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Microglia mediate diesel exhaust particle-induced cerebellar neuronal toxicity through neuroinflammatory mechanisms

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Abstract

In addition to the well-established effects of air pollution on the cardiovascular and respiratory systems, emerging evidence has implicated it in inducing negative effects on the central nervous system. Diesel exhaust particulate matter (DEP), a major component of air pollution, is a complex mixture of numerous toxicants. Limited studies have shown that DEP-induced dopaminergic neuron dysfunction is mediated by microglia, the resident immune cells of the brain. Here we show that mouse microglia similarly mediate primary cerebellar granule neuron (CGN) death *in vitro*. While DEP (0, 25, 50, 100 $\mu\text{g}/2\text{ cm}^2$) had no effect on CGN viability after 24 h of treatment, in the presence of primary cortical microglia neuronal cell death increased by 2–3-fold after co-treatment with DEP, suggesting that microglia are important contributors to DEP-induced CGN neurotoxicity. DEP (50 $\mu\text{g}/2\text{ cm}^2$) treatment of primary microglia for 24 h resulted in morphological changes indicative of microglia activation, suggesting that DEP may induce the release of cytotoxic factors. Microglia-conditioned medium after 24 h treatment with DEP, was also toxic to CGNs. DEP caused a significant increase in reactive oxygen species in microglia, however, antioxidants failed to protect neurons from DEP/microglia-induced toxicity. DEP increased mRNA levels of the pro-inflammatory cytokines IL-6 and IL1- β , and the release of IL-6. The antibiotic minocycline (50 μM) and the peroxisome proliferator-activated receptor- γ agonist pioglitazone (50 μM) attenuated DEP-induced CGN death in the co-culture system. Microglia and CGNs from male mice appeared to be somewhat more susceptible to DEP neurotoxicity than cells from female mice possibly because of lower paraoxonase-2 expression. Together, these results suggest that microglia-induced neuroinflammation may play a critical role in modulating the effect of DEP on neuronal viability.

Keywords

Diesel particles; Microglia, Neuroinflammation; Neuronal death; Oxidative stress

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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), increasing evidence indicates that the central nervous system (CNS) is also a relevant target (Calderon-Garciduenas et al., 2002; Block and Calderon-Garciduenas, 2009; Genc et al., 2012). Epidemiological studies have shown that elevated air pollution is associated with decreased cognitive functions and several other behavioral effects (Chen and Schwartz, 2009; Calderon-Garciduenas et al., 2010; 2011; Ranft et al., 2009; Freire et al., 2010; Fonken et al., 2011; Power et al., 2011; Weuve et al., 2012; Guxens and Sunyer, 2012). Furthermore, a role of air pollution in the etiology of neurodegenerative and neurodevelopmental diseases has also been suggested (Calderon-Garciduenas et al., 2004; 2008; 2012; Levesque et al., 2011a; Raz et al., 2015; Harris et al., 2016).

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; Nelin et al., 2012). PM is broadly characterized by aerodynamic diameter (e.g. PM₁₀ and PM_{2.5}, equivalent to <10 µm and 2.5 µm in diameter, respectively; Brook et al., 2010). Ultrafine particulate matter (UFP; <100 nm), which represents a large percentage of PM_{2.5}, 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; 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). 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). 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 (Block and Calderon-Garciduenas, 2009; Genc et al., 2012). Some *in vitro* studies have shown that PM is cytotoxic, and that toxicity is size-dependent, with UFP being able to better enter the cells and exert toxic effects (Block et al., 2004; Kreyling et al., 2004; Win-Shwe and Fujimaki, 2011; Gillespie et al., 2012).

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 (e.g. organic carbon, metals, poly-aromatic hydrocarbons, aldehydes, etc.), and is a major constituent of ambient PM; indeed, DE exposure is often utilized as a measure of traffic-related air pollution. 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; 2012). Neuroinflammation and oxidative stress also appear to be major mechanisms of DE neurotoxicity (Levesque et al., 2011a; 2011b; Gerlofs-Nijland et al., 2010; Costa et al. 2016; Cole et al. 2016).

A few *in vitro* studies have examined the effects of DE particles (DEP) on the CNS, particularly on dopaminergic neurons (Block et al., 2004; Gillespie et al., 2012; Levesque et

al., 2011b). An earlier study by Block et al. (2004) is of great interest, as it showed that DEP could activate microglia, and that microglia-derived oxidant species caused the demise of dopaminergic neurons (Block et al., 2004). The aim of the present study was to confirm and extend these observations, utilizing a different primary cell system (mouse cerebellar granule cells) and particles prepared from DE utilized in our *in vivo* studies (Cole et al. 2016). We also sought to examine whether a sex-difference was present in the effects of DEP, thereby substantiating and expanding *in vitro* the observations of a gender difference in susceptibility to DE neurotoxicity observed *in vivo* (Cole et al. 2016). This hypothesis stems from our earlier observations that the intracellular antioxidant and anti-inflammatory enzyme paraoxonase-2 (PON2) is expressed at higher levels in females, thereby providing protection against oxidative stress and neuroinflammation (Giordano et al. 2011; 2013).

