Diesel Exhaust Exposure Induces Microglial Activation, Suppresses Adult Neurogenesis, and Increases Levels of Neurodegenerative Markers

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Abstract

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Diesel exhaust (DE) is a major contributor to air pollution globally. Fine and ultrafine particulate matter make up a large portion of its components. For some time, exposure to high levels of traffic related particulate air pollution has been associated with adverse health effects of the respiratory system and the cardiovascular system. More recently, epidemiological studies have shown that high level exposures are also associated with endpoints in the central nervous system. Specifically, exposure may be associated with increased incidence of dementia, Alzheimer's disease (AD), and to a lesser extent, Parkinson's disease (PD), as well as distinctive neuropathology associated with these conditions. Posthumous examination of the brains of individuals who suffer from neurodegeneration have shown an increase in levels of oxidative stress and indices of neuroinflammation, such as increased levels of TNF-α and IL-6. Moreover, while the brains of even healthy elderly people tend to exhibit a more reactive glial phenotype, sufferers of neurodegeneration show evidence of a higher degree of microglial activation compared to their nondiseased counterparts. Significantly, experimental exposures of animals to DE have determined that acute exposures can significantly increase biomarkers of oxidative stress,

microglial activation, and neuroinflammation. High level chronic exposures of animals to DE, which may be considered analogous to high level occupational exposures, have also been shown to increase levels of markers of neurodegeneration such as Tau-pS199, $A\beta_{42}$, and α-synuclein, indices which are significantly increased in individuals with AD and PD.

Conditions of oxidative stress and neuroinflammation, and the intrinsically connected signaling activity of microglia are also factors that affect adult neurogenesis, the birth of new neurons in the postnatal brain. Regulation of adult neurogenesis appears to be strongly connected to cognitive function, as many animal experiments where adult neurogenesis is perturbed show a lack of cognitive function in the domains corresponding to the affected brain regions. This may be due to the preferential involvement of young neurons in the process of long term potentiation, which is a factor in synaptic plasticity, or the strengthening and weakening of connections between neurons over time, a process important to memory. Due to the presence of perturbations of neurogenesis in the brains of individuals suffering from AD in particular, and the absence of cognitive deficits in individuals with AD-type pathology and conserved neurogenesis, the preservation of adult neurogenesis may be an important point of intervention in the prevention of cognitive decline in the elderly.

In this work, we addressed whether exposure to DE suppresses adult neurogenesis, and if so, whether or not male animals would show an increased susceptibility to the neurotoxicity of DE relative to females. Due to the importance of microglial signaling activity in the regulation of adult neurogenesis, we also wanted to see whether attenuation of microglial activation and neuroinflammation through pharmacological intervention could potentially mitigate this effect. We also examined whether or not a subchronic exposure to DE of either 3 weeks or 10 weeks

would be sufficient to cause increases in levels of markers of neurodegeneration in the regions examined.

In response to acute DE exposure, we found that male animals showed reduced proliferation in all regions examined. Similarly, adult neurogenesis, as assessed by survival of adult born neurons, was also impaired in all regions investigated. These effects were mitigated by blocking microglial activation through pretreatment with the PPARγ agonist pioglitazone. By contrast, adult neurogenesis only reduced in females in the olfactory bulb. We also determined that a three-week exposure was only sufficient to significantly increase α-synuclein in midbrain, but that a ten-week exposure was able to increase levels of Dyrk1a, $A\beta_{42}$, and Tau-pS199. This work supports the idea that exposure to high levels of traffic-related particulate air pollution, as modeled by DE exposure, may significantly contribute to the development of neurodegenerative conditions through a mechanism likely to involve neuroinflammation and microglial activation, and could contribute to cognitive decline through dysregulation of adult neurogenesis.

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Dedication

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Chapter 1 : Introduction

1.1 Neurotoxicity of air pollution

As global population increases, anthropogenic pollutants resulting from increased urban density and transportation demands threaten to compromise air quality as never before (Cramer, 2002). In certain megacities such as New Delhi, where use of diesel-powered vehicles predominates, it is estimated that traffic may contribute to up to 72% of air pollution (Goyal et al., 2006). Despite abundant alternative energy sources, diesel remains a fuel of choice for mass transit vehicles, passenger vehicles, heavy machinery, and freight conveyances, and as a consequence, diesel exhaust (DE) is a major contributor to traffic related air pollution (Verboven, 2002). DE contains a vast array of components, including nitrogen and sulfur oxides, carbon monoxide, hydrocarbons, volatile organic compounds (VOCs), metals, organic and elemental carbon, and particulate matter in a variety of sizes, and its makeup varies by engine load conditions and fuel composition (Cooney and Hickey, 2008; Lim et al., 2009). Fortunately, improved diesel engine technology and the use of specialized catalytic converters have changed the composition of DE, resulting in an output lower in concentration of coarse fraction particulates, nitrogen oxides, volatile organic compounds, and carbon monoxide (Su et al., 2008). Additionally, more stringent air quality guidelines in certain localities have reduced allowable sulfur content of diesel fuel, resulting in a lower output of sulfur oxides (Stanislaus et al., 2010). However, the content of $PM_{2.5}$ (particulates smaller than 2.5 μm in diameter) and ultrafine particulate matter (UFPM), is estimated to be unchanged, and the inflammatory potential of DE particulates from these low emission engines is greatly increased, due to the higher proportion of fine particulates (Su et al., 2008). Airborne burden of $PM_{2.5}$ in certain parts of the world may exceed concentrations of 100 $\mu g/m^3$ for long periods of time, particularly in large urban centers of Asia (Allen et al., 2013; Kandlikar and Ramachandran, 2000; Sun et al., 2004). It is precisely these fine particulates that

are most concerning, as they are capable of penetrating the epithelium of the lungs and the olfactory mucosa, where they can then enter into general circulation in the body (Oberdörster et al., 2004).

While exposure to high levels of particulate matter has long been associated with adverse effects on the respiratory system, more recent studies in both humans and animals have shown that exposure is also linked to morbidity concerning the cardiovascular, endocrine, and central nervous systems (Calderón-Garcidueñas et al., 2008, 2012; Costa, 2017; Costa et al., 2014a, 2017; Genc et al., 2012). Although the conditions induced by exposure to particulate air pollution are very diverse in terms of the systems they affect and their pathological features, oxidative stress and inflammation, both of which are consistently reported effects of exposure to particulates, are likely to figure importantly in the etiopathology (Li et al., 2003). Inhaled UFPM may enter the CNS either by way of the olfactory nerve, or through the tight endothelial junctions that comprise the vascular interface of the blood brain barrier, which may be rendered more permeable by higher levels of peripheral oxidative stress and inflammation (Lochhead et al., 2010). Increased peripheral inflammation and macrophage activation may also exacerbate existing neuroinflammation, as these conditions can result in increased permeability of the blood brain barrier to allow both the migration of activated macrophages and the passive diffusion of pathogens and other foreign bodies into the CNS (Elwood et al., 2017; Lochhead et al., 2010; Stranahan et al., 2016). Experimental exposures to DE have been shown to increase levels of proinflammatory cytokines such as IL-6 and TNF-α, and of oxidative stress, as well as increase microglial activation in the brains of acutely exposed mice at environmentally relevant concentrations (Cole et al., 2016).

Importantly, increased levels of oxidative stress and neuroinflammation are conditions that are common to various psychiatric and neurodegenerative conditions, such as schizophrenia,

anxiety, depression, and Alzheimer's disease (AD) (Agostinho et al., 2010; Aricioglu et al., 2016; Furtado and Katzman, 2015; Heneka et al., 2015). Increased levels of both IL-6 and, in particular TNF-α, have been observed in the brains of individuals suffering from AD, suggesting a possible mechanism by which DE exposure might favor the development of the neurodegenerative disorder (Wang et al., 2015).

Exposure to traffic-related particulate air pollution is also notable in its ability to prevent exercise-induced increases in levels of brain-derived neurotrophic factor (BDNF), a powerful stimulator of proliferation and survival of young adult-born neurons originating in the neurogenic regions of the brain (Bos et al., 2011). Lower levels of BDNF, which are associated with poorer performance on cognitive tasks in healthy adults and various neurodevelopmental and psychiatric disorders, may help explain more rapid cognitive decline in geriatric adults exposed to higher levels of particulate air pollution (Jindal et al., 2010; Laske et al., 2011; Shimada et al., 2014). Posthumous examinations of the brains of both humans and animals exposed to high levels of traffic related air pollution show adverse changes relative to control brains from relatively unpolluted regions (Calderón-Garcidueñas et al., 2012). Specifically, this manifests itself in increases in levels of inflammatory and oxidative biomarkers, and histological abnormalities in the CNS consistent with neurodegeneration (Costa et al., 2014a, 2017). Remarkably, children living in areas high in particulate air pollution develop neuropathology in their brains that is more characteristic of elderly adults with AD, a condition noticeably absent in children from areas low in traffic-related air pollution (Calderón-Garcidueñas et al., 2008).

Other studies which have focused on the cognitive effects rather than the histological characteristics of neurodegeneration have uncovered a relationship between exposure to high concentrations of traffic related air pollution and both AD type dementia and PD (Chen et al.,

2017; Palacios, 2017). These findings suggest that exposure to high levels of ambient particulate matter may explain increases in the incidence of neurodegenerative conditions relative to unexposed individuals (Chen et al., 2017; Palacios, 2017). Traffic-related particulate air pollution, particularly $PM_{2.5}$ and finer, has the ability to adversely affect the CNS through a variety of mechanisms, both by directly accessing the CNS through the olfactory nerve, and by inducing peripheral inflammation and oxidative stress, which can, in turn induce infiltration of the brain parenchyma by activated peripheral leukocytes (Rezai-Zadeh et al., 2009).

1.2 Oxidative stress and the brain

Oxidative stress is defined as an imbalance between reactive oxygen species (ROS) and the body's antioxidant defenses (Betteridge, 2000). The damaging activities of ROS are implicated in the etiopathology of a diverse assortment of health conditions, including cancer, autoimmune conditions, cardiovascular disease, neurodegeneration, and psychiatric disorders (Pham-Huy et al., 2008). ROS are a natural by-product of ATP synthesis, generated by the process of oxidative phosphorylation, and can also be generated from the activities of microglia such as the redox cycling of the NADPH oxidases (NOXs) and nitric oxide synthases (NOS) but may also be born from the actions of environmental toxicants such as transition metals, and secreted as part of the immune response to pathogens (Valko et al., 2005; Vatassery et al., 2004). They include chemical species such as the hydroxyl radical, the superoxide radical, and the hypochlorite anion (Haklar et al., 2001).

As the brain is composed largely of lipids, many of which contain unsaturated carbon chains, it is highly vulnerable to attack by reactive oxygen species, a process known as lipid peroxidation (Sultana et al., 2013). Lipid peroxidation results in the formation of reactive aldehydes such as malondialdehyde, 4-hydroxynonenal (4HNE), and acrolein, which are highly

electrophilic and capable of forming adducts to many different kinds of biomolecules (Sultana et al., 2013). Another consequence of oxidative stress may be the formation of isoprostanes and isothromboxanes, prostaglandin-like inflammatory molecules derived from nonenzymatic reaction of ROS with arachidonic acid (Montuschi et al., 2004). They are strongly vasoconstrictive and pro-aggregative, and as such, they potentially set the stage for cerebrovascular events such as thrombotic and ischemic stroke (Cracowski et al., 2001).

Oxidative stress tends to also result in nitrative stress as well, due to the increased synthesis of nitric oxide brought on by inflammatory conditions (Sharma et al., 2007). High levels of ROS may also result in the formation of reactive nitrogen species such as the peroxynitrite anion, which is created from the reaction of superoxide with nitric oxide (Beckman and Koppenol, 1996). Peroxynitrite is also notable in its ability to suppress oxidative phosphorylation, the most productive pathway for the synthesis of ATP (Vatassery et al., 2004). The aging brain tends to lose its capacity for aerobic glycolysis, another important pathway for ATP synthesis in neurons (Goyal et al., 2017; Jones and Bianchi, 2015). Inability to produce sufficient ATP in the brain may result in impaired synaptic transmission in neurons, and may also result in cognitive decline (Owen and Sunram-Lea, 2011; Pathak et al., 2013). Nitration of certain biomolecules, such as the microtubule associated tau protein can disrupt microtubule stability and impair the trafficking of mitochondria as well as vesicles, both of which may be critical to survival of neurons (Zhang et al., 2005). Thus, oxidative and nitrative stress may compromise neuronal viability through the combination of reduced capacity for cellular energy production and the formation of adducts on biomolecules.

Mild oxidative stress, by contrast, may be neuroprotective, and confer greater resistance to oxidative insult (Ristow and Zarse, 2010). Dietary polyphenols like curcumin and resveratrol may have antioxidant properties at minute concentrations, and result in the mitigation of oxidative

damage to the cell in a process known as mitohormesis (Evans et al., 2017; Rainey et al., 2015; Ristow, 2014). The benefit of low levels of oxidative stress may likely come from the induction of antioxidant pathways and proteins such as Nrf2/Keap1, uncoupling protein-1 (UCP1), and the heat shock proteins (HSPs) (Ristow, 2014).

Traffic-related air pollution contains many components with the ability to induce oxidative damage, such as ozone, fine particulate matter, and sulfur and nitrogen oxides (Yang and Omaye, 2009). Additionally, experimental exposures of animals to traffic-related air pollution have shown that exposure may elevate markers of oxidative stress in the brain and peripherally (Cole et al., 2016; Lodovici and Bigagli, 2011). These studies, coupled with the likely involvement of oxidative stress in the process of neurodegeneration and abundant epidemiological studies showing an association between air pollution exposure and neurodegeneration, strongly imply a role for traffic-related air pollution as an environmental component in the etiopathology of neurodegeneration (Cheignon et al., 2018; Chen et al., 2017).

1.3 Neuroinflammation in neurodegeneration and neurogenesis

Neuroinflammation is defined as an increased immune response in the central nervous system, characterized by the release of pro-inflammatory cytokines and activation of both microglia and astrocytes (O'Callaghan et al., 2008; Pekny and Pekna, 2016). When appropriately induced, the state of neuroinflammation may be considered a healthy and normal physiological response to both brain injuries and foreign bodies present in the CNS, as it is necessary for the clearance of particles, pathogens, and necrotic or otherwise nonviable cells (Arcuri et al., 2017). Secretion of pro-inflammatory factors such as certain cytokines during acute timeframes may provoke phagocytic behavior in activated microglia and eventually allow for the regeneration of damaged tissues and cells (Wang et al., 2015). While the phagocytosis of nonviable cells within

the CNS is a desirable outcome, there is evidence that phagocytosis provoked by the prolonged inflammatory state may result in excess neuronal loss, including the phagocytosis of previously viable and unharmed cells, a process called phagoptosis (Neher et al., 2011).

However, elevated and unresolved immune response, when persistent, can have a number of adverse effects on the CNS (Frank-Cannon et al., 2009). Many of the pro-inflammatory factors released during neuroinflammation are also pro-apoptotic, which may inhibit the survival of neurons by sending "death signals." One of these proinflammatory and pro-apoptotic cytokines is TNF-α, which, by binding to TNFR1 and TNFR2 receptors, may promote apoptosis in distinctly different ways (Wang et al., 2015). The prolonged state of neuroinflammation is characterized by elevated levels of pro-inflammatory cytokines such as TNF-α and IL-6, and may exert a sort of one-two punch on the brain, both provoking apoptosis and phagocytosis of previously viable cells, as well as inhibiting the proliferation of neural progenitor cells and their differentiation into new adult born neurons (Iosif et al., 2006; O'Callaghan et al., 2008; Olmos and Lladó, 2014; Rath and Aggarwal, 1999; Vallières et al., 2002).

Increased neuroinflammation may also contribute to loss of neurons by inducing excitotoxicity in vulnerable populations of neurons, particularly through TNF-α signaling (Olmos and Lladó, 2014). High concentrations of TNF-α may inhibit glutamate reuptake by microglia and astrocytes, resulting in binding of the ligand to excitatory receptors, particularly the AMPA receptor, expression and externalization of which is also increased in response to TNF-α (Fine et al., 1996; Leonoudakis et al., 2004).

Due to the separation of the CNS from peripheral circulation by the endothelial layer of the blood-brain-barrier and the presence of endogenous immune cell populations, macrophage migration from the periphery is normally very restricted (Rezai-Zadeh et al., 2009). However,

peripheral inflammation may compromise the integrity of the blood-brain barrier, allowing for migration of activated peripheral macrophages (Persidsky et al., 1999). In particular the cytokines IL-1B, TNF-α, and IL-6, can have a suppressive effect on adult neurogenesis, particularly in the hippocampus ((Iosif et al., 2006; Vallières et al., 2002; Wu et al., 2013). Chronic overexpression of these cytokines not only inhibits the proliferation and maturation of new adult-born neurons, but can also reduce the survival of existing mature neurons, as some of them, in particular TNF-α, are known to induce apoptosis (Yuan and Yankner, 2000). TNF-α may also contribute to neuronal death by reducing reuptake of glutamate, thereby exacerbating existing excitotoxicity in a mechanism that involves the activity of the AMPA receptor (Olmos and Lladó, 2014). Developmentally, TNF-α is recognized as a potent promoter of neurogenesis, in particular acting to stimulate proliferation in the subventricular zone (SVZ) through activation of the TNFR1 receptor (Bernardino et al., 2008). However, in the adult brain, the effect of is quite different, resulting in a reduction in the number of adult-born neurons (Bernardino et al., 2008). A more thorough discussion of the process of adult neurogenesis will be given in Section 1.4.

A state of heightened immune response is observed even in the brains of cognitively healthy elderly individuals (Simen et al., 2011). In general, the elderly brain is characterized by a loss of neurons and an increase in the number of glial cells relative to the younger brain (Conde and Streit, 2006). Additionally, even in non-diseased aged brains, microglia show a more reactive phenotype than in younger brains, with a morphology characterized by shorter thicker processes with less complex dendritic arbors (Spittau, 2017). Numerous studies have shown that neuroinflammation and activation of both microglia and astrocytes can result in impaired adult neurogenesis, due to elevated levels of proinflammatory cytokines (Iosif et al., 2006; Vallières et al., 2002).

However, the mode of glial activation is also important, informing whether the effect is neurotoxic or neuroprotective (Cherry et al., 2014). These cytokines are produced by both astrocytes and microglia, which may be activated and take on a classical (A1/M1) polarization or the alternative (A2/M2) polarization (Liu et al., 2014). The M1 polarization of microglia, which can, in turn, induce A1 classical activation of astrocytes, may be acutely necessary for the clearance of particles and pathogens, and resolving brain injuries (Liddelow et al., 2017). However, because of the release of many pro-inflammatory and pro-apoptotic factors, when microglia and astrocytes remain in the classically activated state for an extended period of time, the phenotype is considered to be neurotoxic (Liao et al., 2012; Liddelow et al., 2017). By contrast, the A2/M2 polarization is neuroprotective, characterized by anti-inflammatory cytokine release and trophic support, favoring resolution of inflammation and survival of neurons (Jha et al., 2016). Neuroinflammation increases expression of the dual specificity tyrosine regulated kinase 1b (Dyrk1b), resulting in an increase in astrocyte activation (He et al., 2018). A1 activated astrocytes and M1 microglia may also engage in excessive pruning of synapses, an activity which is hypothesized to be related to both normal aging and the dementia that epitomizes AD (Hong et al., 2016). Excessive synaptic pruning is also believed to be related to the etiology of schizophrenia, suggesting perhaps that the delusions and hallucinations experienced by individuals who suffer from certain neurodegenerative conditions may be influenced by this process, and be induced at least partly by persistently activated astrocytes and microglia (Liddelow et al., 2017). Thus, the ability of traffic-related air pollution to induce microglial activation, oxidative stress and neuroinflammation tend to be strongly implicated as causative mechanisms for its involvement in the etiopathology of neurodegenerative diseases.

The ability of acute DE exposure to induce neuroinflammation and microglial activation, along with the ability of both pro- and anti-inflammatory signaling molecules to regulate adult neurogenesis, strongly imply that DE could potentially contribute to the development of neurodegenerative disorders in a way that potentially involves the dysregulation of adult neurogenesis, thereby compromising synaptic plasticity and the formation of new memories.

