mitigate DE neurotoxicity.

Acknowledgments

Research by the authors is supported by grants from NIEHS (R01ES22949, P30ES07033, P42ES04696, T32ES07032, R01ES007062), NICHD (U54HD083091) and by funds by the Department of Environmental and Occupational Health Sciences, University of Washington. We thank Drs. Gennaro Giordano, Anna Engstrom, and Chad Weldy for help in some pilot experiments, Dr. Terrance Kavanagh for initially providing the *Gclm* mice, and Dr. Theo Bammler from the Functional Genomics, Proteomics and Metabolomics Facility at the University of Washington for cytokine measurements.

References

- Allen JL, Liu X, Weston D, Prince L, Oberdorster G, Finkelstein JN, Johnston CJ, Cory-Slechta DA. Developmental exposure to concentrated ambient ultrafine particulate matter air pollution in mice results in persistent and sex dependent behavioral neurotoxicity and glial activation. *Toxicol. Sci.* 2014a; 140:160–178. [PubMed: 24690596]
- Allen JL, Liu X, Pelkowski S, Palmer B, Conrad K, Oberdorster G, Weston D, Mayer-Proschel M, Cory-Slechta D. Early postnatal exposure to ultrafine particulate matter air pollution: persistent ventriculomegaly, neurochemical disruption, and glial activation preferentially in male mice. *Environ. Health Perspect.* 2014b; 122:939–945. [PubMed: 24901756]
- Altenhofer S, Witte I, Teiber JF, Wilgenbus P, Pautz A, Li H, Daiber A, Witan H, Clement AM, Forstermann U, Horke S. One enzyme, two functions. PON2 prevents mitochondrial superoxide formation and apoptosis independent from its lactonase activity. *J. Biol. Chem.* 2010; 285:24398–24403. [PubMed: 20530481]
- Anderson JO, Thundiyil JG, Stolbach A. Clearing the air: a review of the effects of particulate matter air pollution on human health. *J. Med. Toxicol.* 2012; 8:166–175. [PubMed: 22194192]
- Block ML, Wu X, Pei Z, Li G, Wang T, Qin L, Wilson B, Yang J, Hong JS, Veronesi B. Nanometer size diesel exhaust particles are selectively toxic to dopaminergic neurons: the role of microglia, phagocytosis, and NADPH oxidase. *FASEB J.* 2004; 18:1618–1620. [PubMed: 15319363]
- Bourquard N, Ng CJ, Reddy ST. Impaired hepatic insulin signaling in PON2-deficient mice: a novel role for the PON2/apoE axis on the macrophage inflammatory response. *Biochem. J.* 2011; 436:91–100. [PubMed: 21361875]