Materials and Methods

Materials

Neurobasal-A medium, Dulbecco's Modified Eagle's Medium, fetal bovine serum, B-27 supplement with and without antioxidant, fungizone, gentamicin, and goat anti-rabbit IgG Alexa 55 were purchased from Invitrogen (Thermo Fisher Scientific, Waltham, MA). Pioglitazone and minocycline hydrochloride were purchased from Cayman Chemical (Ann Arbor, MI). Melatonin, phenyl- α -tert-butyl nitron (PBN) and 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies to Iba1 and to paraoxonase-2 were purchased from Abcam (Cambridge, MA). Falcon 24-well cell culture inserts and PET membranes were purchased from Corning Life Sciences (Tewksbury, MA). For lactate dehydrogenase assays, Promega's CytoTox 96 Non-Radioactive Cytotoxicity kit (Promega Corporation, Madison, WI) was used. The Live/Dead Viability/Cytotoxicity Kit for mammalian cells was purchased from Molecular Probes (Thermo Fisher Scientific, Waltham, MA). All reagents for quantitative polymerase chain reaction (qPCR) were obtained from Biorad Laboratories (Hercules, CA). The Quantikine ELISA Mouse IL-6 Immunoassay kit was from R&D Systems, Inc. (Minneapolis, MN).

Neuronal cell culture

Primary cerebellar granule neurons (CGN) were isolated from 7-day-old (PND7) C57BL/6J mice as previously described (Giordano et al., 2006; Giordano and Costa, 2011), and plated at a density of 0.6×10^6 cells/2 cm² in poly-d-lysine (0.2 mg/mL) pre-coated 24-well plates. Cells were incubated for 4 days in a humidified environment (37°C, 5% CO₂) in antioxidant-containing medium (Neurobasal A medium supplemented with B-27 supplement (2%), glutamax (2 mM), potassium chloride (KCL, 25 mM), fungizone (1 μ g/mL), and gentamycin (100 μ g/mL). On the 4th day, medium was replaced with neuronal medium without antioxidant (B-27 supplement minus AO). After eight days in culture, half of the medium was replaced and CGNs were utilized for co-culture experiments two days after.

Culture of microglia

Primary mixed glia cultures were obtained from the cortices of PND3 mouse pups according to Witting and Möller (2011), with some modifications. Briefly, cortices were dissected and

trypsinized (0.25% in PBS) for 30 minutes at 37°C, dissociated and centrifuged at $250 \times g$ for 10 minutes at room temperature. Cortical cells were resuspended and seeded at a density of 3–6 cortices per flask, pre-coated with poly-d-lysine (40 $\mu\text{g/mL}$). Glial cultures were grown in DMEM supplemented with fetal bovine serum (FBS, 10%) and penicillin and streptomycin. Medium was changed one day after seeding to remove debris and one week later at confluence. Five to seven days after confluence, when microglia are apparent on the surface of the glial layer, they were isolated by dislodging using repetitive tapping of the flask, followed by removal and replacement of the medium. Medium containing microglia was centrifuged ($250 \times g$, 7 minutes, at room temperature), resuspended, counted and sub-cultured for experimental use.

Co-culture and treatment of CGNs and microglia

CGN grown in culture for 10 days were combined with 3.0 μm mesh inserts, or inserts containing microglia sub-cultured for two days (6.0×10^4 cells/insert). Neurons and neurons plus microglia were treated for 24 h with increasing concentrations of DEP (0, 25, 50, 100 $\mu\text{g}/2 \text{ cm}^2$) for the initial lactate dehydrogenase (LDH) experiments. In subsequent experiments only 50 or 100 $\mu\text{g}/2 \text{ cm}^2$ concentrations were used. In additional experiments, cells were treated with DEP plus minocycline (50 μM), pioglitazone (50 μM), melatonin (200 μM) or phenyl- α -tert-butyl nitron (PBN, 100 μM). Separate wells were treated with solvent control, when necessary. To control for the effect of inserts on the co-culture system and small particles of DEP moving through the insert to influence neurons directly, all wells containing neurons were co-cultured with inserts (with or without microglia), and each insert received the corresponding treatment.