1.4 Adult neurogenesis

Adult neurogenesis is the process by which neurons are generated in the postnatal brains of vertebrates and invertebrates alike. In humans and other mammals, neural stem cells proliferate, migrate, and differentiate into fully functional neurons in specific regions of the postnatal brain (Fig 1.2) (Ming and Song, 2011). This process is believed to occur even into old age and up until death, though the existence of adult neurogenesis in humans is still a subject of some controversy (Galvan and Jin, 2007; Sorrells et al., 2018). The slowing rate of neurogenesis, coupled with the gradual loss of neurons and synapses, results in shrinkage of the brain and the subtle decline in cognitive function observed in even healthy elderly adults (Peters, 2006). Many immature neurons do not survive to become integrated into the neural circuitry of the brain; nonetheless, they contribute to long term potentiation, a process whereby synaptic transmission is increased (Pfisterer and Khodosevich, 2017; Schreurs et al., 2017). In certain *ex vivo* models where neurogenesis is impaired, long term potentiation is also reduced (Brown et al., 1988). In most mammals, neurogenesis in the postnatal brain is limited to the subgranular zone of the hippocampus (SGZ) and the subventricular zone (SVZ) (Ming and Song, 2011). While adult born neurons may be found in the mammalian olfactory bulb (OB), they generally originate in the SVZ as neuroblasts, migrating in a catenulate pattern along the rostral migratory stream (RMS) (Wang et al., 2011). In cases of brain injury, migration of neuroblasts and NPCs from the neurogenic

regions may be directed towards the brain lesion, with vasculature serving as a guide for the migrating immature neurons (Kaneko et al., 2017). Certain interventions, whether behavioral or pharmacological, that stimulate neurogenesis can improve recovery time and limit the lasting effects of the injury, and may act by increasing levels of brain derived neurotrophic factor (BDNF) (Griesbach et al., 2009; Lu et al., 2005; McFadden et al., 2011; Waterhouse et al., 2012). BDNF is a potent promoter of adult neurogenesis in both in vitro and in vivo systems, favoring proliferation of neural stem cells as well as the initiation of a neuronal lineage, and the maturation and survival of adult-born neurons (Lim et al., 2008; Lipsky and Marini; Waterhouse et al., 2012).

Lifestyle choices that modulate BDNF, such as frequency and intensity of physical activity, stress level, macronutrient balance, and antioxidant intake, can therefore modulate adult neurogenesis and influence cognitive function in adults of all ages (Mirescu and Gould, 2006; Poulose et al., 2017; Saraulli et al., 2017). Diets high in fat can suppress neurogenesis and impair cognition, while intake of dietary polyphenols such as resveratrol and curcumin can stimulate the generation of new adult-born neurons and improve memory and affect (Dong et al., 2012; Evans et al., 2017; Greenwood and Winocur, 2005; Lindqvist et al., 2006; Park et al., 2010; Torres-Pérez et al., 2015; Zhang et al., 2012). A common characteristic of both moderate exercise and low doses of polyphenols such as curcumin and resveratrol is that they tend to induce mild oxidative stress in a manner that is protective, ultimately having a hormetic effect (Goto and Radák, 2009; Kumar et al., 2016; Rainey et al., 2015). The antagonism of inflammation and oxidative stress in the CNS and peripherally, as well as the induction of mitochondrial biogenesis and mitochondrial protection, may help explain the neurogenesis-promoting and neuroprotective action of physical activity and polyphenols (Evans et al., 2017; Rehman et al., 2013). By contrast, the tendency of high sugar and high fat diets to increase indices of oxidative stress and inflammation as well as

mitochondrial dysfunction, explain the neurotoxic and neurogenesis-suppressing effects (Giugliano et al., 2006). Neuroinflammation may have a suppressive effect on adult neurogenesis through a number of pathways (Ekdahl et al., 2003) (Fig 1.1). In particular, TNF-α is known to negatively regulate adult neurogenesis, primarily through its interactions with the TNFR1 receptor, while IL-6 may act more indirectly to suppress neurogenesis, possibly by affecting the hypothalamic-pituitary-adrenal axis and through the increased peripheral secretion of glucocorticoids, increased levels of which are associated with impaired adult neurogenesis (Iosif et al., 2006; Vallières et al., 2002).

Regulation of adult neurogenesis also appears to be strongly connected to cognitive function as well as affective state (Cameron and Glover, 2015). The hippocampus, one of the brain regions strongly associated with spatial memory and learning, is also one of the recognized sites of neurogenesis in the postnatal brain (Shrager et al., 2007). The low membrane threshold of depolarization that characterizes young neurons makes them more excitable, therefore useful in long-term potentiation, a process which results in the strengthening of synapses over time (Brown et al., 1988). While learning is possible and new memories can be formed in the absence of adult neurogenesis, the process may take longer and certain cognitive domains may be impaired (Jaholkowski et al., 2009; Patzke et al., 2015). Cetaceans, including dolphins, widely considered as highly intelligent animals in many respects, do not show evidence of hippocampal neurogenesis and also display impaired learning in certain hippocampus-associated cognitive domains, such as spatial memory, relative to species in which neurogenesis is observed (Jaholkowski et al., 2009; Patzke et al., 2015). One possible explanation for this is that while immature neurons may play a preferential role in the formation of memories associated with hippocampal learning domains,

older neurons may recruited, though, lacking the lower threshold of depolarization, they may be less effective at long term potentiation (Bischofberger, 2007; Patzke et al., 2015).

Individuals showing extensive AD-type neuropathology but showing conserved adult neurogenesis, a metric which is disrupted in both individuals with mild cognitive impairment and AD, have unimpaired cognitive function relative to controls, while those with impaired neurogenesis and absent neuropathology, showed mild cognitive impairment (Briley et al., 2016)

In various neurodegenerative conditions, perturbations of adult neurogenesis have been noted (Winner and Winkler, 2015). In ALS, hippocampal neurogenesis (SGZ) was observed to be suppressed while SVZ neurogenesis had been increased (Galán et al., 2017). Similarly, in both AD and mild cognitive impairment, neurogenesis may be impaired, while in individuals with even extensive AD type neuropathology with conserved neurogenesis, cognitive function may be equal to that of control subjects (Briley et al., 2016). The involvement of young neurons in synaptic plasticity and the formation of new memories strongly implicates perturbations of adult neurogenesis in the cognitive deficits that exemplify neurodegeneration (Bischofberger, 2007).

Thus, the presence of disrupted neurogenesis may indicate the presence of conditions such as increased inflammation and oxidative stress that may contribute to the development of cognitive decline, which may in turn set the stage for the development of serious neurodegenerative disorders.

1.5 Neurodegenerative diseases and their risk factors

As life expectancy increases in both industrialized and developing countries alike, the burden of neurodegenerative disease on public health resources is expected to grow substantially (Dorsey et al., 2013). In the United States, Alzheimer's disease (AD) disease is the most prevalent

neurodegenerative condition, estimated to affect approximately 5 million elder Americans, with nearly 2 million more suffering from Parkinson's disease (PD), Lewy body dementia, and amyotrophic lateral sclerosis (ALS) collectively (Hogan et al., 2016; Pringsheim et al., 2014; Rocca et al., 2011; Wagner et al., 2016). Though the timing and specific manifestation of symptoms may vary between the disorders, they are all marked by progressive loss of neurons and histopathological abnormalities as well as cognitive impairment, sleep disruptions, emotional lability, and movement disorders, and can also include hallucinations and delusions (Williams and Lees, 2005). While there is currently no cure, both pharmaceutical and non-drug interventions can temporarily alleviate symptoms and perhaps modestly slow cognitive decline, if not ultimately change the course of the disease (Bredesen, 2014; Gauthier et al., 2008). In its later stages, neurodegenerative diseases can severely compromise quality of life and ability to function independently, greatly increasing the economic burden on families of the afflicted, who shoulder the bulk of caregiving costs and labor (Jutkowitz et al., 2017).

The first symptoms of AD are typically failures of short term memory and executive function, with steady global decline in cognitive ability as the disease progresses (Baudic et al., 2006). This loss of cognitive ability appears to be related to dysfunction and death of neurons in the hippocampus and the cortex, the regions of the brain which perform functions associated with the creation of new memories, organization and self-regulation (Ball et al., 1985; Elliott, 2003). While deficits in olfactory discrimination and identification have been noted and are considered part of the prodromal phase of AD, the inability to recognize distinctive odors is a consequence of decline in cognitive function rather than reduced olfactory acuity (Alves et al., 2014). True hyposmia and anosmia resulting from impaired synaptic function and loss of neurons in olfactory

areas such as the entorhinal cortex, the anterior olfactory nucleus, and the olfactory bulb is a feature of later stage AD (Alves et al., 2014; Martinez-Marcos et al., 2011).

As with AD, some of the first symptoms of PD may manifest as olfactory deficits. The decline in olfactory function seen in early PD is characterized by the inability to detect certain distinctive odors such as dill pickle and black licorice, but others can be easily smelled and correctly identified (Bohnen et al., 2008). This form of oddly specific hyposmia results from impaired dopaminergic signaling, and, unlike the olfactory deficits typically observed in AD, may not worsen or change even as other aspects of the disease progress (Bohnen et al., 2008). Other early symptoms of PD tend to be motor disorders, particularly in the upper limbs, and may manifest as micrographia, tremor, bradykinesia, impaired balance, and uncontrollable movements, particularly during sleep (Mazzoni et al., 2012).

Posthumous examinations of brains from AD patients have revealed the presence of characteristic lesions, specifically neurofibrillary tangles (NFTs) and amyloid plaques, also known as senile plaques (Perl, 2010). NFTs are insoluble inclusions composed of aggregations of paired helical or straight filaments, abnormal structural variants of the normally soluble microtubule-associated protein tau, which can occur when tau is excessively and inappropriately phosphorylated at certain residues (Brion et al., 2001). Hyperphosphorylation of tau, particularly toward the N-terminus of the protein, impairs binding of tubulin and compromises microtubule polymerization and stability (Derisbourg et al., 2015). Microtubules are critical to both structure and function of neurons, as they provide a scaffolding allowing for the exaggerated form of the neuron and allow for the trafficking of vesicles along the length of the axon (Dent and Baas, 2014). The loss of microtubule polymerization leads to loss of structure of the neuron function in the neuron. Phosphorylation of tau may be increased threefold in AD patients relative to control

individuals (Iqbal et al., 2010). When sites such as serine 199 (pS199) are excessively phosphorylated, the transport and distribution of mitochondria along the axon of the neuron may be disrupted, compromising cellular viability (Cheng and Bai, 2018). Mitochondria tend to be concentrated in areas where energy requirements are particularly high, such as nodes of Ranvier, growth cones, and in synapses (Chevalier-Larsen and Holzbaur, 2006). The resulting mitochondrial insufficiency in the neuron, especially where a ready source of ATP is most needed, can impair synaptic plasticity and compromise effective propagation of action potentials, thus impairing the ability of the neuron to function properly (Rintoul and Reynolds, 2010).

Like AD, sufferers of PD show characteristic histopathologies of the principle brain regions concerned. However, the brain abnormalities of PD are most associated with the dopaminergic regions of the brain, namely the striatum and the midbrain (Dickson, 2012). Two of the subregions within the midbrain and the brain stem, the substantia nigra pars compacta and the locus cœruleus, are rich in populations of catecholaminergic neurons bearing the black pigment neuromelanin, which gives the regions their distinctive eponymous colors (Isaias et al., 2016). The death of these characteristic neurons results in loss of neuromelanin, an effect detectable by MRI or by unaided visual examination of the post mortem brain (Mandel et al., 2010). Prior to their disappearance, these neurons may be observed bearing proteinaceous nodules known as Lewy bodies (Wakabayashi et al., 2007). Lewy bodies are fibrillary inclusions and, although more than 70 different proteins have been detected inside them, they are largely composed of α-synuclein and ubiquitin (Beyer et al., 2009). Overexpression of α-synuclein appears to inhibit the process of mitochondrial fusion and interfere with vesicular transport, both of which can compromise cellular viability (Gomes et al., 2011; Kamp et al., 2010; Scott and Roy, 2012). Although the appearance of Lewy bodies is associated with neurodegeneration and eventual neuronal death, the

sequestration of α-synuclein in aggresomes that compose them is ultimately protective, preventing apoptosis induced by excess expression of the protein (Johnston et al., 1998).

While advanced age (>65 years) remains the most consistent factor observed among individuals diagnosed with AD and PD, a number of biological, environmental, and genetic risk factors favoring development of the condition have been identified (Guerreiro and Bras, 2015; Launer et al., 1999; Letenneur et al., 2000). Level of education is a variable that appears to modify risk of both PD and AD, with higher attainment appearing to reduce risk of developing the diseases (Kotagal et al., 2015; Letenneur et al., 2000). It has been hypothesized that higher levels of education might protect against cognitive decline by increasing an individual's cognitive reserve, defined as resistance to neuropathology, in terms of the ability to recruit alternative cognitive strategies when the default pathway has become compromised (Tucker-Drob et al., 2009). However, other studies show higher childhood cognitive ability is associated with higher educational attainment as well as reduced risk of dementia, suggesting that education level is a product of cognitive reserve and perhaps innate intelligence, which may be largely determined before an individual reaches adulthood (Cavanagh and Wong, 2018; Wakabayashi et al., 2007).

Certain disorders in which peripheral inflammation is elevated, such as diabetes and obesity, may favor the development of neurodegeneration and increase risk of developing several disorders (Castro et al., 2017; Profenno et al., 2010). One notable exception is rheumatoid arthritis (RA), which is actually associated with reduced risk of developing AD (Policicchio et al., 2017). The reduced risk profile is not likely due to the disease itself, as only those who are under a treatment regime of classical disease-modifying antirheumatic drugs (cDMARDs) such as methotrexate, display lowered risk of developing AD (Policicchio et al., 2017). By contrast, habitual users of nonsteroidal anti-inflammatory drugs (NSAIDs) may not enjoy the same

protections, and in some studies, heavy users of NSAIDS show increased late onset dementia risk (Breitner et al., 2009). Although the reason for this has not been investigated in detail, a likely mechanism by which the cDMARDs reduce risk is suppressed expression of the pro-inflammatory cytokines TNF-α and IL-6 (1996; Wakabayashi et al., 2007). While IL-6 production may be reduced by certain NSAIDS, an increase in the production of TNF-α has been observed, suggesting, perhaps, that TNF-α is more important in the etiopathology of neurodegeneration than IL-6 (Fiebich et al., 1996; Page et al., 2010). Overexpression of these two cytokines in particular is found in the brains of individuals suffering from AD (Jha et al., 2016). Notably, TNF-α is a potent inducer of sleep, and studies have shown increased expression of levels of the cytokine in men deprived of sleep for one night, perhaps suggesting another mechanism by which individuals suffering from sleep disorders experience increased risk of dementia and AD (Chennaoui et al., 2011). Increased levels of TNF-α are also associated with increased presence of neurofibrillary tangles and amyloid plaques (Decourt et al., 2017). These findings may help explain a mechanism by which perturbation of sleep in rodents results in increased AD type pathology in the brain, suggesting a derangement of tau and amyloid metabolism induced by poor quality or insufficient sleep (Di Meco et al., 2014). The cytokines TNF-α, IL-1β, and IL-6, are involved in the induction of different aspects of sleep (Jewett and Krueger, 2012). Thus, poor quality, or insufficient sleep may serve as a potent inducer of neuroinflammation, and may set the stage for the later development of neurodegenerative conditions, particularly when it is combined with certain classes of medications, such as benzodiazepines and anticholinergics,which are independently associated with increased risk of dementia and development of AD in later years (Gage et al., 2014; Gray et al., 2015).

Cumulative use of anticholinergic medications including antihistamine drugs, some soporific drugs, certain antipsychotics, and some anticonvulsants have all been associated with the development of AD and dementia (Gray et al., 2015; Richardson et al., 2018). One of the earlier symptoms of AD is impaired cholinergic signaling, which may result from amyloid beta plaque deposition and the presence of neurofibrillary tangles impinging upon the function of cholinergic neurons (Nunes-Tavares et al., 2012; Rinne et al., 1988). Lower levels of activity of choline acetyltransferase, the enzyme which catalyzes the synthesis of the neurotransmitter acetylcholine, have been noted in various regions of the brain in sufferers of AD (Rinne et al., 1988). As sleep disorders are also associated with increased risk of developing dementia and reduced levels of choline acetyltransferase, the prevalent use of anticholinergics as sleep aids may potentiate risk of experiencing severe cognitive decline in old age (Lie et al., 2015).

Another factor influencing risk of neurodegeneration is exposure to environmental toxicants (Cannon and Greenamyre, 2011). Traffic-related air pollution, of which diesel is rich in many components with a demonstrated ability to induce oxidative stress and neuroinflammation, namely various metals, fine particulates, ozone and other reactive chemicals such as acrolein (Jaganjac et al., 2012; Totlandsdal et al., 2015). Moreover, a number of epidemiological studies show a correlation between exposure to the highest levels of air pollution and severity of cognitive decline and the diagnosis of a neurodegenerative condition (Chen et al., 2017; Weuve et al., 2012). While the evidence linking neurodegeneration and air pollution exposure is strongest for Alzheimer's disease (AD), a few studies have also suggested an association for amyotrophic lateral sclerosis and Parkinson's disease (PD) (Chen et al., 2017; Palacios, 2017; Seelen et al., 2017). The ability of fine particulate air pollution to induce oxidative stress and neuroinflammation, as well

as accelerate plaque formation in a mouse model of AD, strongly implicate the involvement of exposure to air pollution as a causative agent in the etiology of neurodegeneration.

Oxidative stress, or the presence of reactive oxygen species (ROS) in excess of the body's capacity to neutralize them, seems to be present in the brains of AD sufferers (Smith et al., 2000). Exposures to oxidants, such as metals and hydrogen peroxide, can induce the aggregation of Aβ peptides, suggesting that oxidative damage has a role in causing AD as well as being a product of the disease (Liu et al., 2006). Polymorphisms in antioxidant genes can compromise the ability of individuals to quench ROS, making them more vulnerable to pro-oxidant environmental exposures (Doukali et al., 2017; Polonikov et al., 2007). The synthesis of glutathione, an essential endogenous antioxidant, is catalyzed by a number of enzymes, notably glutamate-cysteine ligase (GCL), which is composed of two subunits, a catalytic (Gclc) and a modifier (Gclm) subunit (Franklin et al., 2009). Impaired glutathione synthesis resulting from genetic polymorphisms, especially in the Gclm gene, is relatively common (up to 30% in some populations), and in transgenic animal models of Gclm deficiency, GSH levels may be as little as 10% of that of individuals with the predominant genotype (Nakamura et al., 2002; Yang et al., 2002). Lower GSH levels may be associated with increased risk of certain cardiovascular and cerebrovascular events for individuals, and may also be found in the brains of individuals with amnestic mild cognitive impairment, a condition that precedes AD and full dementia (Mandal et al., 2015; Skvortsova et al., 2017). Some studies have found that heterozygosity in Gclm may confer greater vulnerability to environmental pollutants than homozygous null status, possibly due to upregulation of antioxidant genes such as carbonyl reductase 3 (CBR3) that may occur in the homozygotes but not the heterozygotes (Schaupp et al., 2015). However, both Gclm heterozygous and Gclm-/- animals may show an increased susceptibility to the neurotoxic effects of DE exposure relative to wild-

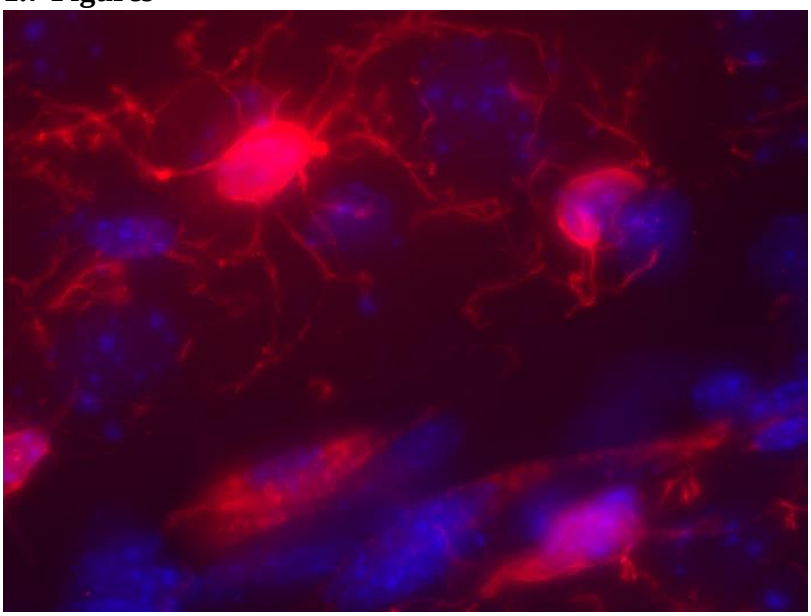
type animals (Cole et al., 2016). Controlled acute exposures to DE resulted in Gclm +/- and -/- mice showing higher levels of oxidative stress as well as neuroinflammation, indicated by higher levels of TNF-α and IL-6, elevated levels of which are observed in the brains of AD sufferers (Cole et al., 2016; Mandel et al., 2010). These studies suggest that perhaps polymorphisms in antioxidant genes may affect inflammation and oxidative stress both peripherally and in the CNS, modulating risk of developing neurodegenerative conditions. Thus, polymorphisms in the Gclm gene cannot be ignored as a risk factor for developing neurodegenerative disorders, as they may increase the vulnerability of individuals bearing the mutation to neuroinflammation and oxidative stress brought on by exposure to environmental toxicants.