- Brook RD, Rajagopalan S. Air pollution and cardiovascular events. *New Engl. J. Med.* 2007; 356:2104–2105.
- Brook RD, Rajagopalan S, Pope A, Brook JR, Bhatnagar A, Diez-Roux AV, Holguin F, Hong Y, Luepker RV, Mittleman MA, Peters A, Siscovick D, Smith SC, Whitese L, Kaufman JD. on behalf of the American Heart Association Council on Epidemiology and Prevention, Council on the Kidney in Cardiovascular disease, and Council on Nutrition, Physical Activity and Metabolism. Particulate matter air pollution and cardiovascular disease. An update to the scientific statement from the American Heart Association. *Circulation.* 2010; 121:2331–2378. [PubMed: 20458016]
- Calderon-Garciduenas L, Azzarelli B, Acuna H, Garcia R, Gambling TM, Osnaya N, Monroy S, Del Rosario Tizapantzi M, Carson JL, Villareal-Calderon A, Rewcastle B. Air pollution and brain damage. *Toxicol. Pathol.* 2002; 30:373–389. [PubMed: 12051555]
- Calderon-Garciduenas L, Reed W, Maronpot RR, Henriquez-Roldan C, Delgado-Chavez, Calderon-Garciduenas A, Dragustinovis I, Franco-Lira M, Aragon-Flores M, Solt AC, Altenburg M, Torres-Jardon R, Swenberg JA. Brain inflammation and Alzheimer's-like pathology in individuals exposed to severe air pollution. *Toxicol. Pathol.* 2004; 32:650–658. [PubMed: 15513908]
- Calderon-Garciduenas L, Solt AC, Henriquez-Roldan C, Torres-Jardon R, Nuse B, Herritt L, Villareal-Calderon R, Osnaya N, Stone I, Garcia R, Brooks DM, et al. Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. *Toxicol. Pathol.* 2008; 36:289–310. [PubMed: 18349428]
- Calderon-Garciduenas L, Franco-Lira M, Henriquez-Roldan C, Osnaya N, Gomzalez-Maciel A, Reynoso-Robles R, Villareal-Calderon R, Herritt L, Brooks D, Keefe S, Palacios-Moreno J, Villareal-Calderon R, Torres-Jardon R, Medina-Cortina H, Delgado-Chavez R, Aiello-Mora M, Maronpot RR, Doty RL. Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. *Exp. Toxicol. Pathol.* 2010; 62:91–102. [PubMed: 19297138]
- Calderon-Garciduenas L, Engle R, Mora-Tiscareno A, Styner M, Gomez-Garza G, Zhu H, Jewells V, Torres-Jardon R, Romero L, Monroy-Acosta ME, Bryant C, Gonzalez-Gonzalez LO, Median-Cortina H, D'Angiulli A. Exposure to severe urban air pollution influences cognitive outcomes, brain volume and systemic inflammation in clinically healthy children. *Brain Cognition.* 2011; 77:345–355. [PubMed: 22032805]