Diesel exhaust particles source and preparation

Diesel exhaust particulate matter (DEP) was generated from a Cummins diesel engine under load, and was provided by the University of Washington's Controlled Inhalation Diesel Exhaust Exposure Facility. DEP was collected from the outflow duct, as previously described (Gould et al., 2008; Weldy et al. 2011) and consisted of previously characterized $\text{PM}_{2.5}$ particles (Gould et al., 2008; Fox et al., 2015). Stock solutions were prepared by suspending particles in PBS (2.5% DMSO) at a concentration of 10 mg/mL . Solutions were mixed by vortexing, and were sonicated for five minutes prior to dilution to treatment concentrations.

Lactate Dehydrogenase (LDH) Assay

Cell death was quantified using the LDH assay, or CytoTox 96 Non-Radioactive Cytotoxicity Assay following the instructions. Briefly, neurons or neurons plus microglia were treated with increasing concentrations of DEP (0–100 $\mu\text{g}/2 \text{ cm}^2$). After 24 h treatment, 1 mL medium was collected from treated, control, and blank wells corresponding to each treatment group, and centrifuged ($12,000 \times g$ at 4°C for 5 minutes) to remove DEP particles. Medium (75 μL) was added to a 96-well plate in triplicate, followed by 50 μL substrate medium, and incubated for 30 min. at room temperature in the dark. At incubation end, stop-buffer was added and the plate was read at 490 nm. Maximum LDH release or maximum cell death was obtained through the addition of lysis buffer (1:10) to triplicate wells 45 minutes prior to the end of the experiment. Lysis buffer was also added to the microglia-

containing inserts. Percentage of cell death was calculated by subtracting background optical density (no cell medium control for each treatment group) from the optical density of the medium from cell treatment, and dividing by the maximum LDH release optical density. Separate maximum LDH values were obtained for neuron only and neurons plus microglia wells.

Live/Dead Assay

CGN cell death was measured using the fluorescent Live/Dead or Calcein-AM/Ethidium homodimer Assay kit (Molecular Probes) as per instructions. Briefly, CGNs seeded on poly-D-lysine (200 µg/mL) pre-coated glass coverslips, were grown and co-cultured with or without microglia as previously described. Co-cultures were treated with DEP (100 µg/2 cm²) for 24 hours. At treatment end, cells were incubated in the dark for 30 minutes with calcein-AM (2 µM) and ethidium homodimer-1 (4 µM) to label live and dead cells, respectively. Coverslips were mounted on slides, and imaged using epifluorescence microscopy (20 ×). Three images each from triplicate coverslips per treatment group were acquired and live (green) and dead cells (red) were manually counted using Image J software.

Microglia Morphology Immunocytochemistry

Microglia were plated on glass coverslips (1×10⁵ cells/coverslip) and incubated overnight. Cells were treated with DEP (50 µg/2 cm²) for 24 hours, then fixed with paraformaldehyde (4%), and immunolabeled overnight with rabbit polyclonal to Iba1 (1:75), fluorescently tagged with goat anti-rabbit Alexa 555 (1:200) for 1 hour at room temperature and counterstained with Hoechst staining (1 µg/mL) for 10 min. Cells were mounted and imaged using an epifluorescence microscope (40×), and morphological changes were qualitatively assessed.

Measurement of Reactive Oxygen Species (ROS)

Microglia were plated in 96-well plates (5.5–6.0 × 10⁴ cells/well). After two days, cells were pretreated for 1 h with 20 µM 2',7'-dichlorodihydrofluorescein diacetate (DCFDA), a cell-permeant, reduced form of fluorescein, used as indicator of ROS. Cleavage of the DCFDA by intracellular esterases and oxidation, results in the conversion of non-fluorescent DCFDA to the highly fluorescent 2',7'-dichlorofluorescein (DCF). After pre-treatment, cells were washed and treated with DEP (0 or 50 µg/2 cm²) or co-treated with DEP and minocycline (50 µM). Relative fluorescence units (RFUs) were obtained at 1 and 2 hours post-treatment using a fluorescent spectrophotometer (485 excitation/530 emission). Readings were adjusted to treatment-matched, no-cell controls.