1.6 Aims of dissertation research

In general, the aging body and brain are characterized by increased levels of oxidative stress, inflammation, and mitochondrial dysfunction. In the brain specifically, this translates into a tendency towards increased neuroinflammation, microglia and astrocytes with a more reactive phenotype, an increase in the number of glia relative to neurons, and reduced mitochondrial function and biogenesis in neurons, a cell type with very high ATP requirements. These factors are all associated with reduced cognitive function as well as certain psychiatric conditions. Some degree of cognitive diminution is virtually inevitable as one ages, although environmental and genetic factors may inform the degree and rapidity of the decline. While some will retain much of their mental faculties throughout life, other individuals may develop mild cognitive impairment, or dementia resulting from a neurodegenerative condition such as AD. Thus, there is a relatively large reference range for normal age-related cognitive decline in the absence of pathological cognitive function. It has been hypothesized that certain biological factors such as sex may potentially increase risk of developing neurodegenerative conditions, particularly in conjunction

with environmental exposures that may tend to cause neuroinflammation and oxidative stress. Epidemiological studies have shown a link between exposures to high levels of fine particulate air pollution and risk of neurodegeneration, particularly AD. These studies are also supported by experimental animal exposures to traffic-related air pollution as modeled by DE, which show increased levels of oxidative stress, neuroinflammation, and microglial activation, higher levels of which may be observed in the brains of individuals suffering from neurodegenerative disorders. Accordingly, the specific aims of this dissertation are (1 determine if acute DE exposure suppresses adult neurogenesis and (2) determine if subchronic DE exposure affects levels of markers of neurodegeneration.

1.7 Figures



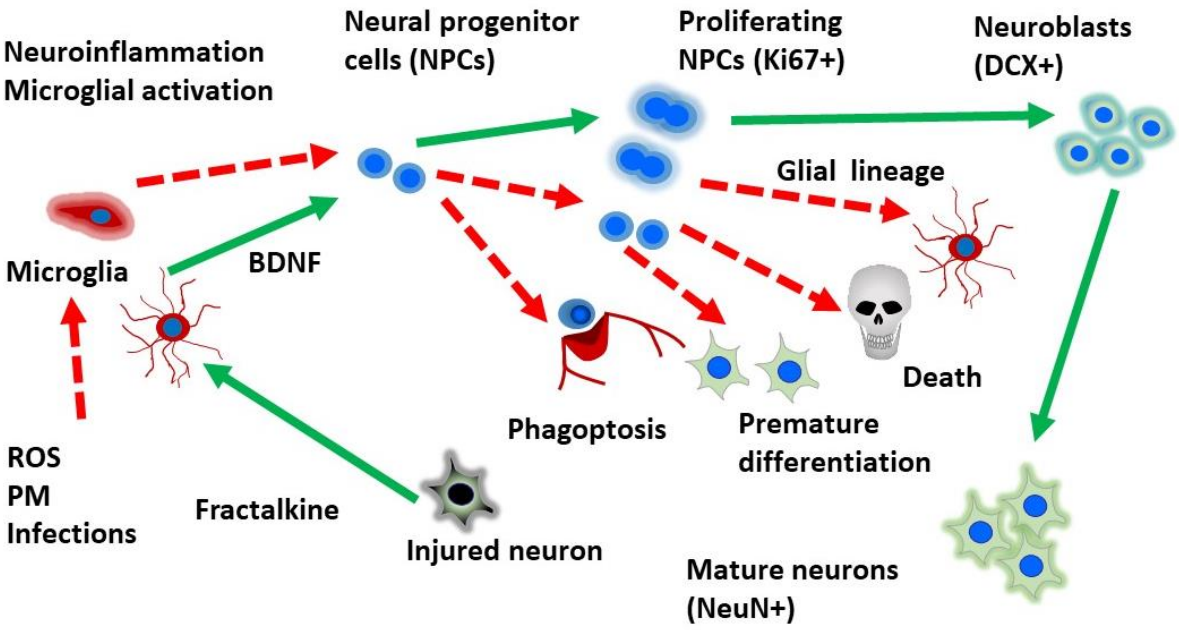


Figure 1.1 Microglia and the process of adult neurogenesis.

Microglia, the macrophages of the CNS (micrograph at top), may become primed or activated and display changes in morphology, as well as release proinflammatory and proapoptotic signals. This condition, called neuroinflammation, may disrupt adult neurogenesis by inhibiting proliferation of NPCs, inducing a glial lineage, causing premature death or differentiation, or causing the phagocytosis of stressed but otherwise viable young neurons in a process called phagoptosis. (Source: J. Coburn; micrograph and diagram)

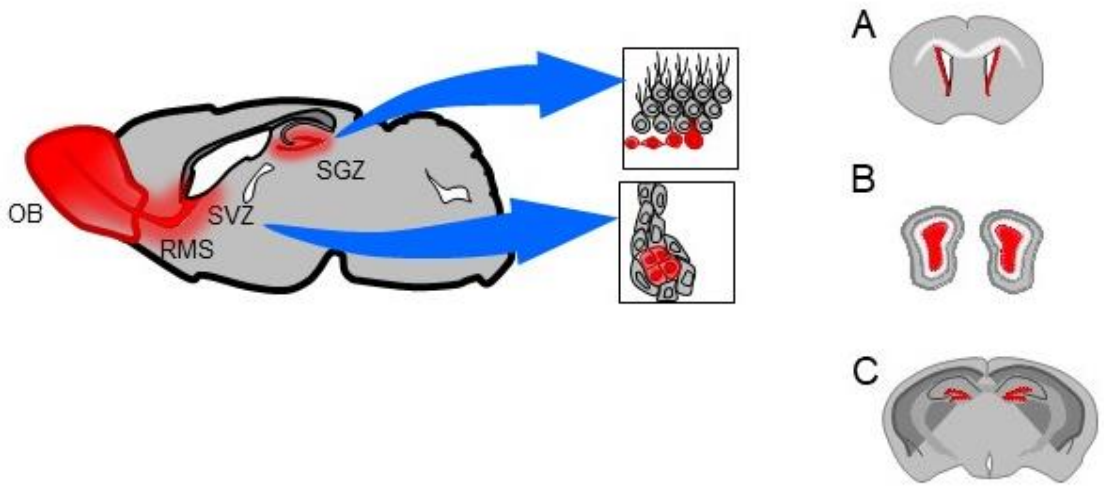


Figure 1.2 Neurogenic centers in the postnatal mouse brain.

The birth of new neurons in the adult brain is restricted to specific regions. Neural progenitor cells in the SVZ (A) and SGZ (C) divide and differentiate into neuroblasts. From the SVZ, neuroblasts migrate through the rostral migratory stream (RMS) to the OB (B), where they become functional local interneurons. (Source: J. Coburn)

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Chapter 2 : Acute exposure to diesel exhaust impairs adult neurogenesis in mice: prominence in males and protective effect of pioglitazone

2.1 Abstract

Adult neurogenesis is the process by which neural stem cells give rise to new functional neurons in specific regions of the adult brain, a process that occurs throughout life. Significantly, neurodegenerative and psychiatric disorders present suppressed neurogenesis, activated microglia, and neuroinflammation. Traffic-related air pollution has been shown to adversely affect the central nervous system. As the cardinal effects of air pollution exposure are microglial activation, and ensuing oxidative stress and neuroinflammation, we investigated whether acute exposures to diesel exhaust (DE) would inhibit adult neurogenesis in mice. Mice were exposed for 6 h to DE at a $PM_{2.5}$ concentration of 250–300 $\mu g/m^3$, followed by assessment of adult neurogenesis in the hippocampal subgranular zone (SGZ), the subventricular zone (SVZ), and olfactory bulb (OB). DE impaired cellular proliferation in the SGZ and SVZ in males, but not females. DE reduced adult neurogenesis, with male mice showing fewer new neurons in the SGZ, SVZ, and OB, and females showing fewer new neurons only in the OB. To assess whether blocking microglial activation protected against DE-induced suppression of adult hippocampal neurogenesis, male mice were pre-treated with pioglitazone (PGZ) prior to DE exposure. The effects of DE exposure on microglia, as well as neuroinflammation and oxidative stress, were reduced by PGZ. PGZ also antagonized DE-induced suppression of neurogenesis in the SGZ. These results suggest that DE exposure impairs adult neurogenesis in a sex-dependent manner, by a mechanism likely to involve microglia activation and neuroinflammation.

2.2 Introduction

Air pollution derives from a variety of sources, including industrial and vehicular emissions and biomass burning, and contains several components such as organic and inorganic particulates,

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

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

Neuroinflammation and microglial activation can adversely influence regeneration of neurons in the brain (Carpentier and Palmer, 2009; Ekdahl et al., 2003). The birth of new neurons in the adult brain, and their survival and functional integration into existing neural circuitries, known as adult neurogenesis, is restricted in rodents to two regions: the subgranular zone (SGZ) in the dentate gyrus of the hippocampus, and the area adjacent to the lateral ventricles and striatum, known as the subventricular zone (SVZ) (Alvarez-Buylla et al., 2001). The SVZ is also the point of origin for immature neuroblasts that migrate along the rostral migratory stream to the olfactory bulb (OB), where they develop into local interneurons (Pignatelli and Belluzzi, 2010). Neurogenesis occurs in the adult brain constantly, though at a rate that decreases with age (Ming and Song, 2011), possibly because of increased neuroinflammation and microglial activation (Schuitemaker et al., 2012). Microglial activation resulting from lipopolysaccharide administration, or increased levels of pro-inflammatory cytokines such as interleukin-6 (IL-6), or tumor necrosis factor-α (TNF-α) can impair hippocampal neurogenesis (Ekdahl et al., 2003; Iosif et al., 2006; Vallières et al., 2002). Even chronic peripheral inflammation may have an adverse effect on the CNS, as increased permeability of the endothelium of the blood–brain barrier allows activated macrophages to migrate into the relatively restricted cerebrospinal compartment, triggering microglial activation and neuroinflammation (Raghavendra et al., 2004; Takeshita and Ransohoff, 2012). Disruption of adult neurogenesis may affect cognitive and olfactory function (Frankland and Miller, 2008), and may lead to severe cognitive impairment in neurodegenerative diseases. Indeed, even in the presence of characteristic Alzheimer's disease (AD) neuropathology, cognitive function is equal to that of healthy individuals when neurogenesis is preserved (Briley et al., 2016). The gradual loss of neurons, coupled with a reduction in young adult born neurons, plays a role in normal diminution of cognitive function due to the unique role of young neurons in

memory (Bishop et al., 2010). Young neurons are very excitable, having a low threshold of depolarization, and they appear to be preferentially activated during the formation of new memories (Bischofberger, 2007). Short-term memory also appears to be dependent on the activities of young neurons, and impairment of short-term memory is one of the first symptoms to emerge in the earliest stages of AD (Baudic et al., 2006). The ability to remember and forget is related to synaptic plasticity, or the weakening and strengthening of neural pathways over time, which also appears to be affected by neurogenesis (Saxe et al., 2006). Decreased neurogenesis, and the resulting impaired synaptic plasticity, may also be involved in depression and anxiety, and explain the therapeutic effectiveness of antidepressants that increase neurogenesis (Hayley and Litteljohn, 2013).

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

2.3 Materials and Methods

Animals

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

Bromodeoxyuridine treatment

A stock solution of bromodeoxyuridine (BrdU) was prepared using 20-mg/mL BrdU (Sigma-Aldrich, St. Louis, Missouri) and 0.007-N sodium hydroxide to facilitate dissolution in sterile normal saline (0.9% sodium chloride). The solution was buffered to pH 7.4 and sterile-filtered prior to use. Each animal was weighed and given an initial dose of 100-mg/kg BrdU by intraperitoneal injection. Mice received four more doses of 100-mg/kg BrdU given at 2-h intervals, resulting in a cumulative dose of 500 mg/kg/day (Pan et al. 2013a; Taupin 2007).

Exposure to diesel exhaust

Individually housed mice were exposed for 6 h to FA or DE (at a $PM_{2.5}$ concentration of 250–300 μg/m3). Exposures to either FA or DE were conducted simultaneously in the University of Washington Controlled Exposure Laboratory's Northlake Diesel Facility, which includes an SPF mouse housing room with Allentown caging systems (Allentown, NJ), with the housing racks modified to ventilate cages with either diluted DE or FA. DE was derived from a Yanmar YDG5500 diesel generator, with a load bank maintaining 75% of rated capacity, using No. 2 undyed, ultra-low sulfur on-highway fuel and Royal Purple Duralec 15W-40 Synthetic crankcase oil to lubricate moving parts, as previously described (Fox et al. 2015; Gould et al. 2008). During exposures, DE concentrations were continuously measured and maintained at steady levels using

a feedback controller monitoring fine particulate levels (Fox et al. 2015; Gould et al. 2008). DE particles had a mean aerodynamic diameter of 100 nm. Characterization of DE is described in detail in a previous publication (Fox et al. 2015). For assays of lipid peroxidation and of TNF-α mRNA levels, mice were euthanized by CO2 asphyxiation within 2 h after the end of the exposure, and brain regions were dissected immediately, flash-frozen in liquid nitrogen, and stored at − 80 °C. For Iba1 and Ki67 immunohistochemistry, mice were euthanized 18 h after the end of exposure, and their brains fixed by transcardial perfusion and then embedded, sectioned, and stored at − 80 °C in cryoprotectant medium. For NeuN/BrdU immunohistochemistry, animals were pre-treated with 500-mg/kg BrdU the day before exposure, exposed for 6 h, then euthanized 21 days following exposure, to label adult-born cells that are still alive. Their brains were fixed, frozen, and sectioned as described above, and stored at − 80 °C in cryoprotectant medium (Pan et al. 2013a).

Lipid peroxidation assay

Lysates were prepared from frozen brain region samples homogenized in CLB lysis buffer (10-mM HEPES; 150- mM NaCl; 1-mM $CaCl_2$; 0.5-mM $MgCl_2$; 10-μg/ml leupeptin; 10-μg/ml aprotinin; 1-mM PMSF; 50-mM NaF). The homogenate was incubated on ice for 10-min, centrifuged at 4 °C and 2000 × g for 5 min, and the resulting supernatant samples were stored at − 80 °C until ready for analysis. The protein content of each sample was determined using the Pierce bicinchoninic acid (BCA) assay (Thermo Scientific, Waltham, MA), with bovine serum albumin (BSA) as a standard, according to the manufacturer's protocol. Lipid peroxidation was measured by quantifying levels of malondialdehyde (MDA), a byproduct of lipid peroxidation, using the Thiobarbituric Acid Reactive Substances (TBARS) assay (Cayman Chemical, Ann Arbor, MI)

according to the manufacturer's instructions, as previously described (Giordano et al. 2013). MDA content was normalized to the amount of protein loaded per well.

RNA extraction and real time PCR analysis

Prior to extraction, all working surfaces and instruments were cleaned with an RNAse inhibitor. Tissue homogenates were prepared using TRIzol RNA extraction reagent (Invitrogen, Carlsbad, California) and purified according to the manufacturer's protocol. Concentration and quality of RNA were determined using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). RNA samples (1 μg) were reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) according to the manufacturer's protocol. The resulting cDNAs were used for PCR amplification in the presence of primers specific to tumor necrosis factor (TNF-α) (forward: GTC GTA GCA AAC CAC CAA GTG; reverse: CTT TGA GAT CCA TGC CGT TGG; 21 bp) and the housekeeping gene hypoxanthine phosphoribosyltransferase (HPRT1) (forward: GAG GAG TCC TGT TGA TGT TGC CAA G; reverse: GGC TGG CCT ATA GGC TCA TAG TGC; 25 bp). Amplification was carried out in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City, California) and quantification was carried out in a Bio-Rad CF384 Real-timer System thermal cycler, using iTaq Universal SYBR Green Supermix.

Pioglitazone treatment

A stock suspension of 1.25-mg/mL pioglitazone hydrochloride (PGZ; 98% pure; Sigma-Aldrich, St. Louis, Missouri) was prepared in a vehicle (VEH) of 0.5% carboxymethylcelluose sodium dissolved in PBS. For 4 days, animals were treated with 10 μL/g of PGZ (12.5 mg/kg) or

10-μL/g VEH by oral gavage, using a 20G stainless-steel curved feeding needle, each morning, up to and including the day of DE exposure (Drew et al. 2015; Maeda et al. 2008).

Transcardial perfusion and post-fixation

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

Olfactory bulb immunohistochemistry

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

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

SGZ and SVZ immunohistochemistry

Every eighth coronal section of the subventricular and hippocampal region of each brain was cut at a thickness of 30 μm using a Reichert-Jung Cryocut-1800 cryostat (Leica) with temperature set at − 25 °C. Sections were stored at − 20 °C in 24-well plates containing cryprotectant medium until processed. Prior to staining, sections were rinsed in PBS to remove cryoprotectant and embedding medium. For Ki67 and Iba1 immunohistochemistry, no antigen retrieval method was used prior to permeabilization. For NeuN/BrdU double-staining, a 30-min DNA-denaturing step with 2N hydrochloric acid and subsequent neutralization with 0.1-M borate buffer was required to improve immunolabeling of BrdU. The sections were then permeabilized with PBST and blocked

overnight in a buffer containing 1% BSA (w/v) and 10% normal goat serum (v/v) in PBST. Sections were then incubated for 48 h with the appropriate primary antibodies [BrdU (rat, 1:1000): MCA2060, AbD Serotec/Bio-Rad; NeuN (mouse, 1:1000): MAB377, EMD Millipore; Iba1 (1:1000): ab107159, and Ki67 (1:1000): ab15580, Abcam, Cambridge, Massachusetts]. Sections were rinsed in PBST then incubated with AlexaFluor 594 goat anti-rat and AlexaFluor 488 goat anti-mouse antibodies (1:1000), AlexaFluor 568 donkey anti-goat (1:1000), or AlexaFluor 555 donkey anti-rabbit antibody (1:1000) for 48 h. The sections were rinsed in PBST and incubated in Hoechst 33,342 to identify nuclei. Following nuclear staining, sections were washed in PBS, and then mounted on gelatin-coated slides. VectaShield Fluorescence Mounting Medium (H-1000, VectaShield, Burlingame, California) was used to mount the sections and prevent photobleaching. No. 1 coverslips (VWR, South San Francisco, California) were placed on top of the sections and secured using nail polish. The slides were then placed in slide boxes and kept in the dark at 4 °C until ready for imaging (Jongbloets et al., 2017; Pan et al., 2012, 2013a).

Image acquisition and analysis

For assessment of adult neurogenesis, images were captured in three channels using a Marianas Imaging System, which included a Zeiss 200M Axiovert microscope with a motorized stage, a 175-W xenon lamp, and a Roper HQ Cool Snap digital camera. The software SlideBook 6.0 (3i Intelligent Imaging Innovations, Denver, Colorado) was used to set parameters of image capture. In the SGZ and SVZ, 40× 3D images of all BrdU-positive cells were collected. Images were uniformly adjusted for brightness and contrast. All BrdU-positive cells that contained a well-defined nucleus were counted, and BrdU-positive nuclei immunoreactive to NeuN were counted as double-labeled cells. The ratio of BrdU+/NeuN+ cells to all BrdU+ cells was calculated for each specimen and expressed as a percentage (Pan et al., 2012, 2013a, 2013b). For adult neurogenesis

in the OB, a 20× montage of each section was taken. The granular cell layer of each section was then defined and stereologically sampled at 12.5% of the cross-sectional area. As with the SGZ and SVZ, three channel 40× images were uniformly adjusted for color and brightness. The number of BrdU+ cells and the number of newborn neurons (NeuN+/BrdU+ cells) were expressed as density (number per cubic mm of region of interest) rather than a total number per region. Newborn neurons were also expressed as a percentage of all BrdU+ cells. To assess proliferation in the SGZ and SVZ, two-channel images of all Ki67+ cells were taken in the SGZ and SVZ. Images were uniformly adjusted for contrast and brightness, and all Ki67+ cells with a well-defined nucleus were counted and expressed as a total number per region. To assess microglial morphology, two-channel 40× images of the hippocampal dentate gyrus were captured using the Marianas Imaging System for imaging and Slide-Book 6.0 to set parameters of image capture. Two-channel 40× images were adjusted uniformly for contrast and brightness, and perimeter of microglial somata on the plane with the sharpest resolution of the nucleus were traced using the irregular drawing tool and analyzed to evaluate shape descriptor parameters using the open-source software FIJI. In addition to the microglial soma area, three other parameters were assessed: circularity ($C = 4\pi$*area/perimeter^2), roundness ($R = 4$*area/π(major axis)^2), and aspect ratio (AR = major axis/minor axis) (Jonas et al., 2012; Morrison and Filosa, 2013; Torres-Platas et al., 2014).

Statistical analysis

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

2.4 Results

As observed previously with this paradigm of DE exposure (Cole et al., 2016), no morbidity was observed in animals exposed to 250–300-μg/m3 DE for 6 h, and there were no salient behavioral or physical differences between DE-and FA-exposed animals of either sex.

Fluorescence immunohistochemistry was used to analyze the neurogenic regions of the brain (OB, SVZ, and SGZ).

Cellular proliferation was assessed by immunohistochemistry using Ki67 in the SGZ and SVZ. In male mice, DE exposure was associated with a significant reduction in the number of Ki67-immunopositive cells in both the SGZ (Fig. 2.1a) and the SVZ (Fig. 2.1b). In contrast, no significant differences were seen between DE- and FA-exposed female mice in the number of Ki67-immunopositive cells in either SGZ (Fig. 2.1a) or SVZ (Fig. 2.1b).