- Calderon-Garciduenas L, Kavanaugh M, Block M, D'Angiulli A, Delgado-Chavez R, Torres-Jardon R, Gonzales-Maciel A, et al. Neuroinflammation, hyperphosphorylated tau, diffuse amyloid plaques, and down-regulation of the cellular prion protein in air pollution exposed children and young adults. *J. Alzheim. Dis.* 2012; 28:93–107.
- Calderon-Garciduenas L, Reynoso-Robles R, Vargas-Martinez J, Gomez-Maqueo-Chew A, Perez-Guille B, Mukherjee PS, Torres-Jardon R, Perry G, Gonzalez-Maciel A. Prefrontal white matter pathology in air pollution exposed Mexico City young urbanites and their potential impact on neurovascular unit dysfunction and the development of Alzheimer's disease. *Environ. Res.* 2016; 146:404–417. [PubMed: 26829765]
- Chang Y, Kalata E, Cole TB, Costa LG. Developmental exposure to diesel exhaust induces autism-related behavioral phenotype in mice. *Toxicologist.* 2016 #1169 (Abstract).
- Costa LG, Aschner M, Vitalone A, Syversen T, Porat-Soldin O. Developmental neuropathology of environmental agents. *Annu. Rev. Pharmacol. Toxicol.* 2004; 44:87–110. [PubMed: 14744240]
- Costa, LG.; Eaton, DL., editors. *Gene-Environment Interactions: Fundamentals of Ecogenetics.* Hoboken, NJ: John Wiley & Sons; 2006. p. 557
- Costa LG, Tait L, de Laat R, Dao K, Giordano G, Pellacani C, Cole TB, Furlong CE. Modulation of paraoxonase 2 (PON2) in mouse brain by the polyphenol quercetin; a mechanism of neuroprotection? *Neurochem. Res.* 2013; 38:1809–1818. [PubMed: 23743621]
- Costa LG, Cole TB, Coburn J, Chang YC, Dao K, Roque P. Neurotoxicants are in the air: convergence of human and in vitro studies on the effects of air pollution on the brain. *Bio Med. Res. Int.* 2014a; 8. ID 736385.
- Costa LG, de Laat R, Dao K, Pellacani C, Cole TB, Furlong CE. Paraoxonase-2 (PON2) in brain and its potential role in neuroprotection. *Neurotoxicology.* 2014b; 43:3–9. [PubMed: 24012887]
- Costa LG, Cole TB, Coburn J, Chang YC, Dao K, Roque P. Neurotoxicity of traffic-related air pollution. *Neurotoxicology.* 2016 (in press).