Measurement of cytokine mRNAs

Relative levels of interleukin-6 (IL-6), interleukin-1β (IL-1β), and tumor necrosis factor-α (TNF-α) mRNAs were determined using real-time, quantitative polymerase chain reaction (qPCR). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene. Primary microglia were plated in 6-well plates at a density of 5.76 × 10⁵ cells per well. After two days, cells were treated with DEP (50 µg/2 cm²) for 24 hours. The

medium was collected, centrifuged, and snap frozen on dry ice and stored at -80°C for use in separate experiments with microglia-conditioned medium. RNA was isolated in Qiazol or Trizol, followed by column purification (Qiagen RNeasy Mini Kit). RNA concentration was quantified by Nanodrop and cDNA was synthesized using the Biorad iScript cDNA synthesis kit, as per instructions. RNA concentrations were held constant for each independent experiment. Biorad iTaq Universal SYBR Green was used for target amplification. Primer sequences were as follows: IL-6 Forward: GTGGAAATGAGAAAAGAGTTGTGC, IL-6 Reverse: CTGCAAGTGCATCATCGTTGT; IL1- β Forward: GCAGCTGGAGAGTGTGGAT, IL1- β Reverse: ACAAAACCGTTTTCCATCTTCTTCT; GAPDH Forward: CCTGGTATGACAATGAATACGGC, GAPDH Reverse: CTCCTTGGAGGCCATGTAGG; TNF- α Forward: GTCGTAGCAAACCACCAAGTG, TNF- α Reverse: CTTTGAGATCCATGCCGTTGG.

Measurement of IL-6 by enzyme-linked immunosorbent assay (ELISA)

Levels of IL-6 in the medium after microglia treatments were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc.). Microglia were plated in 48 well plates at a density of 6.0×10^4 per well and treated with DEP ($50 \mu\text{g}/2 \text{ cm}^2$) for 24 hours. Medium was collected, centrifuged to remove DEP, and cellular protein levels were obtained using the bicinchoninic (BCA) assay. A dilution of 1:10 of medium was used to quantify IL-6 release, and values were normalized to the corresponding protein levels, as measured by the BCA assay.

Treatment of neurons with microglia-conditioned medium (MCM)

Primary microglia were plated in 6-wells plates at a density of 5.8×10^5 cells/well. After two days, cells were treated with DEP ($50 \text{ mg}/2 \text{ cm}^2$) for 24 h. Medium was then collected and centrifuged ($12,000 \times g$ at 4°C for 5 min) to remove particles. The supernatant was snapped-frozen on dry ice and stored at -80°C until use. CGNs, grown for ten days as previously described, were washed with Hank's buffered saline solution and the medium was replaced with unconditioned medium or unconditioned medium plus sterile-filtered MCM from control or DEP-treated microglia. After 24 h cell death was assessed using the LDH assay.

Measurement of paraoxonase-2 protein levels

Paraoxonase-2 (PON2) protein levels were determined by Western blot. Microglia from male and female mice were sub-cultured and plated in poly-d-lysine ($40 \mu\text{g}/\text{mL}$)-coated 6-well plates at a density of 5.76×10^5 cells per well. After two days in culture, cells were collected in lysis buffer, proteins were quantified using the bicinchoninic acid (BCA) assay, and equal amounts of protein were loaded into pre-cast gels (10% Tris-HCL gels). Protein was subjected to gel electrophoresis and transferred to polyvinylidene difluoride (PVDF) membranes and probed for PON2 (1:1000, at 4°C for two nights) and β -actin (1:1,000 for 1 hour at RT). These dilutions were optimized based on our prior work on PON2 protein expression in other cell types (Giordano et al. 2011; 2013). Membranes were incubated in secondary antibodies tagged with horseradish peroxidase (rabbit anti- HRP, 1:1000 for 1 hour, RT) and developed. PON2 levels were normalized to β -actin.

Statistical analysis of data

Results were analyzed for statistical significance by one-way ANOVA followed by the Dunnett's or Bonferroni Multiple Comparison tests when comparing multiple treatments of the same group (e.g. Fig. 3A or 5), and with two-way ANOVA followed by the Bonferroni post-tests when determining the interactions between DEP treatment with or without microglia, or sex differences (e.g. Fig 1 or 6). Results are expressed as mean ($\pm$ SE) of at least three separate experiments.