For assessment of adult neurogenesis, tissue sections containing the OB, SVZ, and SGZ were probed with antibodies specific to the cellular proliferation marker BrdU and the neuronal nuclear marker NeuN. BrdU+ and double-labeled cells (NeuN+/BrdU+) were counted and expressed as a total number and as a percentage of all BrdU+ cells. In male mice, the total number of BrdU+ cells was significantly reduced following DE exposure in both the SGZ (Fig. 2.2a) and the OB (Fig. 2.4a), but not in the SVZ (Fig. 2.3a). The number of double-positive NeuN+/BrdU+ cells was reduced in DE-exposed males in all three regions (Figs. 2.2b, 2.3b, 2.4b) compared to FA-exposed males. NeuN+/BrdU+ double-labeled cells expressed as a percentage of all BrdU+ cells were also significantly reduced in the SGZ (Fig. 2.2c), SVZ (Fig. 2.3c), and OB (Fig. 4c) of DE-exposed males compared to FA controls. No significant differences were seen between DE- and FA-exposed female mice in the SGZ (Fig. 2.2) or SVZ (Fig. 2.3) in either the number of BrdU+ cells (Figs. 2.2a, 2.3a) or the number or percentage of double-labeled NeuN+/BrdU+ cells

(Figs. 2.2b, c, 2.3b, c). However, in the OB (Fig. 2.4), the total number of double-labeled NeuN+/BrdU+ cells was significantly reduced in DE-exposed females compared to FA-exposed controls (Fig. 2.4b).

Pioglitazone (PGZ) has been shown to suppress DE-induced microglial activation in vitro (Roque et al. 2016). Since microglial activation and ensuing neuroinflammation has been shown to affect adult neurogenesis, we assessed the effect of pre-treatment with PGZ on DE-induced inhibition of adult neurogenesis in the SGZ. Male mice were used in this experiment, because they were more sensitive than females to the effects of DE exposure. Mice were pretreated with PGZ or VEH for 4 days, then exposed to DE or FA for 6 h, and sacrificed 18 h later for assessment of microglial activation, or 3 weeks later for assessment of adult neurogenesis. Iba1 immunohistochemistry was used to define the microglial somata in the dentate gyrus of the hippocampus, and shape descriptors associated with reactive microglial phenotypes were used to assess the degree of microglial activation associated with DE exposure. Acute DE exposure resulted in changes in microglial morphology typically associated with microglial activation, and these changes were antagonized by PGZ pre-treatment (Fig. 2.5). Specifically, microglial soma area was increased significantly with DE exposure (Fig. 2.5a), while circularity of microglial soma defined as $4\pi(\text{area}/\text{perimeter}^2)$ and roundness of the soma defined as $4*\text{area}/\pi(\text{major axis})^2$ were significantly reduced by DE exposure (Fig. 2.5b, c). Finally, aspect ratio (Fig. 2.5d), the quotient of the major and the minor axis of the best-fit ellipse, was significantly increased in microglia of the DE-exposed mice compared to FA controls. PGZ decreased the effects of DE exposure on all parameters of microglial morphology (Fig. 2.5a–d), indicating that the treatment was effective at inhibiting microglial activation. This was further confirmed by the findings that the increases in the levels of TNF-α mRNA and of lipid peroxidation in cortex and hippocampus caused by DE

exposure were antagonized by pre-treatment with PGZ (Fig. 2.6a, b). As expected, DE exposure significantly reduced cellular proliferation in the SGZ of the VEH-treated mice (Fig. 2.7). PGZ pre-treatment antagonized this effect of DE. In animals pre-treated with PGZ DE exposure did not affect proliferation (Fig. 2.7). With regard to neurogenesis, in VEH-treated control animals, the total number of BrdU+ cells in the SGZ was reduced by DE exposure, and this effect was antagonized by PGZ (Fig. 2.8a). Similarly, NeuN+/BrdU+ cells expressed both as a total number and as percentage of all BrdU+ cells were reduced by DE exposure in VEH-treated controls, and this was also antagonized by pre-treatment with PGZ (Fig. 2.8b, c).

2.5 Discussion

The main findings of this study are that acute exposure to DE causes an impairment of adult neurogenesis in mice, which is likely secondary to neuroinflammation and oxidative stress that follow microglial activation, and that this effect is more pronounced in male animals. Young adult mice (8 weeks) were used in this study, and the exposure was for 6 h to a moderate/high concentration of DE (250–300-$\mu g/m^3$ $PM_{2.5}$). Such levels of $PM_{2.5}$ can often be reached and even exceeded in several cities worldwide, particularly in India and China (Allen et al., 2013; Costa, 2017; Kandlikar and Ramachandran, 2000; Sun et al., 2004). Traffic-related air pollution is a major contributor of $PM_{2.5}$ levels and DE is the major source of $PM_{2.5}$ and ultrafine particles (Calderón-Garcidueñas et al., 2015; Ghio et al., 2012). In some megacities such as New Delhi, where diesel fuel powers many passenger vehicles in addition to heavy machinery and public transportation conveyances, the contribution of traffic to air pollution is estimated to be as high as 72% (Goyal et al., 2006). The use of diesel fuel to power heavy machinery and vehicles of mass transit results in high daily exposures for those who work in certain occupations; miners and mechanics in bus garages regularly experience some of the very highest levels of particulate

air pollution, where PM2.5 May range from 300 to 1000 μg/m^3 (Pronk et al., 2009). It should also be noted that DE contains several other components that were not measured in this study, and their potential contribution to neurotoxicity cannot be discounted. In future studies, it would be of much interest to also measure PM deposition in brain over time.

A short-term (6 h) exposure to DE was sufficient to affect adult neurogenesis in different brain regions. We had previously shown that the same DE exposure caused activation of microglia, increased oxidative stress (elevated lipid peroxidation), and neuroinflammation (increased levels of pro-inflammatory cytokines; (Cole et al., 2016). These findings were confirmed in the present study, as lipid peroxidation and TNF-α mRNA levels were increased in cerebral cortex and hippocampus of DE-exposed mice (Fig. 2.6) and hippocampal microglia were activated (Fig. 2.5). Our earlier findings (Cole et al., 2016) also indicated that male mice are more sensitive to DE-induced oxidative stress and neuroinflammation. We had formulated the hypothesis of a possible higher susceptibility of male mice on the basis of a series of findings related to the levels of expression of the intracellular enzyme paraoxonase-2 (PON2). This enzyme has antioxidant and anti-inflammatory properties (Giordano et al., 2011; Schweikert et al., 2012), and is believed to have a neuroprotective role (Costa et al., 2014b). In vitro and in vivo studies have shown that males express lower levels of PON2 in brain and other tissues (Giordano et al., 2011, 2013); as such, they are more susceptible to oxidative stress and neuroinflammation than female mice (Costa et al., 2014b; Giordano et al., 2011, 2013), though additional mechanisms may also be involved in the differential susceptibility of male and female mice to DE-neurotoxic effects (Cole et al., 2016). Since it has been shown that microglial activation and increased levels of pro-inflammatory cytokines such as IL-6 or TNF-α impair hippocampal neurogenesis (Ekdahl et al., 2003; Iosif et al., 2006; Vallières et al., 2002), we

hypothesized that DE exposure would inhibit adult neurogenesis. Furthermore, based on our previous findings, we also hypothesized that DE-induced inhibition of neurogenesis would be more pronounced in male mice. Results of this study confirm both hypotheses. DE exposure impaired proliferation in the SGZ and SVZ only in male mice (Fig. 2.1); in the same two regions, adult neurogenesis was also impaired only in males (Figs. 2.2, 2.3). In the OB, the total number of adult-born neurons was reduced in males (Fig. 2.4), and to a minor extent was reduced in females (Fig. 2.4). Gender differences in adult neurogenesis have been observed in some studies. Male rodents show higher hippocampal neurogenesis (as we also observed, see Fig. 2.2), accompanied by better performance in tests of spatial memory (Perfilieva et al., 2001). Anxiety disorders, which are also associated with reduced neurogenesis, are more prevalent and disabling in women than in men (McLean et al., 2011). These differences may, in part, be due to the effect of the differential levels of androgens; indeed, androgens increase adult hippocampal neurogenesis by promoting survival of young neurons in the dentate gyrus (Hamson et al., 2013). Gender differences also appear to extend to the effect of environmental factors on adult neurogenesis; for example, hippocampal neurogenesis is impaired by high-fat diet and stress in male, but not female, rodents (Lindqvist et al., 2006). To test the hypothesis that inhibition of adult neurogenesis was secondary to microglia activation and ensuing oxidative stress and neuroinflammation, we utilized the PPAR-γ agonist PGZ. PGZ and other thiazolidinediones are recognized for their antidiabetic and hypolipidemic properties (Smith, 2001), effects that may be mediated by the modulation of different parameters of mitochondrial function, and the promotion of mitochondrial biogenesis (Bogacka et al., 2005; Corona and Duchen, 2016; Miglio et al., 2009; Smith, 2001). PGZ also has neuroprotective activities that are ascribed to attenuation of microglial activation (Ji et al., 2010). Indeed, PGZ can reduce inflammation and microglial

activation by activating the PPAR-γ of microglia, inhibiting the release of a number of pro-inflammatory substances (Carta and Pisanu, 2013; Drew et al., 2015; Ji et al., 2010). We had previously shown that PGZ inhibits the neurotoxicity of DE particles in a microglia–neuron coculture (Roqué et al., 2016). In the present study, we found that administration of PGZ to male mice antagonizes DE-induced microglial activation, as well as increases in lipid peroxidation and neuroinflammation in the hippocampus and the cerebral cortex (Figs. 2.5, 2.6). Upon PGZ pre-treatment, DE-induced inhibition of proliferation and adult neurogenesis in the SGZ was also inhibited (Figs. 2.7, 2.8).

The precise mechanisms by which DE exposure may inhibit adult neurogenesis are still elusive, though activation of microglia appears to play a relevant role. Microglia undergoes extensive cytoskeleton remodeling to better carry out certain functions such as phagocytosis (Arcuri et al., 2017; Perry and Teeling, 2013). While the default state of microglia is silent vigilance, in which they survey their territory with fine, highly branched processes (Ito et al., 1998), certain chemical signals (e.g., extracellular ATP and subtle changes in concentration of potassium), that may indicate damaged or stressed neurons, can induce chemotaxis, and may activate microglia, triggering them to release TNF-α (Gehrmann et al., 1995; Hide et al., 2000). Reactive microglia have greater cell body area and thicker, shorter processes (Jonas et al., 2012; Morrison and Filosa, 2013; Torres-Platas et al., 2014). It should be noted that microglia may either promote or inhibit adult neurogenesis (Sato, 2015), depending on the activation state (Aarum et al., 2003; Xu et al., 2017). Notably, the M2 activation state, which is induced by anti-inflammatory cytokines such as IL-4 and can be induced through activation of the PPAR-γ receptor, favors proliferation, and can direct neural progenitor cells (NPC) towards neurogenesis (Bouhlel et al., 2007; Cherry et al., 2014; Choi et al., 2017; Zhao et al., 2017). In contrast, the

inflammatory cytokines such as interferon-γ and TNF-α , as well as oxidative stress, induce the M1 (classical activation) state, provoking killing behavior in microglia and other macrophages (Colton, 2009). Thus, the two polarities of microglial activation have drastically different effects upon neurogenesis in the adult brain. Specific assessment of M1 (classical) and M2 (alternative) polarization upon exposure to DE would be useful to elucidate this issue. The mode of microglial activation may promote or inhibit NPC proliferation, cause them to embark upon a glial rather than a neuronal lineage, or favor either survival or apoptosis (Yuan et al., 2017). Another possibility is the phagocytosis of stressed but viable developing neurons by microglia, in a process called "phagoptosis" (Brown and Neher, 2014). The ability of PGZ to inhibit both microglial activation and the effects of DE on neurogenesis suggests that this event is critical to DE-induced inhibition of neurogenesis. However, PGZ may also have other mechanisms of neuroprotection (e.g., improving the number and efficiency of mitochondria); hence, additional or alternative mechanisms for the effects of DE on neurogenesis are possible.

Another potential mechanism relates to the ability of traffic-related air pollution to suppress production of brain-derived neurotrophic factor (BDNF) (Bos et al., 2011, 2012). BDNF is a neurotrophin with stimulant effects on proliferation of NSCs, favoring differentiation of NSCs into neuroblasts rather than glial cells, and promoting the survival of adult-born neurons (Binder and Scharfman, 2004). Activation of the PPAR-γ receptor with thiazolidinediones such as pioglitazone may act to increase neurotrophins such as BDNF (Prakash and Kumar, 2014). Additionally, factors such exercise or dietary polyphenols may increase BDNF signaling, and thus enhance neurogenesis (Sleiman et al.; Zhang et al., 2012). However, the increase in BDNF levels caused by exercise may be abrogated by exposure to particulate air pollution in both human and animal models (Bos et al., 2011, 2012). Increased lipid peroxidation and reduced

BDNF resulting from a high-fat diet impair proliferation in the SGZ, and direct treatment of NPCs with malondialdehyde in vitro also significantly reduced proliferation, which was then restored by treatment with BDNF (Park et al., 2010). Independent of the specific mechanism, suppression of neurogenesis by DE is of interest with regard to the reported reduced cognitive function and depression associated with traffic-related air pollution that has been observed in elderly adults (Chen et al., 2017; Lim et al., 2012; Weuve et al., 2012). Notably, impaired neurogenesis is an early event in the etiology of Alzheimer's disease (AD) (Demars et al., 2010). In particular, older adults with high levels of exposure to particulate air pollution show poorer cognitive outcomes than non-exposed individuals (Ailshire and Clarke, 2015). Aberrant neurogenesis is associated with poor cognitive functioning, and even individuals having characteristic AD neuropathology show no impairment of cognitive functions when neurogenesis is conserved (Briley et al., 2016). These considerations should, however, be tempered by the fact that in the present study, the persistence over time of the effect of DE on neurogenesis was not assessed and that an assessment of cognitive behavior was not carried out. A follow-up of this study should also likely investigate neuroinflammation at timepoints beyond what done here, to determine whether persistent recurrent neuroinflammation remains, or waves of neuroinflammatory responses are seen.

In conclusion, results of this study show that short-term exposures to moderate/high concentrations of DE induce neuroinflammation, oxidative stress, and microglial activation, and suppress adult neurogenesis and cellular proliferation in male mice, as evidenced by a reduced number of Ki67+ cells in the SVZ and SGZ, and a reduced number of surviving NeuN/BrdU double-labeled cells in the OB, SVZ, and SGZ. The effects were significantly less pronounced in female mice. When microglial activation was blocked by pre-treatment with the PPAR-γ agonist

PGZ, DE-induced suppression of adult neurogenesis was significantly antagonized. These findings underscore the importance of considering sex/gender when measuring neurotoxic effects, including alterations in neurogenesis, and reinforce the hypothesis that traffic-related air pollution may contribute to cognitive decline and perhaps also neurodegenerative diseases (Cacciottolo et al., 2017).

2.6 Figures

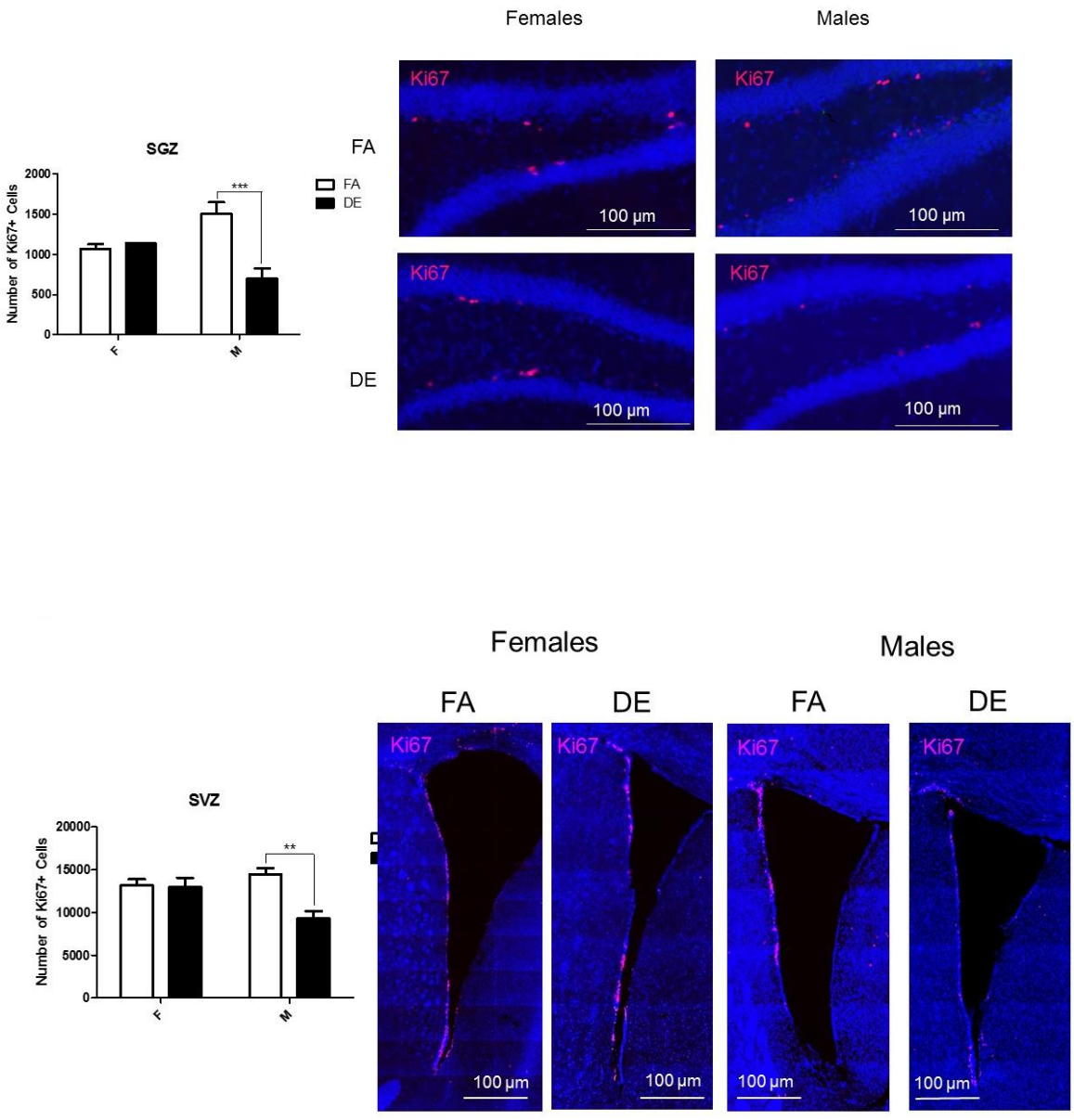

Figure 2.1 Acute DE exposure decreases proliferation in the SGZ (a**) and SVZ (**b**) of male mice.**

Eight-week-old male and female C57BL/6J mice were exposed to DE (250 µg/m^3) or FA for 6 h, and then sacrificed 18 h later. Images shown are representative micrographs of Ki67 immunohistochemistry in female and male mice. Results represent the mean (± SE) with $n = 3$ per group. Significantly different from FA, $^{**}p < 0.01$, $^{***}p < 0.001$. *SGZ* subgranular zone of the hippocampus, *SVZ* subventricular zone

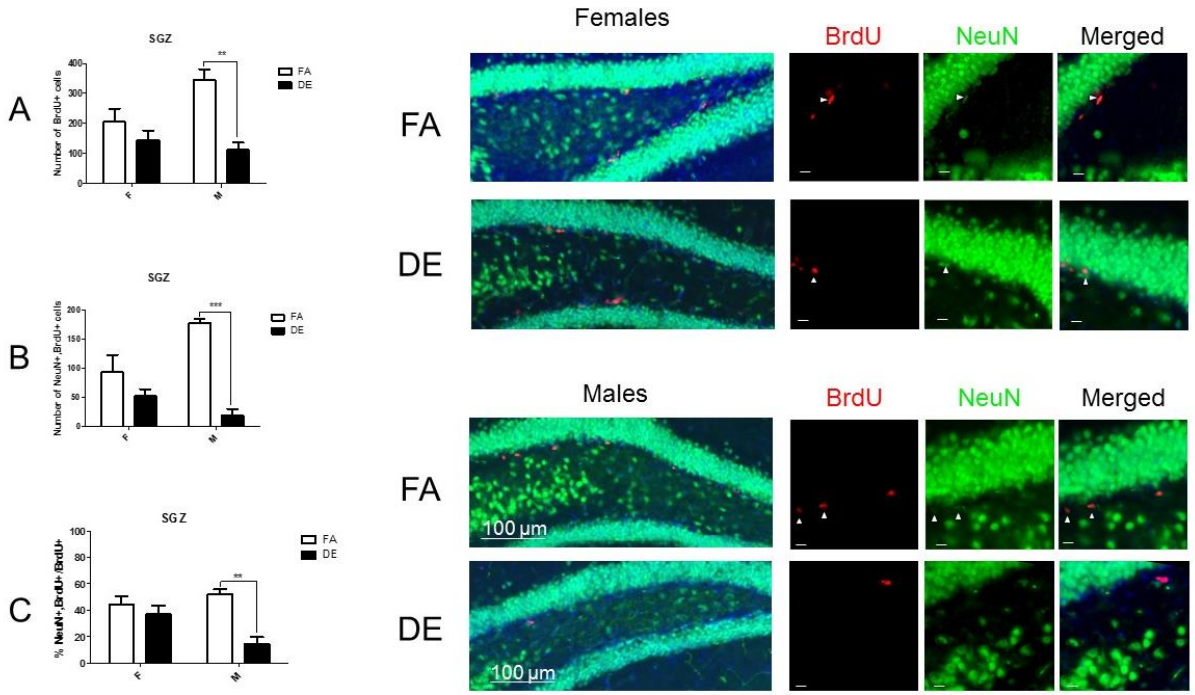

Figure 2.2 Effect of acute DE exposure on adult hippocampal neurogenesis in mice.