- Crüts B, Driessen A, van Etten L, et al. Exposure to diesel exhaust induces changes in EEG I human volunteers. *Particle Fibre Toxicol.* 2008; 5 art. 4.
- Datta SC, Opp MR. Lipopolysaccharide-induced increases in cytokines in discrete mouse brain regions are detectable using Luminex xMAP technology. *J Neurosci Methods.* 2008 Oct 30;175:119–124. [PubMed: 18771691]
- El-Ansary A, Al-Ayadhi L. Neuroinflammation in autism spectrum disorders. *J. Neuroinflammat.* 2012; 9:265.
- Fonken LK, Xu X, Weil ZM, Chen G, Sun Q, Rajagopalan S, Nelson RJ. Air pollution impairs cognition, provokes depressive-like behaviors and alters hippocampal cytokine expression and morphology. *Mol. Psych.* 2011; 16:987–995.
- Fox JR, Cox DP, Drury BE, Gould TR, Kavanagh TJ, Paulsen MH, Sheppard L, Simson CD, Stewart JA, Larson TV, Kaufman JD. Chemical characterization and in vitro toxicity of diesel exhaust particulate matter generated under varying conditions. *Air Qual. Atmos. Health.* 2015; 8:507–519. [PubMed: 26539254]
- Garrick JM, Dao K, de Laat R, Elsworth J, Cole TB, Marsillach J, Furlong CE, Costa LG. Developmental expression of paraoxonase 2. *Chem Biol. Interact.* 2016 (in press).
- Genc S, Zadeoglulari Z, Fuss SH, Genc K. The adverse effects of air pollution on the nervous system. *J. Toxicol.* 2012; ID 782462:23.
- Gerlofs-Nijland ME, van Berlo D, Cassee FR, Schins RPF, Wang K, Campbell A. Effect of prolonged exposure to diesel engine exhaust on proinflammatory markers in different regions of the rat brain. *Particle Fibre Toxicol.* 2010; 7:12.
- Ghio AJ, Smith CB, Madden MC. Diesel exhaust particles and airway inflammation. *Curr. Op. Pulm. Med.* 2012; 18:144–150.
- Gill EA, Curl CL, Adar SD, Allen RW, Auchincloss AH, O'Neill MS, Park SK, Ven Hee VC, Diez Roux AV, Kaufman JD. Air pollution and cardiovascular disease in the Multi-Ethnic Study of Atherosclerosis. *Progr. Cardiovasc. Res.* 2011; 53:353–360.
- Giordano G, White CC, McConnachie LA, Fernandez C, Kavanagh TJ, Costa LG. Neurotoxicity of domoic acid in cerebellar granule neurons in a genetic model of glutathione deficiency. *Mol. Pharmacol.* 2006; 70:2116–2126. [PubMed: 17000861]
- Giordano G, Cole TB, Furlong CE, Costa LG. Paraoxonase 2 (PON2) in the mouse central nervous system: a neuroprotective role? *Toxicol. Appl. Pharmacol.* 2011; 256:369–378. [PubMed: 21354197]
- Giordano G, Tait L, Furlong CE, Cole TB, Kavanagh TJ, Costa LG. Gender differences in brain susceptibility to oxidative stress are mediated by levels of paraoxonase-2 (PON2) expression. *Free Rad. Biol. Med.* 2013; 58:98–108. [PubMed: 23376469]
- Gould T, Larson T, Stewart J, Kaufman JD, Slater D, McEwen N. A controlled inhalation diesel exhaust exposure facility with dynamic feedback control of PM concentration. *Inhal. Toxicol.* 2008; 20:49–52. [PubMed: 18236222]
- Guxens M, Sunyer J. A review of epidemiological studies on neuropsychological effects of air pollution. *Swiss Med. Wkly.* 2012; 141:w13322. [PubMed: 22252905]
- Hanamsagar R, Bilbo SD. Sex differences in neurodevelopmental and neurodegenerative disorders: focus on microglial function and neuroinflammation during development. *J. Steroid Biochem. Mol. Biol.* 2016; 160:127–133. [PubMed: 26435451]
- Hartz AMS, Bauer B, Block ML, Hong JS, Miller DS. Diesel exhaust particles induce oxidative stress, proinflammatory signaling, and P-glycoprotein up-regulation at the blood-brain barrier. *FASEB J.* 2008; 22:2723–2733. [PubMed: 18474546]
- Horke S, Witte I, Wilgenbus P, Kruger M, Starnd D, Forstermann U. Paraoxonase-2 reduces oxidative stress in vascular cells and decreases endoplasmic reticulum stress-induced caspase activation. *Circulation.* 2007; 115:2055–2064. [PubMed: 17404154]
- Hougaard KS, Saber AT, Jensen KA, Vogel U, Wallin H. Diesel exhaust particles: effects on neurofunction in female mice. *Basic Clin. Pharmacol. Toxicol.* 2009; 105:139–143. [PubMed: 19389041]
- Kraft AD, Harry GJ. Features of microglia and neuroinflammation relevant to environmental exposure and neurotoxicity. *Int. J. Environ. Res. Public Health.* 2011; 8:2980–3018. [PubMed: 21845170]