Results

Neuronal toxicity of DEP is mediated by microglia

To determine the role of microglia in mediating DEP-induced neuronal death, CGNs were treated with increasing concentrations of DEP (0, 25, 50, 100 $\mu\text{g}/2\text{ cm}^2$) for 24 hours in the absence or presence of primary cortical microglia (Fig. 1). Microglia were sub-cultured at a density of 10% of the neuronal population, to reflect the average *in vivo* microglia-to-neuron ratio (Block et al. 2007), and plated into porous inserts two days prior to co-culture. All neuronal wells were co-cultured with either empty inserts or inserts containing microglia, and both inserts and neuronal wells were co-treated with the corresponding treatment. At treatment end, cytotoxicity was measured using the lactate dehydrogenase (LDH) assay. Results show that DEP had no effect on the viability of CGNs when neurons were cultured alone; in contrast, in the presence of microglia, cell death (LDH release) increased by more than two-fold (Fig. 1A). Since the assay cannot discriminate between the LDH released from dying neurons from that of microglia, calcein and ethidium homodimer labeling of neurons was used, to assess neuron-specific cell death. As observed in the LDH assay, the highest concentration of DEP (100 $\mu\text{g}/2\text{ cm}^2$ for 24 h) had no effect on neuronal death in the absence of microglia, but induced a greater than 2-fold increase in neuronal death when neurons shared their environment with DEP treated microglia (Fig. 1B, 1C). Together, these findings suggest that the presence of microglia is essential to promote neuronal cell death induced by DEP.

Activation of microglia by DEP

When activated, microglia change from a ramified state to an amoeboid shape, moving through a series of changes from increased proliferation, the retraction of extensions, increased mobility, and finally, the ability to phagocytose debris (Stence et al., 2001). To determine the effect of DEP on microglia, cells were immunocytochemically labeled for Iba1 and imaged using fluorescence microscopy. Compared to control microglia, those treated with DEP were qualitatively larger, more spherical, lacked extensions and contained DEP particles, suggestive of a phagocytotic state (Fig. 2). These results suggest that DEP induces morphological changes indicative of microglia activation.

Potential role of ROS in DEP-induced, microglia-mediated neuronal death

Reactive microglia release both cytotoxic and protective factors, which may influence neuronal viability (Block et al. 2007; Ransohof and Perry, 2009; Luo and Chen, 2012). Microglia-released factors that contribute to oxidative stress have been shown to play a role

in microglia-induced neurotoxicity (Block et al. 2004). To test whether DEP treatment of microglia results in the generation of reactive oxidative species we measured ROS upon DEP (50 $\mu\text{g}/2\text{ cm}^2$) treatment. DEP induced a greater than 2-fold increase in ROS production after 1 h of treatment, which was sustained at 2 h (Fig. 3A). Inhibition of microglia activation with the antibiotic minocycline (50 μM) attenuated the increase in ROS (Fig. 3A). However, the antioxidant phenyl- α -tert-butyl nitron (PBN; 100 μM) was unable to rescue neurons after co-culture treatment with DEP (Fig. 3B). Similar results were found with melatonin (200 μM ; not shown). These findings suggest activation of microglia by DEP causes an increase in ROS, but that these do not appear play a significant role in microglia-mediated DEP neurotoxicity in the present experimental system.

Potential role of pro-inflammatory cytokines in DEP-induced, microglia-mediated neuronal death

Activated microglia have also been shown to release pro-inflammatory cytokines which may influence neuronal death. To test whether the pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α were differentially expressed in microglia treated with DEP, we measured relative levels of mRNA by qPCR. Lipopolysaccharide (LPS; 500 ng/mL) was used as a positive control in these experiments. As shown in Fig. 4, DEP (50 $\mu\text{g}/2\text{ cm}^2$ for 24 h) induced 18- and 4-fold increases in IL-6 and IL-1 β mRNA, respectively, with only a two-fold increase of TNF- α . LPS also caused large increases in IL-6 (100-fold) and IL-1 β (45 fold), but not of TNF- α (Fig. 4). Using ELISA, we also measured levels of IL-6 protein released to the medium; after 24 h, DEP (50 $\mu\text{g}/2\text{ cm}^2$) induced an approximate 12-fold increase in IL-6 release from microglia relative to untreated cells (Fig. 4G). These findings indicate that DEP induces the expression and release of pro-inflammatory cytokines from microglia, which may be involved in microglia-mediated DEP-induced neuronal death.

To test whether these microglia-released factors are sufficient to induce neuronal death on their own, microglia conditioned medium (MCM) was collected from control and DEP (50 $\mu\text{g}/2\text{ cm}^2$)-treated microglia. The addition of MCM from DEP-treated microglia to CGNs, grown as in previous experiments, induced a greater than 3-fold increase in cell death, as measured by the LDH assay (Fig. 5A). Additionally, when neurons were co-treated with MCM in the presence of minocycline (50 μM), no protective effect was observed (Fig. 5A). However, when neurons were co-treated with DEP and minocycline in the presence of microglia, DEP-induced neuronal cell death was significantly decreased (Fig. 5B). These findings suggest that factors released from microglia after DEP treatment are capable of inducing neuronal death, and that the effect of minocycline is due to an action on microglia rather than on the neurons (Henry et al. 2008). To further confirm that microglia activation is necessary for the increase in cell death observed in the co-culture system, CGNs and microglia were co-cultured in the presence of an alternative pharmacological inhibitor of microglia activation, the PPAR- γ agonist pioglitazone (Ji et al. 2010; Drew et al. 2015). As shown in Fig. 5C, pioglitazone (50 μM) significantly attenuated DEP-induced neurotoxicity.