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

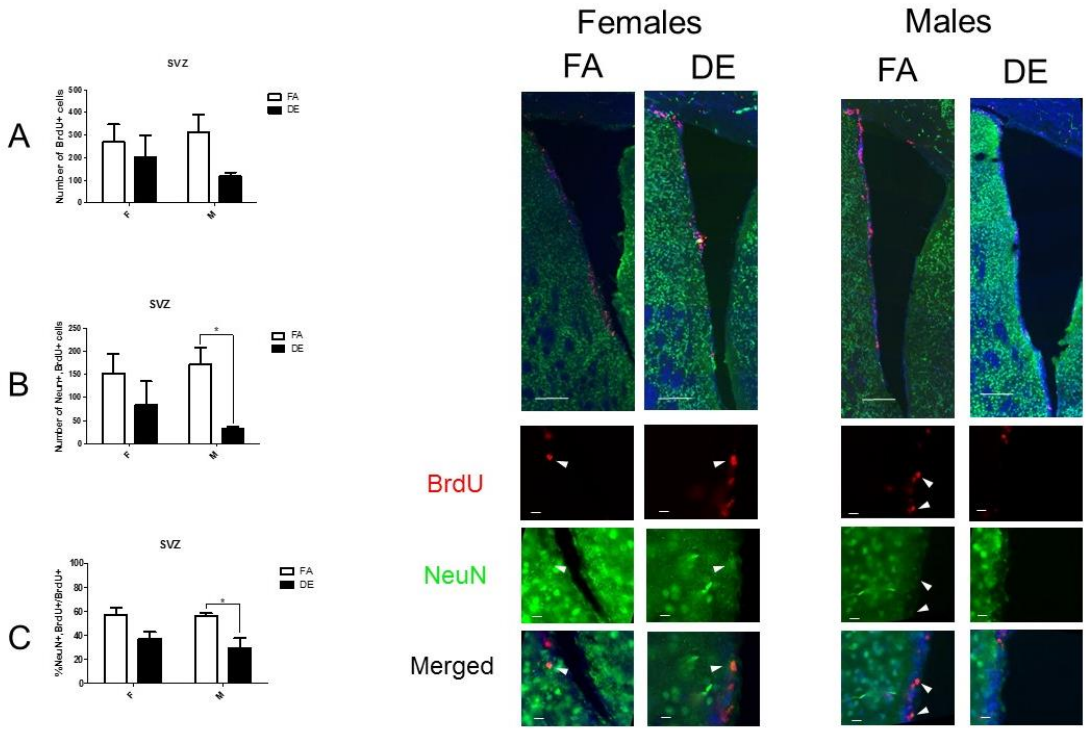

Figure 2.3 Effect of acute DE exposure on adult neurogenesis in the SVZ of mice.

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

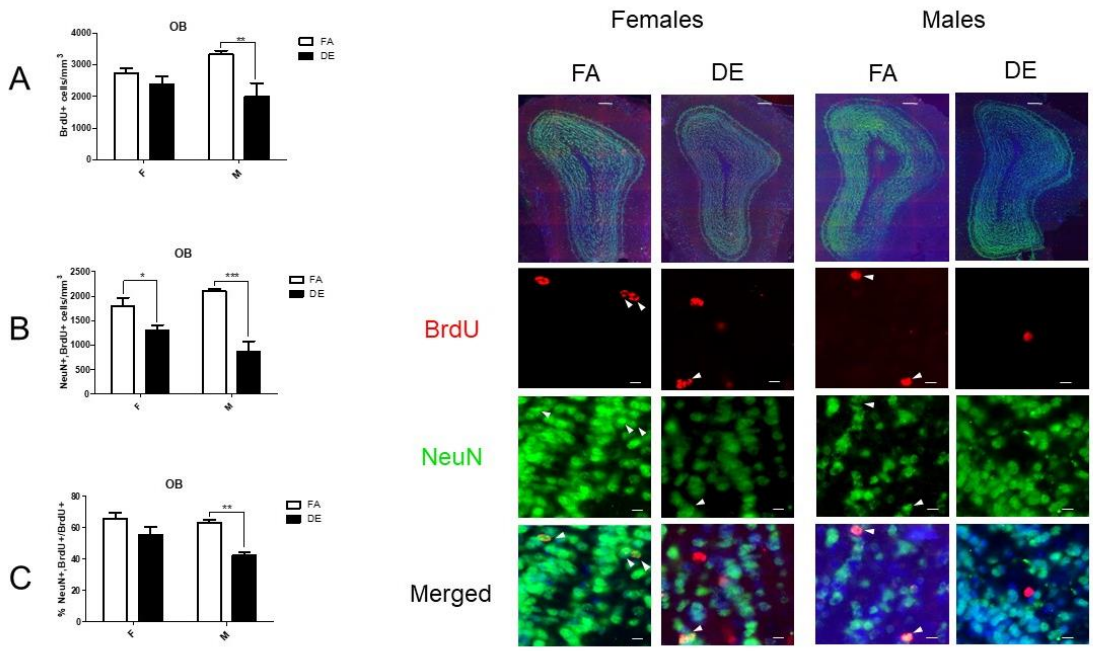

Figure 2.4 Effect of acute DE exposure on adult neurogenesis in the OB of mice.

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

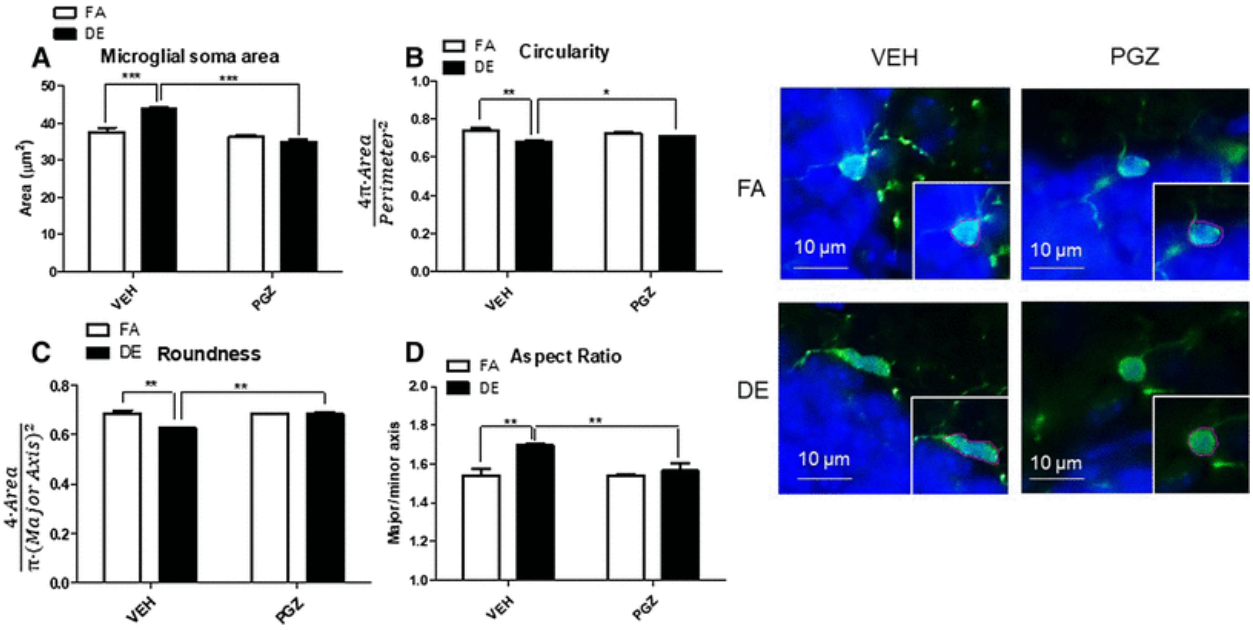

Figure 2.5 Pioglitazone (PGZ) pre-treatment suppresses microglial activation in the hippocampus of male mice.

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

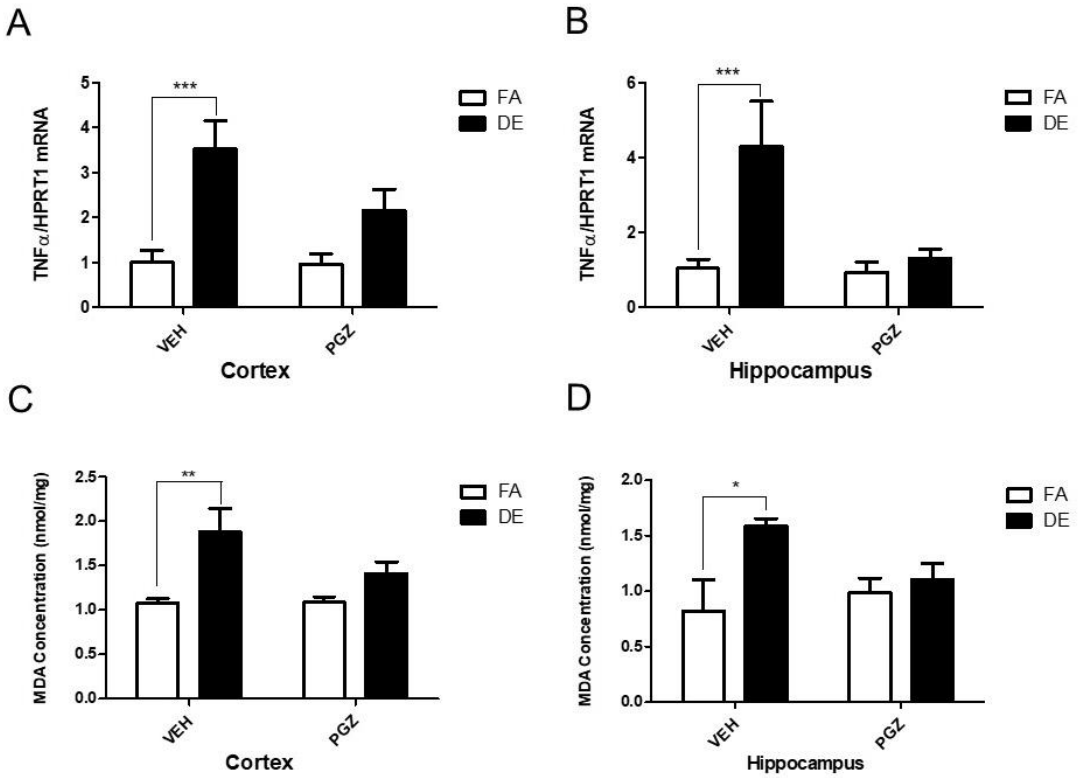

Figure 2.6 Effect of pioglitazione on oxidative stress and cytokine expression.

Lipid peroxidation (levels of malondialdehyde) and levels of TNF-α mRNA were assessed in the cerebral cortex (**a, c**), and the hippocampus (**b, d**) of male mice following 4 days of pre-treatment with either 12. 5-mg/kg/day PGZ or with VEH, followed by a 6-h exposure to DE (250 μg/m^3) or FA. Results represent the mean (± SE) with $n = 6$ per group. Significantly different from FA control, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

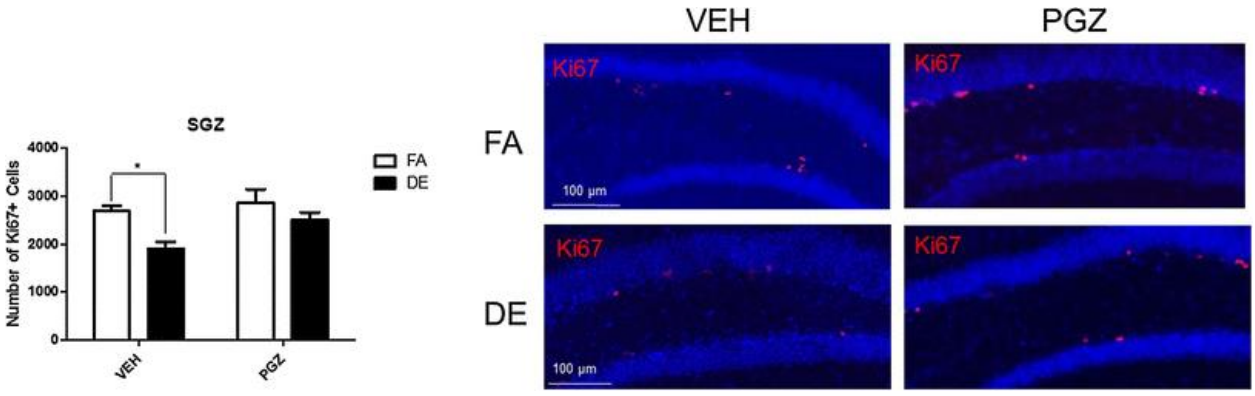

Figure 2.7 Effect of PGZ pre-treatment on DE-induced inhibition of proliferation in the SGZ of male mice.

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

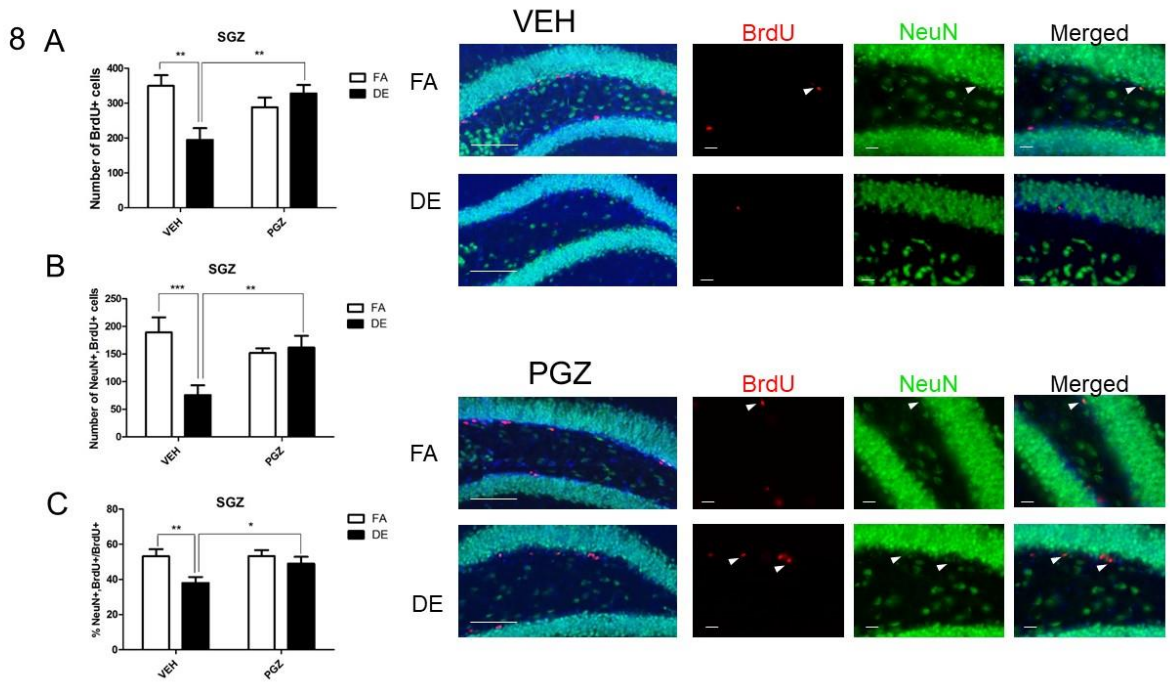

Figure 2.8 Effect of PGZ pre-treatment on DE-induced inhibition of adult hippocampal neurogenesis in male mice.

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

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Chapter 3：Effect of subchronic diesel exhaust exposure on levels of early neurodegenerative markers

3.1 Abstract

Neurodegenerative disorders afflict nearly five million older Americans today, with Alzheimer's disease (AD) being the most prevalent. Neurodegeneration is characterized by cognitive and behavioral changes, movement disorders, and characteristic histopathology upon posthumous examination. A number of genetic and environmental factors have been associated with increased incidence of these diseases. Recent epidemiological studies have shown an association between exposure to traffic-related air pollution, and neuropathology and cognitive decline in humans. DE, a major component of air pollution, is rich in particulate matter, metals and nitrogen oxides, exposures to all of which are identified as risk factors in the development of neurodegenerative disorders. Some of these components are also known to induce oxidative stress and neuroinflammation. We hypothesized that subchronic exposures to DE would induce increases in certain early neurodegenerative markers. To test this hypothesis, we exposed male and female C57BL/6J mice to DE for three or 10 weeks and assessed levels of Dyrk1a, $A\beta_{42}$, tau (pS199), and α-synuclein in cortex, and α-synuclein in midbrain. Three weeks of exposure was insufficient to elevate any of the neurodegenerative markers except for α-synuclein in midbrain in male animals, but 10 weeks was sufficient to see elevations in Dyrk1a, Tau pS199, and $A\beta_{42}$ in cortex. These findings suggest that exposure to traffic-related air pollution may favor the development of neurodegeneration by contributing to oxidative stress and neuroinflammation.

3.2 Introduction

Air pollution originates from a variety of natural events and human activities, including industrial and vehicular emissions and biomass burning, and it contains a multitude of components such as organic and elemental carbon particles, metals, volatile organic compounds, and gases (Monks et al., 2009). A relatively recent discovery is that high levels of air pollution have been associated with increased risk of adverse health endpoints affecting the central nervous system (CNS) (Calderón-Garcidueñas et al., 2008, 2012; Costa et al., 2014c, 2017; Genc et al., 2012). Epidemiological studies have shown an association between air pollution and cognitive deficits, as well as the presence of AD-type histopathology in the brains of exposed children (Calderón-Garcidueñas et al., 2008; Chen et al., 2017; Weuve et al., 2012). Of the many components of air pollution, $PM_{2.5}$ (particulate matter having an aerodynamic diameter of 2.5 micrometers or less) is one of the most concerning. Due to its minute dimensions and unusual aerodynamic behavior, $PM_{2.5}$ is capable entering the circulatory system through the pulmonary or olfactory mucosa, and can also enter the brain through the olfactory nerve (Oberdörster et al., 2004). Airborne burden of $PM_{2.5}$ may reach or even surpass concentrations of 100 μg/m3 with some regularity in some regions of the world, particularly in certain regions of India and China (Kandlikar and Ramachandran, 2000; Sun et al., 2004). Traffic-related air pollution is a major contributor to ambient air pollution, and diesel exhaust (DE) is among the most abundant of its components (Ghio et al., 2012).

Air pollution exerts much of its systemic and central nervous system toxicity through the mechanisms of increased inflammation and oxidative stress (Costa et al., 2017; Genc et al., 2012). Studies involving experimental exposures of mice to DE show increased priming and activation of microglia and subsequent neuroinflammation and oxidative stress (Cole et al., 2016; Levesque

et al., 2011a). The neurotoxicity of DE and its particulates (DEP) appears to be dependent upon microglial activation, as monocultured neurons lacked the neurotoxic response observed in neurons co-cultured with microglia. In addition, blocking microglial activation with the PPAR-γ agonist pioglitazone (PGZ) attenuated DE neurotoxicity both *in vivo* and in *vitro* (Coburn et al., 2018; Roqué et al., 2016).

Conditions of persistent neuroinflammation and glial activation can have significantly adverse effects in the brain. Even peripheral inflammation induced by the administration of lipopolysaccharide is capable of increasing levels of amyloid beta peptides in the brain (Marottoli et al., 2017). Notably, activation of both astrocytes and microglia, as well as increased levels of proinflammatory cytokines, are present in the brains of individuals suffering from AD (Heneka et al., 2015; Mrak and Griffin, 2001). Activated astrocytes and microglia may also engage in excessive and inappropriate pruning of synapses, an activity that may contribute to the cognitive decline representative of both normal aging and neurodegenerative diseases (Chung et al., 2015; Mottahedin et al., 2017; Pekny and Pekna, 2016).