- Lee YJ, Han SB, Nam SY, Oh KW, Hong JT. Inflammation and Alzheimer's disease. *Arch Pharm. Res.* 2010; 33:1539–1556. [PubMed: 21052932]
- Levesque S, Surace MJ, McDonald J, Block ML. Air pollution and the brain: subchronic diesel exhaust exposure causes neuroinflammation and elevates early markers of neurodegenerative disease. *J. Neuroinflammat.* 2011; 8:105.
- Levy E, Trudel K, Bendayan M, Seidman E, Delvin E, Elchebly M, Lavoie JC, Precourt LP, Amre D, Sinnett D. Biological role, protein expression, subcellular localization, and oxidative stress response of paraoxonase 2 in the intestine of human and rats. *Am. J. Physiol. Gastrointest. Liver Physiol.* 2007; 293:G1252–G1261. [PubMed: 17916643]
- Lodovici M, Bigagli E. Oxidative stress and air pollution exposure. *J. Toxicol.* 2011 Article ID 487074.
- Loth MK, Choi J, McGlothlan JL, Pletnikov MV, Pomper MG, Guilarte TR. TSPO in a murine model of Sandhoff disease: presymptomatic marker of neurodegeneration and disease pathophysiology. *Neurobiol. Dis.* 2016; 85:174–186. [PubMed: 26545928]
- Lucchini RG, Dorman DC, Elder A, Veronesi B. Neurological impacts from inhalation of pollutants and the nose-brain connection. *Neurotoxicology.* 2012; 33:838–841. [PubMed: 22178536]
- McConnachie LA, Mohar I, Hudson FN, Ware CB, Ladiges WC, Fernandez C, Chatterton-Kirchmeier S, White CC, Pierce RH, Kavanagh TJ. Glutamate cysteine ligase modifier subunit deficiency and gender as determinants of acetaminophen-induced hepatotoxicity in mice. *Toxicol. Sci.* 2007; 99:628–636. [PubMed: 17584759]
- MohanKumar SMJ, Campbell A, Block M, Veronesi B. Particulate matter, oxidative stress and neurotoxicity. *Neurotoxicology.* 2008; 29:479–488. [PubMed: 18289684]
- Moller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, Vesterdal LK, Forchhammer L, Wallin H, Loft S. Role of oxidative damage in toxicity of particulates. *Free Rad. Res.* 2010; 44:1–46.
- Nakamura SI, Kugiyama K, Sugiyama S, et al. Polymorphism in the 5'-flanking region of human glutamate-cysteine ligase modifier subunit gene is associated with myocardial infarction. *Circulation.* 2002; 105:2968–2973. [PubMed: 12081989]
- Napoli E, Wong S, Giulivi C. Evidence of reactive oxygen species-mediated damage to mitochondrial DNA in children with typical autism. *Mol. Autism.* 2013; 4:2. [PubMed: 23347615]
- Ng CJ, Wadleigh DJ, Gangopadhyay A, Hama S, Grijalva VR, Navab M, Fogelman AM, Reddy ST. Paraonase-2 is a ubiquitously expressed protein with antioxidant properties and is capable of preventing cell-mediated oxidative modification of low density lipoprotein. *J. Biol. Chem.* 2001; 276:44444–44449. [PubMed: 11579088]
- Oberdoerster G, Sharp Z, Atudorei V, Elder A, Gelein R, Lunts A, Kreyling W, Cox C. Extra-pulmonary translocation of ultrafine carbon particles following whole body inhalation exposure of rats. *J. Toxicol. Environ. Health A.* 2002; 65:1531–1543. [PubMed: 12396867]
- Oberdoerster G, Sharp Z, Atudorei V, Elder A, Gelein R, Kreyling W, Cox C. Translocation of inhaled ultrafine particles to the brain. *Inhal. Toxicol.* 2004; 16:437–445. [PubMed: 15204759]
- Peters A, Veronesi B, Calderon-Garciduenas L, Gehr P, Chen LC, Geiser M, Reed W, Rothen-Rutishauser B, Schurch S, Schulz H. Translocation and potential neurological effects of fine and ultrafine particles a critical update. *Particle Fibre Toxicol.* 2006; 3:13.
- Power MC, Weisskopf MG, Alexeeff SE, Coull BA, Avron S III, Schwartz J. Traffic-related air pollution and cognitive function in a cohort of older men. *Environ. Health Perspect.* 2011; 119:682–687. [PubMed: 21172758]
- Pronk A, Coble J, Stewart PA. Occupational exposure to diesel engine exhaust; a literature review. *J. Exp. Sci. Environ. Epidemiol.* 2009; 19:443–457.
- Qian L, Flood PM, Hong JS. Neuroinflammation is a key player in Parkinson's disease and a prime target for therapy. *J. Neural Transm.* 2010; 117:971–979. [PubMed: 20571837]
- Ranft U, Schikowski T, Sugiri D, Krutmann J, Kramer U. Long-term exposure to traffic-related particulate matter impairs cognitive function in the elderly. *Environ. Res.* 2009; 109:1004–1011. [PubMed: 19733348]