Sex differences in the effects of DEP

Previous work in our laboratory has shown that the intracellular antioxidant/anti-inflammatory enzyme paraoxonase-2 (PON2) is differentially expressed in the brains of

male and female mice, with levels higher in females than males, and a 1.9-fold difference in CGNs (Giordano et al. 2011; 2013). Here we found that a sex difference in PON2 levels was also present in microglia, with female mice displaying 1.5-fold higher levels compared to males (Fig. 6A, 6B). To investigate whether microglia-mediated neurotoxicity after DEP treatment differs between males and females, we carried out parallel experiments with co-cultures of primary microglia and CGNs from mice of either sex. A comparison of male and female control co-cultures showed that there is no sex difference in baseline cell viability. A 24 h treatment with DEP (50 $\mu\text{g}/2\text{ cm}^2$) increased cell death in cultures of both sexes (Fig. 6C). Cell death in male co-cultures increased 2.1-fold relative to controls, while in female co-cultures there was a 1.6-fold increase in cell death, though the difference between sexes was not statistically significant. Nevertheless, higher PON2 levels in cells from female mice may afford some protection from DEP neurotoxicity.

Discussion

The main finding of the present study is that microglia play an important role in mediating the neurotoxicity of DEP *in vitro*, confirming and expanding previous results (Block et al. 2004). When using mixed neuron-glia cultures, the microglial content is usually low because of the differential development of most neuronal populations (prenatally in mice) and microglia (early postnatally) (Ransohoff and Cardona, 2010). For this reason, a neuron-microglia co-culture system has been suggested as the best choice for investigating the role of microglia in neurotoxicity and for exploring neuroprotective strategies (Gresa-Arribas et al., 2012). In the present study we utilized a co-culture system consisting of mouse CGNs, a well-established neuronal model to investigate *in vitro* neurotoxicity, and microglia isolated from mouse cerebral cortex. Microglia are known to differ between brain regions, particularly with regard to their distribution, with cerebral cortex expressing higher microglial content than cerebellum, and the *substantia nigra* displaying the greatest number relative to neurons (Lawson et al., 1990; Block et al. 2007; Yang et al., 2013). The choice of cortical microglia was mainly dictated by convenience, specifically a higher yield compared to cerebellar microglia, as we did not aim to search for particular region-specific effects of DEP. Nevertheless, we chose to set the neuron:microglia ratio as 9:1, which is the approximate average ratio in brain (Block et al. 2007). In addition, we also carried out an experiment, similar to the one shown in Fig. 1A, comparing cortical and cerebellar microglia, and found no differences between the two systems in response to DEP (not shown).

Our findings indicate that DEP was not toxic to neurons when cultured alone, and that neuronal cell death was significant only in the presence of microglia (Fig. 1). We confirmed that the observed mortality was due to death of neurons rather than microglia, and that the latter is more resistant to DEP cytotoxicity (unpublished observation; Block et al. 2004). DEP was capable of activating microglia, as previously observed *in vitro* (Block et al. 2004; Levesque et al. 2011b; 2013) and *in vivo* (Levesque et al. 2011a; Durga et al. 2015; Cole et al. 2016). Activated microglia are known to release both cytotoxic and protective factors, which may influence neuronal viability (Block et al. 2007; Ransohoff and Perry, 2009; Luo and Chen, 2012). In particular, activated microglia contribute to oxidative stress and to neuroinflammation, which are believed to play primary roles in neurotoxicity and in

neurodevelopmental and neurodegenerative disorders (Koutsilieri et al., 2002; Kraft and Harry, 2011; Graeber et al., 2011). We found that DEP caused an increase of microglial ROS, and that this was prevented by an inhibitor of microglia activation, minocycline (Fig. 3A). However, two antioxidants (PBN and melatonin) failed to protect neurons from DEP-induced, microglia-mediated, toxicity (Fig. 3B). In contrast, others have previously shown that PM increases oxidative stress in the brain (Mohan Kumar et al. 2008; Sagrillo Fagundes et al. 2015), and that oxidative stress plays a primary role in DEP neurotoxicity (Block et al. 2004). Thus, the possibility that some forms of oxidative stress may be involved in the microglia-mediated effects of DEP warrants further investigations.