Glial activation appears to be a significant risk factor and possible mechanism in PD and Parkinsonism as well (Booth et al., 2017). Heavy users of methamphetamines experience increased risk of developing PD, and though amphetamine-induced excitotoxicity remains a major mechanism behind the death of dopaminergic neurons in the nigrostriatal regions and the locus cœruleus in the brainstem, this is not the only factor involved in amphetamine-induced neurodegeneration (Curtin et al., 2015). Methamphetamine users, who experience a greater risk of developing Parkinson's type neurodegeneration, show microglial and astrocyte activation that is selectively increased in dopaminergic regions but not elsewhere in the brain (Curtin et al., 2015; Sekine et al., 2008). It has been observed, however, that the activation of microglia into the M1

polarity can induce the activation of astrocytes, through the release of the proinflammatory cytokines IL-1α, TNF-α, and C1q (Mottahedin et al., 2017). TNF-α is itself implicated in the loss of neurons to excitotoxicity, as it tends to reduce the reuptake of glutamate by microglia (Olmos and Lladó, 2014). The accumulation of extracellular glutamate may induce excitotoxicity by affecting the expression of, and binding to, various glutamatergic receptors, in particular the AMPA receptor (Bernardino et al., 2005)

Another consequence of neuroinflammation is the suppression of adult neurogenesis, or the birth of neurons in the post-development brain (Carpentier and Palmer, 2009; Ekdahl et al., 2003). Importantly, suppressed neurogenesis appears to be a condition present in the earlier stages of neurodegenerative conditions such as AD, and the subsequent lack of synaptic plasticity may help drive the progression of cognitive decline that characterizes the disorders (Demars et al., 2010; Saxe et al., 2006).

One of the hallmarks of neurodegeneration is excessive loss of neurons in the brains of those afflicted (Gorman, 2008). Neuronal death in neurodegeneration appears to result from not one distinct cause, but from a multitude of mechanisms, including excitotoxicity, apoptosis, necrosis, and autophagic cell death (Gorman, 2008).

Neuroinflammation may induce changes that promote the formation of lesions in the brain that are characteristic of both AD and PD (Cai et al., 2014). One consequence of prolonged neuroinflammation is increased secretion of amyloid peptides (Cai et al., 2014). While the function of amyloid peptides is not completely understood, they appear to have antimicrobial activity both *in vivo* and *in vitro* in animal models (Vijaya Kumar et al., 2016). Accumulation and aggregation of these peptides at the site of nerve terminals, however, can itself trigger microglial activation and neuroinflammation, and their presence in the brain can further fan the flames of existing

neuroinflammation, resulting in a vicious cycle of amyloid plaque aggregation (Cai et al., 2014). Neuroinflammation may also increase the levels of dual specificity tyrosine kinase 1b (Dyrk1b), elevated levels of which are also associated with astrocyte activation (He et al., 2018). Prolonged glial activation may result in excessive synaptic pruning, an activity that is believed to be associated with the hallucinations of schizophrenia and the cognitive deficits and delusions of AD and other neurodegenerative conditions (Hong et al., 2016; Ota et al., 2013). The presence of amyloid plaques and neuroinflammation may also induce the formation of neurofibrillary tangles (Metcalfe and Figueiredo-Pereira, 2010). Neuroinflammation may result in increased nitration and phosphorylation of amyloid beta and tau, due to the increased presence of peroxynitrite originating from the reaction of reaction of nitric oxide with superoxide radicals (Brion et al., 2001; Horiguchi et al., 2003; Kumar and Walter, 2011). Dyrk1a, overexpression of which is associated with the cognitive deficits and AD-type histopathology associated with trisomy 21, is largely responsible for the increased phosphorylation of tau, and appears to play a central role in AD-type neurodegeneration (Latour et al., 2018). Excessive phosphorylation of tau, a microtubule-associated protein, may result in the formation of paired straight and helical filaments (Santa-Maria et al., 2012). These formations may compromise microtubule stability and interfere with neuronal function by disrupting vesicular trafficking within the axon (Niikura et al., 2006; Theofilas et al., 2018).

Recent epidemiological studies show a relationship between levels of traffic related particulate air pollution and neurodegeneration or cognitive decline (Cacciottolo et al., 2017), Moreover, there is a well-established association between neurodegeneration and oxidative stress and neuroinflammation (Fischer and Maier, 2015). Acute exposures to DE induce suppression of adult neurogenesis and increase oxidative stress, neuroinflammation, and microglial activation,

and male animals have shown increased susceptibility to the adverse CNS endpoints of DE exposure relative to female animals (Coburn et al., 2018; Cole et al., 2016). Based on these studies, we had hypothesized that a subchronic exposure of male and female animals to DE would result in increases in levels of neurodegenerative markers, an effect which would be more pronounced in male animals.

3.3 Materials and Methods

Animals

Male and female 11-week-old C57BL6/J mice were purchased from Jackson Laboratories (Bar Harbor, ME). All animals were housed in specific pathogen-free facilities with a 12-h dark–light cycle, with feed and water available ad libitum. The animals were randomly assigned to either filtered air (FA) or DE exposures.

Diesel exposure

Group-housed mice were exposed for 6 h to FA or DE (at a $PM_{2.5}$ concentration of 250–300 $\mu g/m^3$) for 5 days a week Monday through Friday for either 3 weeks or 10 weeks. Exposures to either FA or DE were conducted simultaneously in the University of Washington Controlled Exposure Laboratory's Northlake Diesel Facility, which includes an SPF mouse housing room with Allentown caging systems (Allentown, NJ), with the housing racks modified to ventilate cages with either diluted DE or FA. DE was derived from a Yanmar YDG5500 diesel generator, with a load bank maintaining 75% of rated capacity, using No. 2 undyed, ultra-low sulfur on-highway fuel and Royal Purple Duralec 15W-40 Synthetic crankcase oil to lubricate moving parts, as previously described (Fox et al. 2015; Gould et al. 2008). During exposures, DE concentrations were continuously measured and maintained at steady levels using a feedback controller monitoring fine particulate levels (Fox et al. 2015; Gould et al. 2008). DE particulates had a mean aerodynamic diameter of 100 nm. Characterization of DE is described in detail in a previous

publication (Fox et al. 2015). Mice were euthanized by CO_2 asphyxiation within 2 h after the end of the exposure, and brain regions were dissected immediately, then flash-frozen in liquid nitrogen, and stored at −80 °C.

Western blot

Brain regions were dissected out from the frozen brains, then homogenized using a dounce homogenizer in RIPA buffer (pH 7.4) composed of 50 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), 150 mM sodium chloride, 1 mM ethylenediamenetetracetic acid (EDTA), 0.5% sodium deoxycholate, 0.1% Triton X-100, and 0.1% sodium dodecylsulfate (SDS) in water. To inhibit proteases, 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and one Protease and Phosphatase Inhibitor Mini Tablet (A32961, Thermo Scientific, Waltham, Massachusetts) per 10 mL RIPA were added 30 min prior to use. . Each lysate was sonicated for 20 seconds using a Qsonic sonicator with amplitude set at 10. The lysates were analyzed for protein content, and samples containing 25 µg protein were prepared. Six µL 4x Sample Loading Buffer and 4µ LSample Reducing Agent were added to each sample, and brought to equal volume with RIPA buffer containing protease inhibitors. Samples were heated for 7 min and spun for 1 min at RT at 13400 × *g*. Protein samples were kept on ice until loaded into the gel. They were electrophoresed using 1 x MOPS buffer and 10% Bis-Tris gels (Invitrogen, Carlsbad, California) for 30 minutes at 30 mA and then for 60 minutes at 60 mA using an XCell SureLock® Mini-Cell electrophoresis chamber (Invitrogen, Carlsbad, California). The proteins were transferred to polyvinylidene fluoride (PVDF) membrane for 18 hours at 35 V. Following transfer, the membranes were rinsed in TBST, then blocked in 5% dry skim milk in TBST for 1 hour at room temperature. The membranes were probed with antibodies specific to Tau pS199 at 1:8000 and total tau at 1:10000 (rabbit, ab80579 and mouse, ab4749, Abcam for 2 h) or 24 hours for Dyrk1a

(rabbit, 1:1000) and α-synuclein (rabbit, 1:4000) (#2771 and #2642 respectively, Cell Signaling Technology, Danvers, Massachusetts) diluted in 3% BSA in TBST. The blots were rinsed in TBST, then probed with horseradish peroxidase-conjugated secondary (α-mouse IgG 1:5000 for Tau, α-rabbit IgG (1:4000 for Tau (pS199), 1:1000 for Dyrk1a, and 1:2000 for α-synuclein)). Membranes were rinsed with TBST and then were developed with a chemiluminescent substrate (ECL kit from Thermo Scientific, Waltham, MA). Band intensity was measured by optical density using the open source software ImageJ (provided by the National Institutes of Health), and the intensity of the bands was normalized to β-actin content.

$A\beta_{42}$ ELISA Assay

Animals were euthanized by CO_2 asphyxiation and their brains were resected whole and flash frozen in liquid nitrogen. Guanidine extracts of cortex were prepared for the ELISA assay using a solution of 5M guanidine and 0.5M Tris HCl. Frozen tissue was weighed and placed in a douncing homogenizer, to which eight microliters of 5 M guanidine/0.5 M Tris HCl per milligram of tissue was added. Samples were homogenized manually, then transferred to 1.7 mL microcentrifuge tubes. The tubes containing the stable guanidinium extracts were placed on an orbital shaker and allowed to mix at room temperature for 3 hours. Each lysate was sonicated for 20 seconds using a Qsonic sonicator with amplitude set at 10. Aliquots of the guanidine extracts were diluted 1:2 with PBS containing one Protease and Phosphatase Inhibitor Mini Tablet (A32961, Thermo Scientific, Waltham, Massachusetts) per 10 mL liquid, 1 mM phenylmethylsulfonlylfluoride (PMSF), 1 mM sodium orthovanadate, and 10 mM sodium fluoride. They were then spun down in a microcentrifuge at 9200 × *g* at 4º C for 15 minutes, then the supernatant transferred to a fresh tube and the pellet discarded. The diluted extracts were analyzed for protein content using the bicinchoninic acid (BCA) assay. The mouse $A\beta_{42}$ ELISA

kit (KMB3441) was purchased from Thermo Fisher (Waltham, Massachusetts). Lyophilized $A\beta_{42}$ standard was first diluted in a reconstitution buffer consisting of 55 mM sodium bicarbonate in water that had been sterile filtered. Dilute guanidine extracts of mouse cortex were further diluted with Standard diluent buffer (provided with kit) containing 1 mM PMSF, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and one Protease and Phosphatase Inhibitor Mini Tablet, (A32961, Thermo Scientific, Waltham, Massachusetts) per 10 mL of Standard diluent buffer. A 200 ng/mL stock solution of $A\beta_{42}$ was prepared and further diluted to a concentration of 2 ng/mL, which was further diluted 1:10 to a concentration of 200 pg/mL. From the 200 pg/mL solution, 1:2 serial dilutions were prepared at concentrations of 100 pg/mL, 50 pg/mL, 25 pg/mL, 12.5 pg/mL, 6.25 pg/mL, and 3.125 pg/mL using the protease inhibitor-spiked Standard Diluent Buffer as a diluent. The guanidine extracts diluted with PBS were standardized to a concentration of 1 mg/mL protein with protease inhibitor-spiked Standard Diluent Buffer. 100 µL of each standard or sample were pipetted into the wells of the ELISA plate, with each standard or sample assayed in triplicate.

Two wells were left empty to serve as the chromogen blank. The plate was incubated at room temperature for two hours on an orbital shaker. Samples and standards were aspirated from the wells and then the wells were rinsed four times with 1x ELISA wash buffer, prepared from 20x concentration provided with the kit. 100 µL Ms $A\beta_{42}$ Detection Antibody (containing 0.1% sodium azide and blue dye) were added to each well except for the chromogen blanks. The plate was incubated at room temperature for one hour on an orbital shaker. The antibody solution was aspirated from the wells and then the wells were rinsed four times with 1x ELISA wash buffer. All wash buffer was thoroughly aspirated from the wells. The Anti-Rabbit IgG HRP (100X) was diluted 1:100 in HRP Diluent (containing 3.3 mM thymol and yellow dye). 100 µL was added to each well with the exception of the chromogen blanks. The plate was incubated at room

temperature for 30 minutes on an orbital shaker. The secondary antibody solution was aspirated from the wells, which were then rinsed four times with 1x ELISA wash buffer. All wash buffer was aspirated from the wells. 100 µL of TMB chromogen was added to each well and allowed to incubate for 30 minutes at room temperature in the dark. 100 µL of stop solution was added to each well, and then the plate was read using a SpectroMax 190 Plate Reader (Molecular Devices, San Jose California) using SoftMax Pro v. 5.3 software (Molecular Devices, San Jose California), set to the pre-programmed HRP with TMB ELISA protocol.

RNA extraction and real time PCR analysis

Prior to extraction, all working surfaces and instruments were cleaned with an RNAse inhibitor. Tissue homogenates were prepared using TRIzol RNA extraction reagent (Invitrogen, Carlsbad, California) and purified according to the manufacturer's protocol. Concentration and quality of RNA were determined using a NanoDrop ND-100 Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware). RNA samples (1 µg) were reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, California) according to the manufacturer's protocol. The resulting cDNAs were used for PCR amplification in the presence of primers specific to tumor necrosis factor (TNF-α) (forward: GTC GTA GCA AAC CAC CAA GTG; reverse: CTT TGA GAT CCA TGC CGT TGG; 21 bp), IL-6 (forward: GTG GAA ATG AGA AAA GAG TTG TGC; 24 bp; reverse: CTG CAA GTG CAT CAT CGT TGT; 21 bp), IL-1β (forward: GCA GCT GGA GAG TGT GGA T; 19 bp; reverse: ACA AAC CGT TTT TCC ATC TTC TTC T; 25 bp), and the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (forward: CCT GGT ATG ACA ATG AAT ACG GC; 23 bp; reverse: CTC CTT GGA GGC CAT GTA GG; 20 bp). Amplification was carried out in a SimpliAmp Thermal Cycler (Applied Biosystems, Foster City,

California) and quantification was carried out in a Bio-Rad CF384 Real-timer System thermal cycler, using iTaq Universal SYBR Green Supermix.

Lipid peroxidation assay

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 lysate was 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 concentration 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).

Statistical analysis

Statistical analysis of all data was by one-way ANOVA with Bonferroni's post-test, or using Student's t-test where appropriate, with significance set at $p<0.05$. All graphs show the mean and SEM. Prism 5.02 (GraphPad, San Diego, California) was used for the preparation of graphs as well as statistical analysis of data.

3.4 Results

No morbidity was observed in animals exposed to 250–300-μg/m3 DE for 6 h a day for either 3 weeks or 10 weeks, and there were no salient behavioral or physical differences between DE-and FA-exposed animals of either sex.

Western blot was used to analyze levels of neurodegenerative markers following exposure to DE for either 3 weeks or 10 weeks in cortex (CTX), olfactory bulb (OB), midbrain (MB), and cerebellum (CB).

In CTX, no statistically significant change was seen in any neurodegenerative marker following a 3 week DE exposure (Fig. 3.1 A, B, C) The only neurodegenerative marker that was changed following a 3 week DE exposure was α-synuclein in MB (Fig. 3.2 A). A statistically significant increase was seen in male C57BL6/J mice (Fig. 3.2 A). However, 3 week exposure to DE comparing Gclm +/+ and Gclm +/- mice failed to reproduce previous results and or show increased vulnerability in Gclm +/- mice (Fig. 3.2 B). A possible explanation for this is the smaller n (3-4 vs. 5) and the availability of younger animals (1-2 months vs. 3 months) at the time of exposure.

In the OB, no difference was seen in the male mice exposed for 10 weeks in any of the neurodegenerative markers (Tau (pS199), Dyrk1a, or α-synuclein) assessed (Fig. 3.3), although in all cases there was a trend of higher levels of expression. Similarly, no difference was seen in the CB either in any of the measured markers (Tau (pS199), Dyrk1a, or α-synuclein) (Fig. 3.4) By contrast, a 10 week DE exposure was sufficient to cause a statistically significant increase in levels of neurodegenerative markers in CTX (Fig. 3.5). Specifically, a statistically significant increase in Tau (pS199), Dyrk1a, and $A\beta_{42}$ was observed, while α-synuclein was unchanged.

Neuroinflammation and oxidative stress were assessed using qPCR and the TBARS assay respectively. In the CTX of C57BL6/J mice exposed 3 weeks, TNF-α, IL-6, and IL1β were significantly increased in both males and females. Similarly, oxidative stress was increased in both sexes (Fig. 3.6). In the OB, TNF-α and IL-1β were significantly increased in male mice only. Oxidative stress, however, was increased in both sexes (Fig 3.7). In the CB, TNF-α, IL1β and

oxidative stress were significantly increased in male mice exposed to DE. IL-6, IL1β, and oxidative stress were increased in female mice (Fig. 3.8).

3.5 Discussion

The main findings of this study show that subchronic DE exposure may adversely affect the CNS of mice at high but environmentally relevant levels; specifically, that DE exposure at a concentration of 250-300μg/m^3 is sufficient to cause increases in the levels of select neurodegenerative markers in male mice, provided that the exposure is of sufficient duration. In particular, a 10-week exposure increased levels of phosphorylated tau (pS199), Dyrk1a, and $A\beta_{42}$, in the cerebral cortex, while a three week exposure was only sufficient to increase levels of α-synuclein in midbrain in male animals. Young adult mice (12 weeks) were used in this study, and the exposure was either 3 weeks (6 h a day, 5 days a week) or 10 weeks (6 h a day, 5 days a week) to a high but environmentally relevant concentration of DE (250–300-μg/m^3 $PM_{2.5}$). These levels of $PM_{2.5}$ may be reached and even surpassed in a number of cities worldwide, particularly in India, Mongolia, and China (Allen et al., 2013; Costa, 2017; Kandlikar and Ramachandran, 2000; Sun et al., 2004). Vehicular emissions contribute significantly to $PM_{2.5}$ levels, and DE is one of the most prominent sources of $PM_{2.5}$ and ultrafine particles (Calderón-Garcidueñas et al., 2015; Ghio et al., 2012).

In some megacities such as New Delhi, where diesel fuel powers many passenger vehicles in addition to heavy machinery and public transportation conveyances, the contribution of traffic to air pollution is estimated to be as high as 72% (Goyal et al., 2006). The use of diesel fuel as an energy source for heavy machinery and mass transit conveyances may result in potentially hazardous daily exposures for individuals in certain lines of work(Pronk et al., 2009). Specifically, miners and mechanics in bus garages on average receive exposures to some of the very highest

levels of particulate air pollution, where $PM_{2.5}$ may range from 300 to 1000 $\mu g/m^3$ (Pronk et al., 2009). It should also be noted that DE contains a multitude of other substances that were not measured in this study, and their possible contribution to neurotoxicity cannot be ruled out without a more thorough investigation. Certain components of DE, such as nitrogen oxides, $PM_{2.5}$, and metals and metalloids, including lead, arsenic, and manganese, may contribute to neurodegeneration in a manner that likely involves oxidative and nitrative stress as part of the mechanism (Bakulski et al., 2012; Cholanians et al., 2016; Zatta et al., 2003). Because of this, they aggravate existing neuroinflammation and contributing to the exacerbation of existing lesions (Karthikeyan et al., 2013; Lim et al., 2009; Talebi and Abedi, 2005; Zatta et al., 2003).

While genetic factors, such as variants in APOE, PSEN, and amyloid precursor protein (APP), may result in a higher risk of developing AD, the majority of cases are sporadic, and have no determinate genetic trigger (Cacciottolo et al., 2017; Guerreiro et al., 2012). Some studies have shown that exposure to $PM_{2.5}$ may increase levels of certain neurodegenerative markers for both wild-type and polymorphic mice; however, certain genotypes appear to have more susceptibility to the neurotoxicity of DE (Cacciottolo et al., 2017). This is further supported by experimental data showing increases in IL-1β and $A\beta_{42}$ following subchronic exposure to high levels of DE in a mouse model of AD (Hullmann et al., 2017). These findings also reinforce the results of existing studies linking high levels of particulate air pollution to increased levels of neurodegeneration in both humans and animals (Calderón-Garcidueñas et al., 2008). Specifically, post mortem examinations of children and animals from areas with high concentrations of traffic-related air pollution reveal histological abnormalities, increased levels of neurodegenerative markers such as $A\beta_{42}$ and hyperphosphorylated tau, and pro-inflammatory cytokines such as IL-1β (Calderón-Garcidueñas et al., 2008). Similarly, increases in early markers of neurodegeneration have been

seen in rats after a six month exposure to high levels of DE (Levesque et al., 2011b). (Cacciottolo et al., 2017). These studies certainly support a role for $PM_{2.5}$ and UFPM in the development of AD and related dementias, though many risk factors, such as educational level, lifestyle choices, and medical conditions and related medications, may also play a part in determining risk of AD (Chew et al., 2006; Griesbach et al., 2009; Judge et al., 2017; Letenneur et al., 2000).

Increased microglial activation and neuroinflammation are strongly implicated as mechanisms for the increases in markers of neurodegeneration observed in mice subchronically exposed to DE. Notably, DE is capable of increasing neuroinflammation and oxidative stress even following an acute DE exposure at similar levels, i.e.,250-300 $\mu g/m^3$ (Cole et al., 2016). Neurodegenerative disorders affect the sexes differentially, particularly with respect to the most commonly diagnosed conditions, namely AD and PD (Miller and Cronin-Golomb, 2010; Viña and Lloret, 2010). PD is diagnosed more commonly in men and at a younger age (Miller and Cronin-Golomb, 2010). Perhaps significantly, PON2, an anti-oxidant enzyme that is more highly expressed in females, is also most highly expressed in the brain regions affected by PD (substantia nigra, nucleus accumbens, and the striatum), possibly offering premenopausal women some protection against the illness (Giordano et al., 2013).