- Roberts AL, Lyall K, Hart JE, Laden F, just AC, Bobb JF, Koenen KC, Ascherio A, Weisskopf MG. Perinatal air pollutant exposures and autism spectrum disorder in the Children of Nurses's Health Study II participants. *Environ. Health Perspect.* 2013; 121:978–984. [PubMed: 23816781]
- Roqué PJ, Dao K, Costa LG. Microglia mediate diesel exhaust particle-induced cerebellar neuronal toxicity through neuroinflammatory mechanisms. *Neurotoxicology.* 2016; 56:204–214. [PubMed: 27543421]
- Schweikert EM, Amort J, Wilgenbus P, Forsterman U, Teiber JF, Horke S. Paraoxonases-2 and -3 are important defense enzymes against *Pseudomonas aeruginosa* virulence factors due to their anti-oxidative and anti-inflammatory properties. *J. Lipids.* 2012; ID 352857
- Suzuki T, Oshio S, Iwata M, Saburi H, Odagiri T, Udagawa T, Sugawara I, Umezawa M, Takeda K. In utero exposure to a low concentration of diesel exhaust affects spontaneous locomotor activity and monoaminergic system in male mice. *Particle Fibre Toxicol.* 2010; 7:7.
- Teeling JL, Perry VH. Systemic infection and inflammation in acute CNS injury and chronic neurodegeneration: underlying mechanisms. *Neuroscience.* 2009; 158:1062–1073. [PubMed: 18706982]
- Thirtamara Rajamani K, Doherty-Lyons S, Bolden C, Willis D, Hoffman C, Zelikoff J, Chen LC, Gu H. Prenatal and early life exposure to high level diesel exhaust particles leads to increased locomotor activity and repetitive behaviors in mice. *Autism Res.* 2013; 6:248–257. [PubMed: 23495194]
- Tiffany-Castiglioni E, Venkatraj V, Qian Y. Genetic polymorphisms and mechanisms of neurotoxicity: overview. *Neurotoxicology.* 2005; 26:641–649. [PubMed: 16026840]
- Tsukue N, Watanabe M, Kumamoto T, Takano H, Takeda K. Perinatal exposure to diesel exhaust affects gene expression in mouse cerebrum. *Arch. Toxicol.* 2009; 83:985–1000. [PubMed: 19629445]
- Van Berlo D, Albrecht C, Knaapen AM, Cassee FR, Gerlofs-Nijland ME, Kooter IM, Palomero-Gallagher N, Bidmon HJ, van Schooten FJ, Krutman J, Schins RPF. Comparative evaluation of the effects of short term inhalation exposure to diesel engine exhaust on rat lung and brain. *Arch. Toxicol.* 2010; 84:553–562. [PubMed: 20467864]
- Van Donkelaar A, Martin RV, Brauer M, Boys BL. Use of satellite observations for long-term exposure of global concentrations of fine particulate matter. *Environ. Health Perspect.* 2015; 123:135–143. [PubMed: 25343779]
- Volk HE, Lurmann F, Penfold B, Hertz-Picciotto I, McConnell R. Traffic-related air pollution, particulate matter, and autism. *JAMA Psychiat.* 2013; 70:71–77.
- Weiss B. Same sex, no sex, unaware sex in neurotoxicology. *Neurotoxicology.* 2011; 32:509–517. [PubMed: 20875453]
- Weldy CS, White CC, Wilkerson HW, et al. Heterozygosity in the glutathione synthesis gene *Gclm* increases sensitivity to diesel exhaust particulate induced lung inflammation in mice. *Inhal. Toxicol.* 2012; 23:724–735.
- Weuve J, Puett RC, Schwartz J, Yanosky JD, Laden F, Grodstein F. Exposure to particulate air pollution and cognitive decline in older women. *Arch. Int. Med.* 2012; 172:219–227. [PubMed: 22332151]
- Win-Shwe TT, Yamamoto S, Fujitani Y, Hirano S, Fujimaki H. Spatial learning and memory function-related gene expression in the hippocampus of mouse exposed to nanoparticles-rich diesel exhaust. *Neurotoxicology.* 2008; 29:940–947. [PubMed: 18926851]
- Win-Shwe TT, Fujimaki H. Nanoparticles and neurotoxicity. *Int. J. Mol. Sci.* 2011; 12:6267–6280. [PubMed: 22016657]
- Win-Shwe TT, Fujitani Y, Kyi-Tha-Thu C, Furuyama A, Michikawa T, Tsukahara S, Nitta H, Hirano S. Effects of diesel engine exhaust origin secondary organic aerosols on novel object recognition ability and maternal behavior in BALB/c mice. *Int. J. Environ. Res. Public Health.* 2014; 11:11286–11307. [PubMed: 25361045]
- Wu YC, Lin YC, Yu HL, Chen JH, Chen TF, Sun Y, Wen LL, Yip PK, Chu YM, Chen YC. Association between air pollutants and dementia risk in the elderly. *Alzheimers Dement. (Amst).* 2015; 1:220–228. [PubMed: 27239507]