DEP also caused a significant increase of IL-6 and IL-1 β mRNA, but surprisingly, had a relatively small effect on TNF- α (Fig. 4). The positive control LPS had similar effects. Block et al. (2004) also found a lack of effect of DEP on TNF- α , and Levesque et al. (2011a; 2013) did not find any effect on TNF- α nor on IL-1 β . In contrast, Gresa-Arribas et al. (2012) reported a significant increase of TNF- α (and of IL-6) release upon LPS treatment of microglia. It is likely that different experimental conditions and timing of testing may be at the basis of the observed differences (Manzano-Leon et al., 2016). For example, Nakamura et al. (1999) showed that over a 24 h period, a LPS-induced increase in TNF- α was first observed at 1 h, peaked at 6 h, and then declined, while IL-6 and IL-1 β increased later in the 24 h time-frame, with IL-6 reaching its peak at 24 h.

In addition to increasing cytokine mRNA expression, DEP also caused increased release to the medium of IL-6 (Fig. 4G), which we chose to measure as it showed the largest mRNA increase upon DEP exposure (Fig. 4A). Interestingly, conditioned medium from DEP-treated microglia was highly toxic to CGNs, indicating that pro-inflammatory cytokines and other factors secreted by activated microglia play a central role in DEP neurotoxicity. Of interest is also that minocycline was ineffective in protecting neurons against this conditioned medium, but protected neurons against the toxicity of DEP in a co-culture system, indicating that the target for minocycline neuroprotection is microglia. Indeed, another reported inhibitor of microglia activation, the PPAR- γ agonist pioglitazone (Ji et al. 2010; Drew et al. 2015), also protected neurons from DEP-induced, microglia-mediated neurotoxicity.

Among the factors that can affect neurotoxic outcomes, gender is considered of much relevance (Weiss, 2011). We have recently shown that the enzyme paraoxonase-2 (PON2) displays a significantly different level of expression between genders in mice and in other species including human (Giordano et al., 2011; 2013; Costa et al. 2014), with females exhibiting a 2- to 4-fold higher expression. PON2 exerts potent antioxidant and anti-inflammatory effects (see Costa et al. 2014 for a review), and lack of PON2 (as in PON2^{-/-} mice) or lower expression levels (as in males vs. females), increases susceptibility to neurotoxicity (Giordano et al. 2011; 2013). In a recent study (Costa et al. 2016; Cole et al. 2016) we found that *in vivo* exposure to DE caused oxidative stress (increased lipid peroxidation) and neuroinflammation (increased levels of IL-6, IL-1 β and of other pro-inflammatory cytokines) which were more pronounced in male mice. In order to determine whether a similar sex-dependent difference may be observed in the present *in vitro* co-culture system, we compared male and female microglia and CGNs. In both cell types PON2 levels were <2-fold higher in females (Fig. 6A, B; Giordano et al. 2011; 2013). We

found that DEP toxicity was more pronounced in co-cultures of male mice, as expected, though results did not reach statistical significance, suggesting that other factors may be also involved in the sex-dependent, differential effects seen *in vivo* (Costa et al. 2016; Cole et al. 2016).

In summary, we implemented an *in vitro* co-culture system to investigate the neurotoxicity of DEP, and found that DEP-induced microglia activation is an early event in DEP neurotoxicity. Increase synthesis and release of pro-inflammatory cytokines by microglia appear to be key mediators in modulating DEP neurotoxicity, which is somewhat more pronounced in males. This co-culture system will allow identification of additional mediators, testing of cells from transgenic animals, and identification of potential novel neuroprotective agents. Modification of the experimental conditions, for example using neurons and microglia from other brain regions, or using DEP restricted to UFP, would allow extension of current findings and further our understanding of the effects of DE on the nervous system

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HIGHLIGHTS

- Diesel particles are toxic to CGNs only in the presence of microglia
- Microglia release pro-inflammatory cytokines in response to diesel particles
- Blocking microglia activation protects neurons from diesel particle toxicity

Figure 1.

Effect of microglia on DEP-induced neuronal cell death. **A.** CGNs alone, or co-cultured with microglia, were treated with increasing concentrations of DEP for 24 h. Cell death was determined by measuring LDH released to the medium compared to maximum LDH of each group, after normalization to no-cell treatment controls. Values represent mean ($\pm$ SE) of five independent experiments. Significantly different from control neurons + microglia, ** $p < 0.01$, *** $p < 0.001$. Significantly different from the respective neurons only, ### $p < 0.001$.