Oxidative stress and neuroinflammation, and by consequence, nitrative damage are implicated as mechanisms in the development of neurodegeneration (Cobb and Cole, 2015). As part the neuroinflammatory cascade, the reactive nitrogen species peroxynitrite is formed from chemical reaction of nitric oxide with the superoxide radical (Beckman and Koppenol, 1996). The peroxynitrite radical is capable of inflicting nitrative damage on different classes of biomolecules, and may cause single strand breaks and nitrative lesions in DNA as well as the nitration of proteins at tyrosine residues (Radi, 2013; Szabó and Ohshima, 1997). Thus, examination of alterations in

the relative levels of 3-nitrotyrosine could provide an insight into the degree of nitrative stress that may occur following DE exposure (Beckman and Koppenol, 1996). While nitrated tau and amyloid beta would not be measured directly, an increase in 3-nitrotyrosine would imply increased nitration of proteins and other peptides across the proteome, and therefore, by extension, increased nitration of the markers of interest (Radi, 2013). Similarly, assessing differences in levels of inducible nitric oxide synthase (iNOS/NOS2) could, along with the data on oxidative stress, indirectly provide information on the degree to which DE exposure may contribute to neurodegeneration by inducing nitrative damage, as well as oxidative stress (Evans et al., 1996).

Previous studies have shown that blocking microglial activation by activating the PPARγ receptor with the thiazolidinedione pioglitazone may abrogate DE-exposure-induced suppression of adult neurogenesis, as well as mitigate oxidative stress and the expression of certain proinflammatory cytokines such as TNF-α (Coburn et al., 2018). Due to the strong association of these biomarkers with neurodegenerative disease and cognitive deficit, it would be informative to pretreat animals with pioglitazone and assess endpoints of neurodegenerative markers as previously performed (Coburn et al., 2018; Fischer and Maier, 2015).

Another endpoint that could be worth investigating is the M1 and M2 activation of microglia using the technique of multiplex fluorescence immunohistochemistry. The M1 polarity of microglial activation is considered neurotoxic, while the M2 state is considered more neuroprotective, associated as it is with the processes of tissue repair and regeneration, and has the ability to stimulate neurogenesis (Choi et al., 2017). The microglia specific marker Iba1 would serve to identify cells as microglia, to distinguish them from astrocytes, which may also undergo classical (A1) or alternative (A2) activation, and express the same markers. To indicate microglia in an M1 activation state, the pro-inflammatory marker inducible nitric oxide synthase (iNOS)

would be used. For the M2 polarization state, CD206, the mannose receptor would be used. Unlike other markers of M1 and M2 polarization which are not relevant in a human model such as arginase-1 and Ym1, iNOS and CD206 may be observed in both human and mouse models (Raes et al., 2005; Rőszer, 2015). Staining for the two different polarities of microglial activation would give a fuller picture of the way in which DE exerts its neurotoxicity and specify further the mechanisms by which DE exposure suppresses neurogenesis. The PPAR-γ agonist pioglitazone was shown to mitigate suppression of adult neurogenesis induced by DE exposure, and to block microglial activation as assessed by both Iba1 immunohistochemistry and the expression of Iba1 (Coburn et al., 2018; Cole et al., 2016). While the suppression of the M1 state of activation may be sufficient to ameliorate DE-induced suppression of adult neurogenesis, pioglitazone at certain doses may also cause M2 activation, an event which may have a stimulating effect on the proliferation of neural stem cells and favor their survival (Bouhlel et al., 2007). Similarly, it has not been investigated whether or not morphological differences exist between M1 and M2 activated microglia. Four days of pretreatment with 12 mg/kg pioglitazone was apparently sufficient to block the induction of the M1 neurotoxic phenotype by acute DE exposure, but this level of treatment may not be sufficient to induce the neuroprotective M2 state (Coburn et al., 2018).

As described earlier, one of the most significant factors modifying risk of neurodegeneration is age, with the majority of cases occurring in individuals well above the age of 65 years (Lindsay et al., 2002). This study focused on the levels of neuroinflammation and oxidative stress immediately following a subchronic exposure. However, it would also be informative to also assess whether or not exposed animals recover to baseline levels observed in controls, with respect to the endpoints observed, or whether the 3-week exposure is sufficient to

induce an enduring elevation in markers of neurodegeneration for an extended period of time. There is some indication that events happening earlier in life may shape the risk of developing neurodegeneration or dementia in a persistent way (Borenstein et al., 2006). Given the way that certain hallmarks of neurodegeneration may exacerbate existing neuroinflammation or inhibit its resolution by triggering microglial activation and the generation of ROS, it seems biologically plausible that a lasting artifact of the exposure could be observed long after the neurotoxic insult has occurred (Brion et al., 2001; Cai et al., 2014). A two-year-old mouse is approximately equivalent to a 70 year old human (Dutta and Sengupta, 2016). Thus, an assessment of the levels of neurodegenerative markers at an age of two years following a three-week exposure at three months may provide a model for the consequences later in life of an earlier life subchronic exposure.

In conclusion, results of this study show that 10 week exposures to moderate/high concentrations of DE at environmentally relevant levels induce elevations of Dyrk1a, Tau (pS199) and $A\beta_{42}$ in cortex of mice. These limited findings support the hypothesis that traffic-related air pollution as modeled by DE exposure, may contribute to cognitive decline and perhaps also contribute to the development of neurodegenerative diseases (Cacciottolo et al., 2017).

3.6 Figures

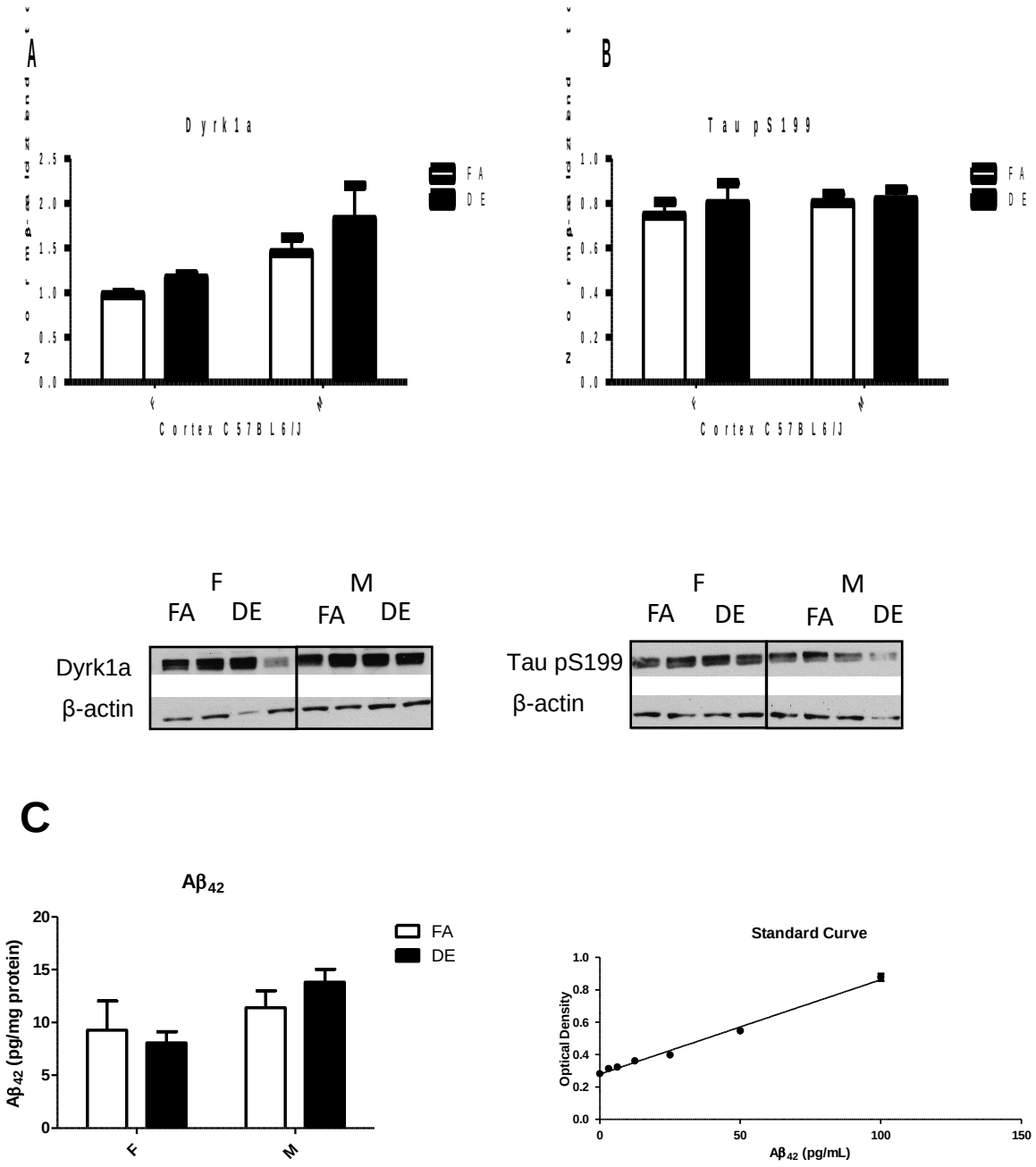

Figure 3.1Neurodegenerative markers in CTX following 3 week subchronic DE exposure.

Levels of Tau pS199, Dyrk1a, and alpha synuclein in CTX were not significantly changed in male mice or female mice exposed to DE exposure for 10 weeks. N=5, P>0.05

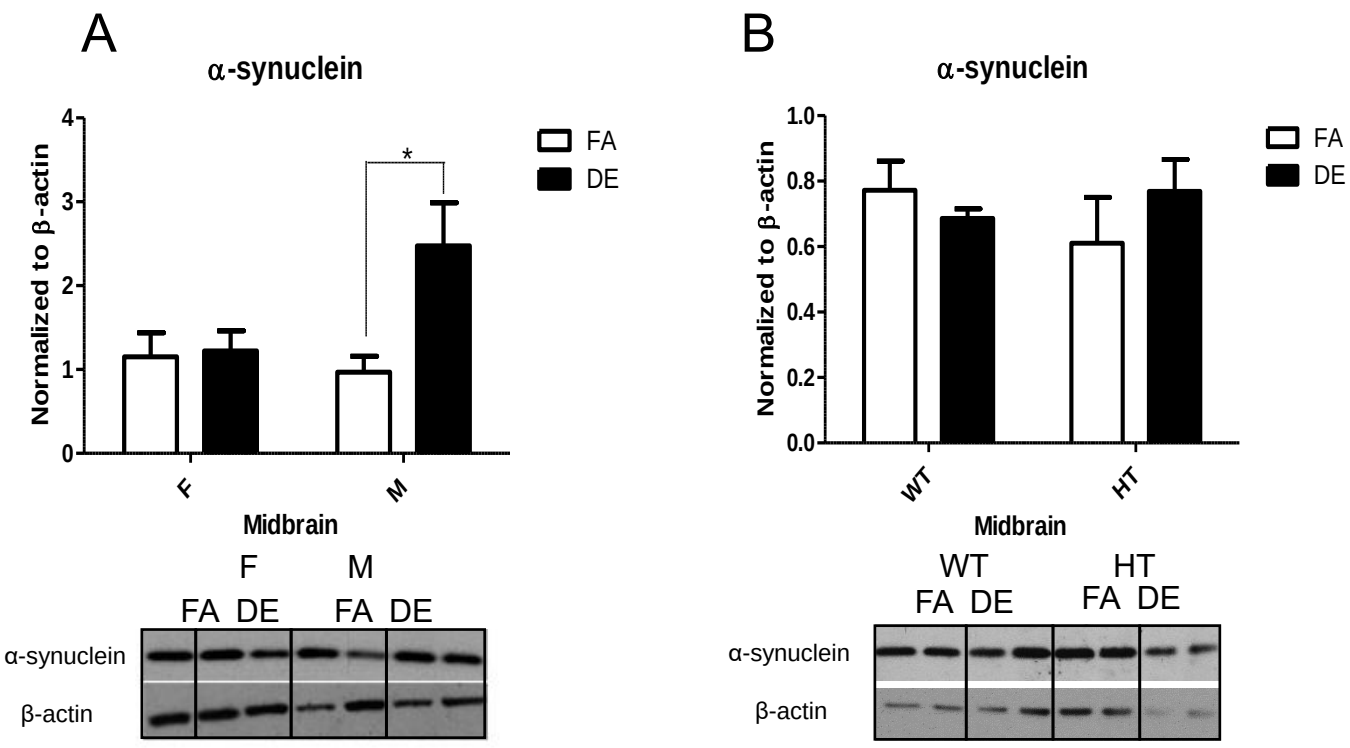

Figure 3.2*α*-synuclein in MB following 3 week DE exposure.

Levels of α-synuclein were found to be increased in 3-month old male C57BL6/J mice but not female animals (A). Younger (1- 2 month old) male Gclm +/- and +/+ mice were exposed, but possibly due to age differences and variability, no change was seen (B). N=5, *p<0.05, MB - midbrain

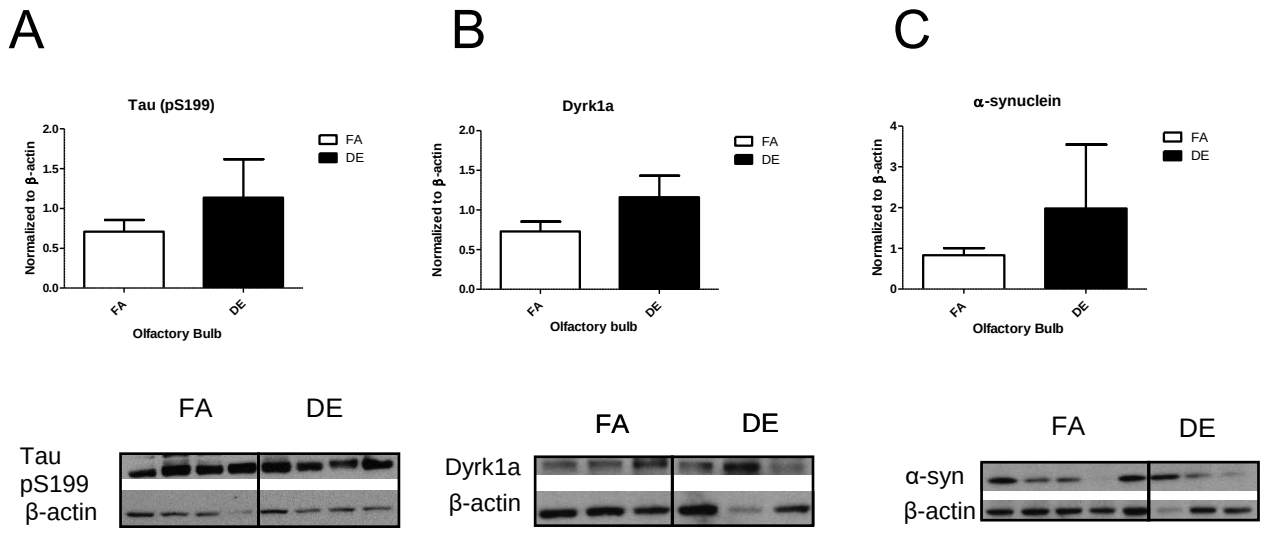

Figure 3.3 Neurodegenerative markers in OB following 10 week subchronic DE exposure.

Levels of Tau pS199, Dyrk1a, and alpha synuclein in OB were not significantly changed in male mice exposed to DE exposure for 10 weeks. P>0.05, OB – olfactory bulb

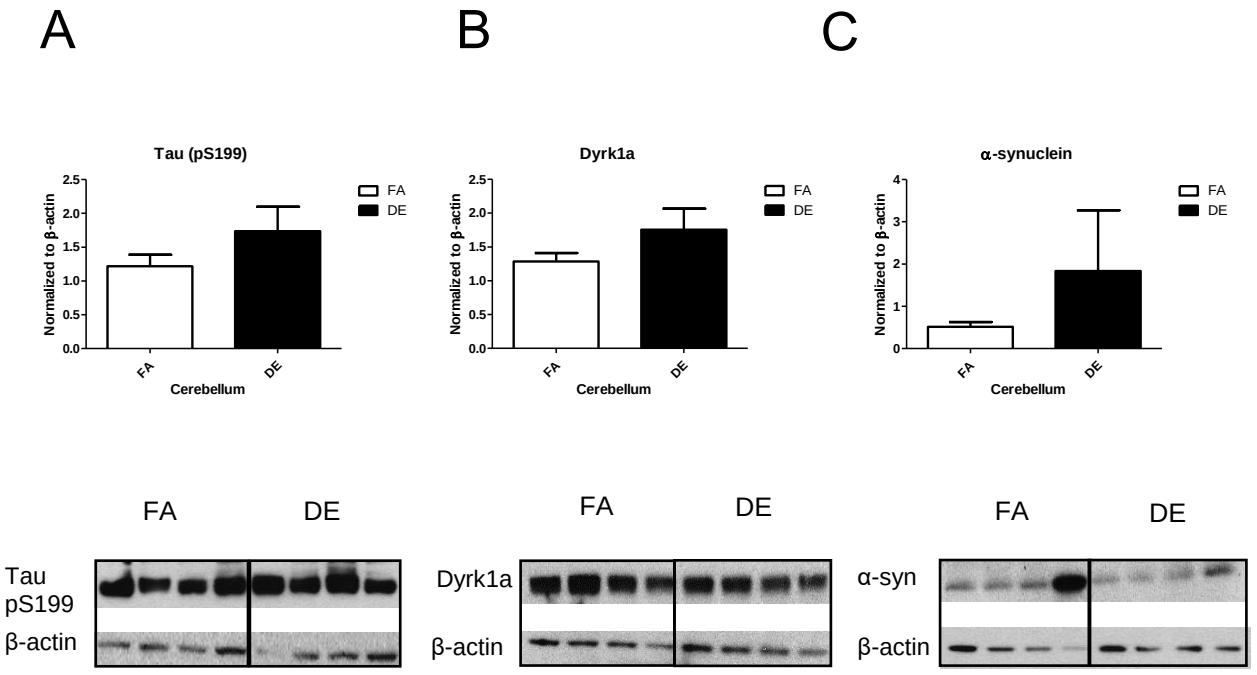

Figure 3.4 Neurodegenerative markers in CB following 10 week subchronic DE exposure.

Levels of Tau pS199, Dyrk1a, and α-synuclein in CB were not significantly changed in male mice exposed to DE exposure for 10 weeks. N=5, P>0.05, CB – cerebellum

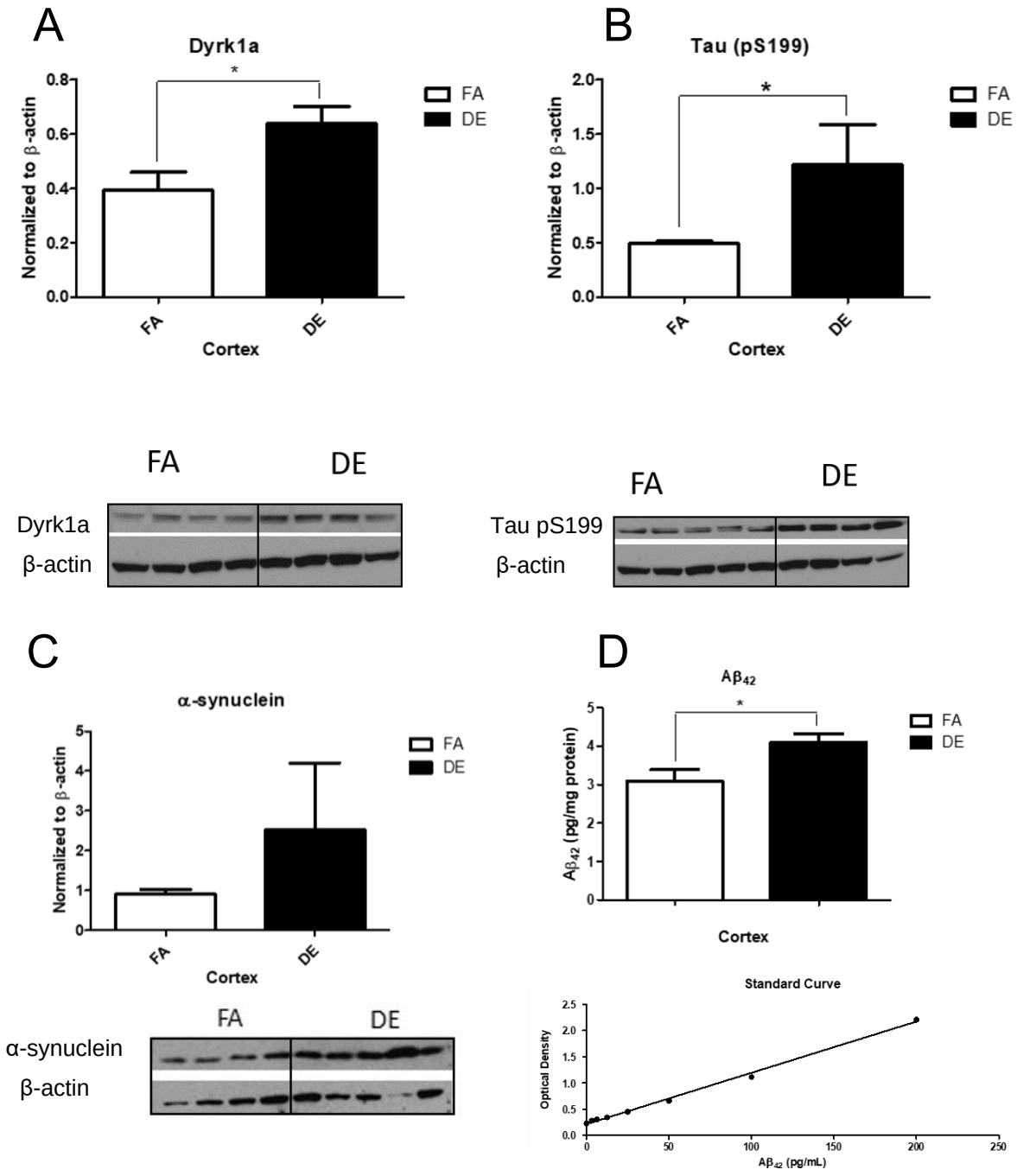

Figure 3.5Neurodegenerative markers in CTX following 10 week subchronic DE exposure.