Yokota S, Mizuo K, Moriya N, Oshio S, Sugawara I, Takeda K. Effect of prenatal exposure to diesel exhaust on dopaminergic system in mice. *Neurosci. Lett.* 2009; 449:38–41. [PubMed: 18938223]

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 1. Diesel exhaust exposure is associated with microglial activation

Iba1 quantification by Western blot in olfactory bulb (OB) and hippocampus (HIP) of male and female mice exposed to diesel exhaust (DE, 250–300 $\mu\text{g}/\text{m}^3$) or filtered air (FA) for six hours. *Top*: Iba1 band intensity (optical density standardized to β -actin; mean $\pm$ SE; $n=9-11$) in OB (left) and HIP (right). Significant different from control, ** $p<0.01$; *** $p<0.001$

Bottom: Representative Western blots for Iba1 and β -actin in the olfactory bulb (left) and the hippocampus (right).

Fig. 2. Phenotypic changes in microglia induced by diesel exhaust exposure

Left: Microglia of DE-exposed mice showed morphological changes indicating a heightened state of reactivity. Area of microglial soma (µm²) increased, while circularity

($C = 4\pi \cdot \frac{\text{Area}}{(\text{Perimeter})^2}$, a measure of inverse aspect ratio) was reduced. Results represent the mean (± SE) of six mice per group. Significant difference between FA and DE, ***p<0.001, *p<0.05. *Right:* Micrographs of Iba1 immunohistochemistry of adult mouse dentate gyrus following exposure to FA (top) or DE (bottom) are shown at 20× (left) or 40× (right).

Fig. 3. Microglial activation in brain sub-regions of mice exposed to diesel exhaust measured by TSPO autoradiography

Autoradiography was performed on horizontal sections, using [^3H]-DPA 713 as the TSPO ligand. *Upper left:* Representative image of horizontal brain section showing regions analyzed for TSPO autoradiography. Regions analyzed included 1) Limbic cortex, 2) Orbital cortex, 3) Somatosensory cortex, 4) Entorhinal cortex, 5) Caudate Putamen, 6) Thalamus, 7) Hippocampus, 8) Periaqueductal Grey, and 9) Cerebellum. Each region was analyzed on the right and left hemisphere. *Lower left:* Tendency towards increased TSPO in the cerebellum of males ($p=0.064$) and females ($p=0.126$). Top right and bottom right: Tendency towards increased TSPO in multiple cortical regions in males ($p=0.278$, limbic Cx; $p=0.053$, Orbital Cx; $p=0.059$, Somatosensory Cx; $p=0.416$ Entorhinal Cx), but not females. Results are the mean ($\pm$ SE) of six animals for each gender and treatment.

Table 1

Cytokine levels in olfactory bulb and hippocampus after acute exposure to DE

Brain region/ Sex	Treatment	IL-1 α	IL-1 β	IL-3	IL-6	TNF- α	IL-9
Olfactory Bulb							
M	FA	33.3 (2.0)	12.7 (1.6)	0.33 (0.10)	1.9 (0.3)	1.3 (0.3)	1133.2 (43.0)
	DE	45.3* (10.4)	31.1* (7.1)	3.85** (1.31)	32.4*** (7.4)	17.6*** (1.1)	699.0** (27.2)
F	FA	33.5 (1.4)	14.4 (1.9)	0.31 (0.10)	1.8 (0.6)	0.9 (0.1)	1234.9 (27.1)
	DE	51.5* (6.9)	23.2* (5.1)	0.95*** (0.32)	21.6*** (1.7)	4.0** χ (2.1)	769.7** (67.7)
Hippocampus							
M	FA	20.3 (6.0)	13.4 (1.2)	0.26 (0.09)	1.7 (0.4)	1.4 (0.4)	1033.1 (75.7)
	DE	53.9*** (3.8)	32.8*** (3.3)	2.56** (0.81)	30.3*** (3.6)	9.8** (1.9)	587.8*** (48.3)
F	FA	32.9 (0.1)	13.1 (0.6)	0.28 (0.10)	1.2 (0.3)	0.7 (0.1)	1231.0 (8.5)
	DE	47.3** (5.2)	20.6*** $\wedge$ (0.3)	0.75* χ (0.19)	9.6*** χ (0.6)	1.3** $\wedge$ (0.2)	591.5*** (85.7)

Cytokine levels were analyzed by the Luminex xMAP® method (Datta and Opp, 2008) in 96-well plates. Results are expressed as pg/ml, and represent the mean ($\pm$ SE) of three animals analyzed in triplicate.