B. CGNs alone or co-cultured with microglia, were treated with DEP ($100\text{ }\mu\text{g}/2\text{ cm}^2$) for 24

h. Neurons were incubated with calcein-AM to label live cells, and ethidium homodimer (EH) to label dead cells. Coverslips were mounted, and cells were imaged using an epifluorescence microscope. Percent neuronal death was determined by the ratio of the number of dead cells (red) to the number of total cells (live-green, and red). Values represent the mean ($\pm$ SE) of 24–27 neuronal images from three independent experiments.

Significantly different from control, $**p < 0.01$, and from neurons alone, $^{##}p < 0.01$. **C.**

Representative images of control and DEP treated neurons after co-culture with microglia.

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Figure 2.

DEP induces morphological changes in microglia. Primary microglia were plated at a density of 1.2×10^5 on glass coverslips, and treated for 24 h with DEP ($50 \mu\text{g}/2 \text{ cm}^2$). Cells were fixed, immunocytochemically labeled for Iba1, and imaged using fluorescent microscopy. Shown are representative greyscale images of control (left) and treated (right) microglia. Cell morphology is indicated by dotted white lines, and arrows indicate the presence of DEP that has been phagocytosed by microglia.

Figure 3.

A. Effect of DEP on ROS formation in microglia. ROS levels were measured after treatment with DEP (50 $\mu\text{g}/2\text{ cm}^2$) for 1 or 2 h. Minocycline (50 μM) was added 60 min before DEP. Results represent the mean ($\pm$ SE) of three separate experiments. Significantly different from control, *** $p < 0.001$. Significantly different from DEP, ## $p < 0.01$, ### $p < 0.001$. **B. Effect of the antioxidant PBN on DEP- induced, microglia-mediated cytotoxicity.** CGN, alone or co-cultured with microglia were treated

with DEP in the presence of the antioxidant PBN (100 μ M). Results show the mean ($\pm$ SE) of three determinations. Significantly different from neurons only, [#]p<0.05.

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Figure 4.
Effect of DEP on levels of cytokine mRNA. Expression of IL-6, IL-1 β and TNF- α mRNA in primary microglia was determined by qPCR analysis. Microglia were treated for 24 hours with 50 μ g/2 cm² DEP (A, C, E) or 500 ng/mL LPS (B, D, F). Relative levels of IL-6 (A, B), IL-1 β (C, D), and TNF- α (E, F) mRNA were compared after normalization to GAPDH mRNA. Results are the mean ($\pm$ SE) of 4–8 separate determinations. Significantly different from control, * p <0.05, ** p <0.01; *** p <0.001. **G. Effect of DEP on IL-6 release from microglia.** Levels of IL-6 released from microglia after DEP treatment (50 μ g/2 cm², 24 h),

were measured by ELISA. Values represent the means ($\pm$ SE) of six separate experiments. Significantly different from control, *** $p < 0.001$.

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Figure 5.

A. Effect of microglia-conditioned medium on neuronal cell death. CGNs were exposed for 24 h to medium obtained from primary microglia treated for 24 h with DEP (50 $\mu\text{g}/2\text{ cm}^2$; MCM-DEP) or to medium from untreated microglia (MCM-cntrl), in the absence or presence of minocycline (50 μM). MCM-DEP caused a significant increase in neuronal cell death. Results represent the mean ($\pm$ SE) of three separate experiments. Significantly different from control and MCM-cntrl, * $p < 0.05$. **B, C. Effect of blocking microglia activation on DEP-induced cell death.** CGNs co-cultured with microglia were treated with DEP (50 $\mu\text{g}/2\text{ cm}^2$) in the absence or presence of minocycline (MC; 50 μM) or pioglitazone (PIO; 50 μM). Cytotoxicity was determined by the LDH assay. Values represent mean ($\pm$ SE) of 3–6 separate experiments. Significantly different from control, * $p < 0.05$, *** $p < 0.001$. Significantly different from DEP50, # $p < 0.05$.

Figure 6.

Effect of sex on DEP-induced, microglia-mediated neurotoxicity. **A, B.** Paraoxonase-2 (PON2) protein expression in male and female microglia. Cell lysate of microglia was collected and probed for levels of PON2 by Western blot. Results show the mean optical density of PON2 normalized to β -actin by sex (mean $\pm$ SE, $n = 4$; **A**), and a representative blot of PON2 and β -actin levels (**B**). There is a significant difference between sexes, $*p < 0.05$. **C.** Co-cultures of CGNs and microglia of either sex were treated with DEP ($50 \mu\text{g}/2 \text{ cm}^2$) for 24 h and cytotoxicity was measured using the LDH assay. Results are the mean ($\pm$ SE) of three separate experiments. Significantly different from same sex control, $*p < 0.05$.