Abbreviations: M, males; F, female; FA, filtered air; DE, diesel exhaust. Results were analyzed for statistical significance by one-way ANOVA with Bonferroni correction for multiple comparisons; DE vs. FA:

- * p<0.05;
- ** p<0.01;
- *** p<0.001; M vs. F;
- # p<0.05;
- $\wedge$ p<0.01;
- χ p<0.001.

Table 2

Levels of pro-inflammatory cytokines IL-3 and IL-6 in olfactory bulb and hippocampus of male *Gclm*^{+/+}, *Gclm*^{+/-}, and *Gclm*^{-/-} mice following acute diesel exhaust exposure

Olfactory bulb	<i>Gclm</i> ^{+/+}	<i>Gclm</i> ^{+/-}	<i>Gclm</i> ^{-/-}
IL-3 (pg/ml)			
FA	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.0
DE	3.8 ± 1.3 **	8.3 ± 0.2 ***,#	5.3 ± 0.1 ***
IL-6 (pg/ml)			
FA	1.9 ± 0.3	6.0 ± 0.3	4.0 ± 0.1
DE	32.4 ± 7.4 ***	403.7 ± 2.9 ***,###	181.9 ± 5.3 ***,###
Hippocampus			
IL-3 (pg/ml)			
FA	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.1
DE	2.6 ± 0.8 **	5.5 ± 0.2 ***,#	2.7 ± 0.1 ***
IL-6 (pg/ml)			
FA	1.7 ± 0.4	6.7 ± 0.1	4.0 ± 0.2
DE	30.3 ± 3.6 ***	314.1 ± 12.8 ***,###	169.2 ± 2.4 ***,###

Male mice of each genotype were exposed for 6 h to diesel exhaust (DE, 250–300 µg/m³) or filtered air (FA) and levels of IL-3 and IL-6 were measured in the olfactory bulb and the hippocampus. Results represent the mean of three separate animals done in triplicate. Results were analyzed for statistical significance by one-way ANOVA with Bonferroni correction for multiple comparisons; DE vs FA:

**
p<0.01;

p<0.001;

Gclm^{+/+} vs. *Gclm*^{+/-} or *Gclm*^{-/-}:

p<0.05;

p<0.01;

p<0.001.

Table 3

Effect of acute exposure to diesel exhaust on lipid peroxidation in mouse brain

Sex	Treatment	OB	HIP	CB	CX	ST
M	FA	5.0 ± 0.1	4.4 ± 0.1	3.6 ± 0.2	3.0 ± 0.2	2.2 ± 0.1
	DE	30.1 ± 1.7**	13.0 ± 0.3*	6.3 ± 0.1*	4.1 ± 0.2	6.1 ± 0.1*
	(% of FA)	602	295	175	137	277
F	FA	2.3 ± 0.1	1.1 ± 0.1	5.9 ± 0.1	4.4 ± 0.1	2.6 ± 0.1
	DE	8.4 ± 0.1*##	2.5 ± 0.1*#	8.8 ± 0.1*	6.7 ± 0.1	3.1 ± 0.2#
	(% of FA)	365	227	149	152	119

Levels of malonyldialdehyde (MDA, nmol/g) are shown as a measurement of lipid peroxidation. Wild-type male (M) and female (F) mice were exposed to diesel exhaust (DE; 250–300 µg/m³) or filtered air (FA) for 6 h, as described in Methods. Results represent the mean (± SE) of three animals/group analyzed in duplicate. Results were analyzed for statistical significance by one-way ANOVA with Bonferroni correction for multiple comparisons; DE vs FA:

* p<0.05;

** p<0.01; Female vs. male,

p,0.05,

p<0.01.

OB, olfactory bulb; HIP, hippocampus; CB, cerebellum, CX, cerebral cortex; ST, striatum.