Levels of Tau (pS199), Dyrk1a, and $A\beta_{42}$ in CTX were significantly increased in male mice exposed to DE for 10 weeks. There was no change in α-synuclein levels. *$p<0.05$, CTX – cortex

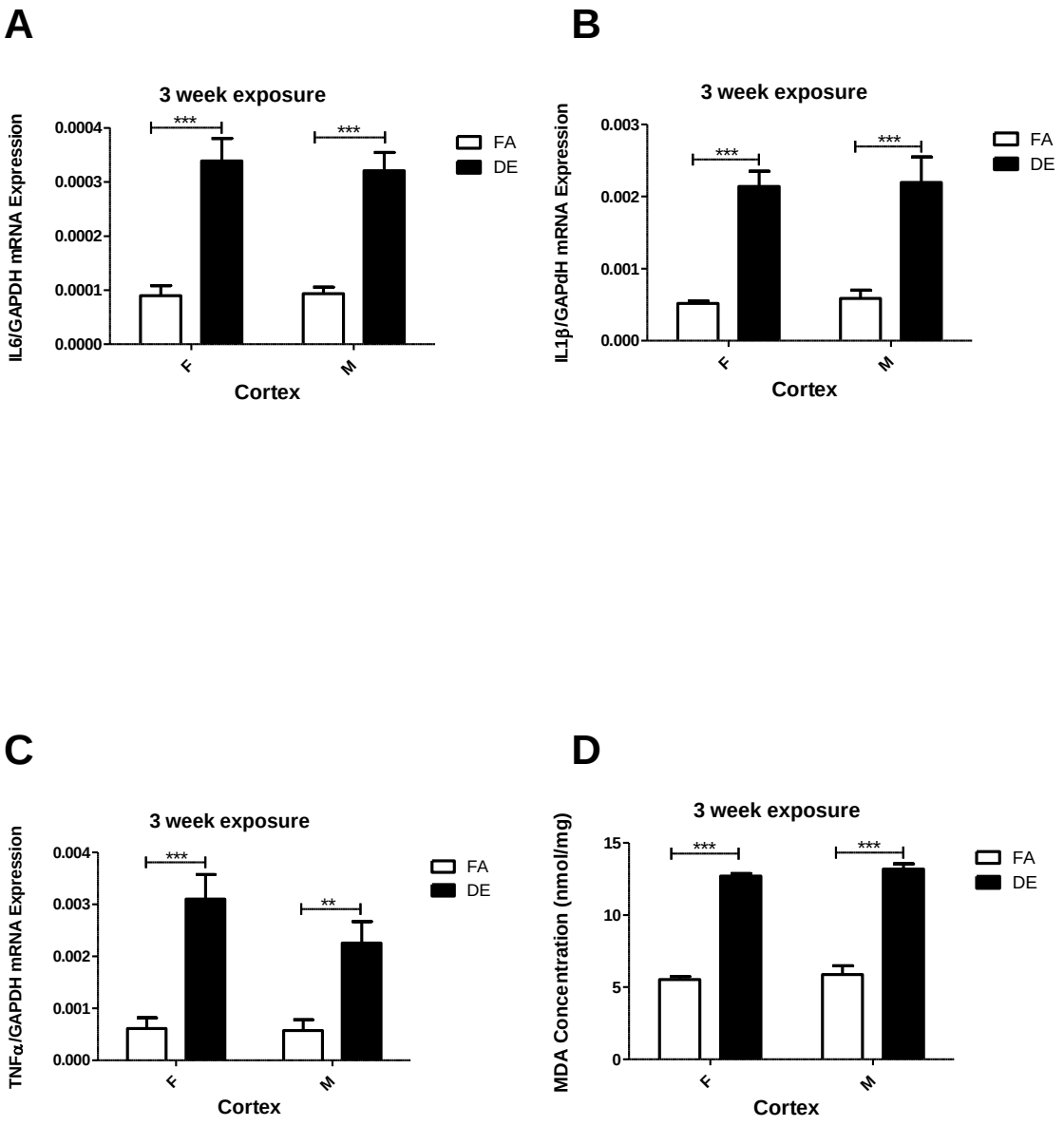

Figure 3.6 Neuroinflammation and oxidative stress following 3 week DE exposure.

TNF-α, IL-6, and IL-1β were found to have statistically significant increases in both males and females. Similarly, oxidative stress was increased in both sexes. *p<0.05, **p<0.01, ***p<0.005

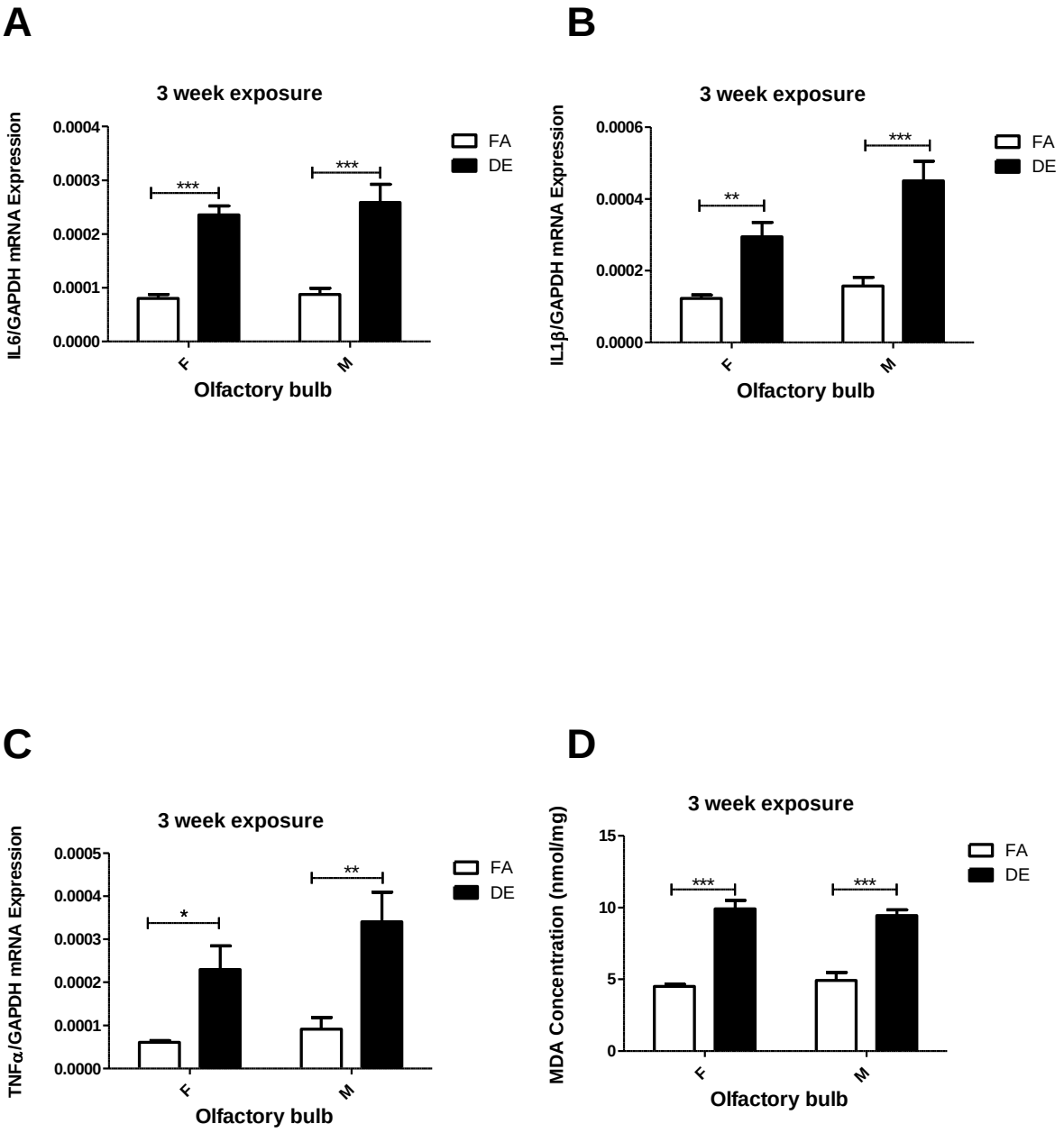

Figure 3.7 Neuroinflammation and oxidative stress in OB following 3 week DE exposure of male and female C57BL6/J mice.

TNF-α, IL-6, and IL1β as well as oxidative stress were increased in both sexes. *p<0.05, **p<0.01, ***p<0.005

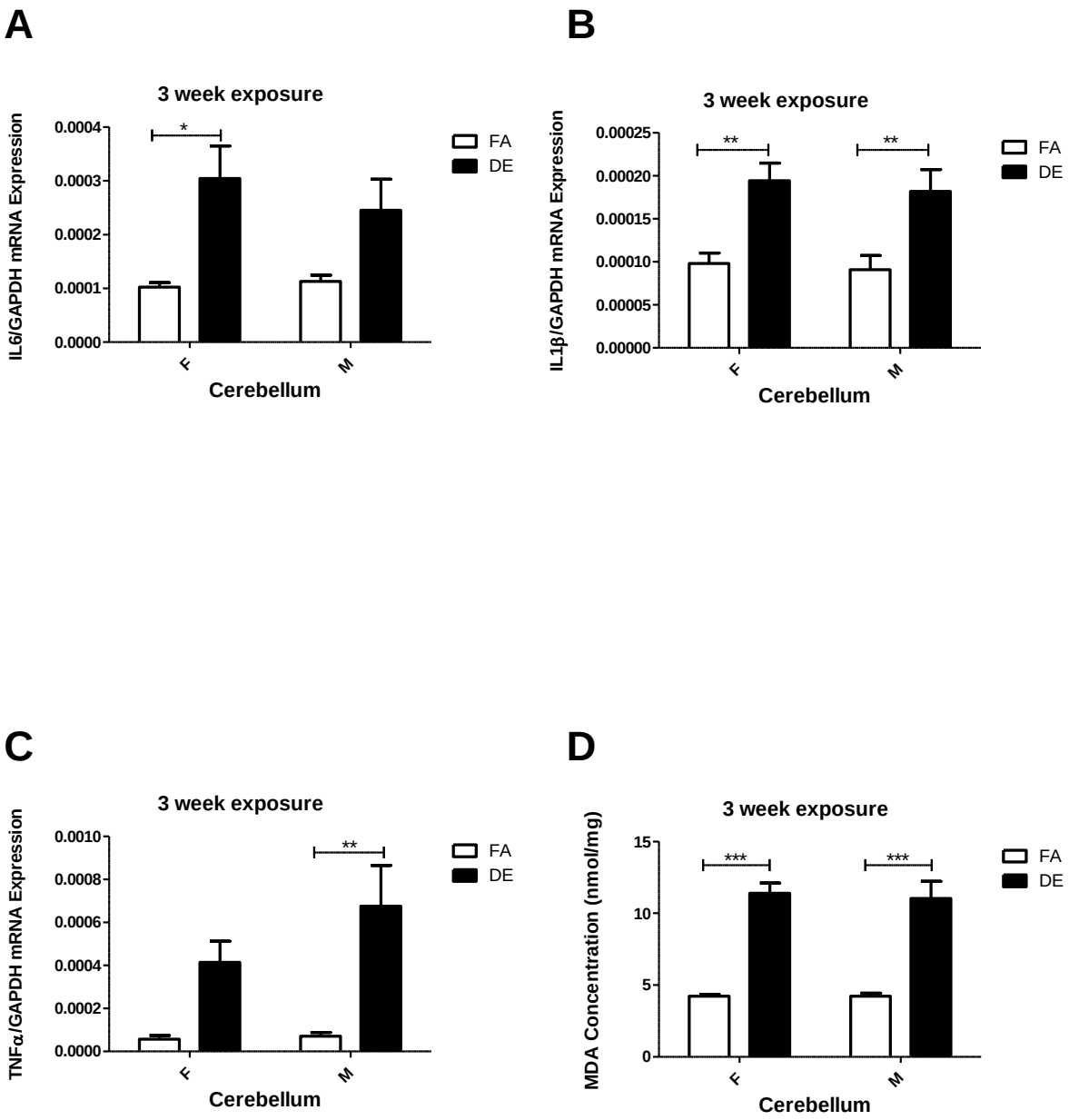

Figure 3.8 Neuroinflammation and oxidative stress in CB following 3 week DE exposure of male and female C57BL6/J mice.

IL1β and oxidative stress were found to have statistically significant increases in both sexes. IL-6 was increased in females, while TNF-αwas increased in males. *p<0.05, **p<0.01, ***p<0.005

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Chapter 4: Conclusions and future research

Until the use of less polluting alternative energy sources for various kinds of vehicles and heavy machinery is sufficiently incentivized and adopted, it is likely that exposures to unhealthy levels of traffic related particulate air pollution will remain commonplace. Exposure to these particulates, especially $PM_{2.5}$ and finer, has a demonstrated capacity to induce inflammation and oxidative stress, both peripherally and in the CNS, thereby setting the stage for the development of a number of adverse health endpoints in various systems throughout the body (Karthikeyan et al., 2013). The mechanisms of oxidative stress and inflammation, and in the case of the CNS, neuroinflammation, seem to be at the heart of the damaging effects of exposure to DE and other traffic related particulate air pollution (Costa et al., 2017; Wei et al., 2016). The increased presence of markers of oxidative stress and proinflammatory cytokines such as TNF-α in the brains of individuals suffering from AD strongly implicate the involvement of neuroinflammation in the etiopathology of the disease, and the ability of acute DE exposure to elevate levels of these markers suggest that exposure to traffic related air pollution contributes to its development (Coburn et al., 2018; Fischer and Maier, 2015).

As population increases globally, there is a shift towards higher density living in ever larger urban centers, as it is estimated that by 2050, 70% of the projected 9 billion humans on earth will be living in cities (Gross, 2016). Unquestionably, dense metropolitan areas have high energy demands, especially where modes of transport are concerned, and without mass adoption of less polluting alternative fuel sources, many individuals may receive high levels of particulate air pollution exposure (Kammen and Sunter, 2016). An increasingly elderly and overweight population exposed to high levels of inflammatory particulate matter appear to portend a looming public health crisis, as converging risk factors will likely increase the incidence of

neurodegenerative conditions and severe cognitive decline in elderly adults (Ailshire and Clarke, 2015; Guerreiro and Bras, 2015; Hruby and Hu, 2015). Thus, the need to understand the role of particulate air pollution in contributing to cognitive deficits in the geriatric population has never been more pressing.

The principal findings of these studies support the hypotheses that DE exposure contributes to the development of neurodegeneration and the suppression of adult neurogenesis, in a way that is primarily sex-dependent. The mechanism is likely through the induction of oxidative stress and the induction of inflammation both peripherally and within the CNS. These studies are well supported by previous research showing that DE exposure may tend to favor the development of conditions of neuroinflammation and oxidative stress, as well as inducing the M1 state of microglial activation and the A1 polarity of astrocyte activation (Cole et al., 2016). While certain endpoints are inducible by an acute exposure as brief as 6 hours, for other endpoints, an exposure of 3 or even as many as 10 weeks is necessary for a statistically significant change (Cobb and Cole, 2015; Coburn et al., 2018).

One of the findings was that adult neurogenesis was suppressed in male mice by a six hour exposure to DE. Adult neurogenesis is a process of multiple phases, consisting of the proliferation of neural progenitor cells, differentiation, and the survival of fully mature adult-born neurons, followed by integration into existing neural circuitry. Suppression of proliferation is an important endpoint in the investigation of cognitive function, due to the important contributions of immature neurons to the process of long term potentiation, in which synaptic connections become stronger over time (Bischofberger, 2007; Brown et al., 1988)

These studies support the hypothesis that traffic related air pollution as modeled by DE exposure contributes to the development of neurodegenerative disease through a mechanism

involving microglial activation, neuroinflammation, and oxidative stress, and possibly through the disruption of synaptic plasticity by suppressing adult neurogenesis.

One of the issues surrounding neurodegeneration that our study fails to explain is the increased incidence of AD in women (Viña and Lloret, 2010). As specified earlier, age greater than 65 years old is one of the most consistent risk factors involved in the development of AD, but even the increased life expectancy of women relative to men is not sufficient to explain the increased risk that women have of developing the disease (Viña and Lloret, 2010).

There is considerable overlap between the factors increasing risk of death from cardiovascular disease and risk factors for AD, such as obesity, diabetes, high triglycerides, metabolic syndrome and insulin resistance (Agyemang et al., 2009). Because heart disease is a major cause of death for men above the age of 40 years old, it is likely that the least healthy of these men, who might otherwise survive to develop AD, succumb before they can develop the condition (Beltrán-Sánchez et al., 2015).

In many of our experiments, female animals seem to show more resistance to the neurotoxic effects of DE exposure, either showing no evidence of neurotoxicity following exposure to DE or showing a reduced, but still statistically significant effect relative to male animals (Cole et al., 2016). Neither male nor female animals showed much of a change in levels of neurodegenerative markers following a 3-week exposure, with the exception of α-synuclein in midbrain in male animals. Unfortunately, female animals were not available for the 10 week exposure, and we cannot exclude the possibility that they might demonstrate an increased susceptibility relative to male animals. One of the possible mechanisms behind the increased robustness of female animals to the neurotoxic effects of DE is a higher concentration of paraoxonase-2 (PON2), a mitochondrial lactonase and arylesterase type enzyme with potent

antioxidant properties (Giordano et al., 2013). PON2 can mitigate endoplasmic reticular stress and provide resistance to induction of oxidative stress-induced apoptosis and favor survival (Witte et al., 2011). Lower levels of oxidative stress may also favor reduced generation of reactive nitrogen species, thus mitigating microglial activation and neuroinflammation (Peterson and Flood, 2012; Qin and Crews, 2012).

One of the important caveats for this study is that the many factors that influence probability of developing neurodegenerative conditions may tend to potentiate each other, or even mitigate risk. Living in an urban setting may expose an individual to higher levels of particulate air pollution, but may also be associated with greater levels of stress and mental health issues for reasons that may be unrelated to environmental toxicants, such as sleep cycle disruption due to light pollution (Chepesiuk, 2009; Gruebner et al., 2017; Strosnider et al., 2017). However, in cities, educational attainment, recognized to be a protective factor in neurodegeneration, tends to be higher (Letenneur et al., 2000; Ulubaşoğlu and Cardak, 2007).

Another possibility for the apparently higher number of women with AD is that women of the age in which AD is typically observed traditionally have lower educational attainment (Margriet van Hek et al., 2016). Some studies have even shown that educational attainment modifies the risk of developing AD for women, but not for men (Letenneur et al., 2000). If higher educational attainment does, in fact reduce AD risk in women, it is likely that the gender difference in AD incidence will vanish, as the number of women with college degrees has reached parity with, and more recently, even surpassed the number of men having graduated from college (Margriet van Hek et al., 2016).

Nonetheless, the results of this study suggest that exposure to airborne $PM_{2.5}$ and other finer particulates may favor the development of neurodegenerative conditions as assessed by a

number of endpoints, as modeled by DE (Coburn et al., 2018). These findings also support existing epidemiological studies showing a relationship between traffic related particulate air pollution exposure and AD and cognitive decline (Cacciottolo et al., 2017; Chen et al., 2017; Shin et al., 2018). While they support previously observed greater susceptibility of male animals to the neurotoxic effects of DE, and to some extent support the greater incidence of PD in men relative to women, they do not explain the greater prevalence of AD in women, which is likely affected by factors beyond the scope of this research, possibly biochemical or behavioral, and more likely a combination (Launer et al., 1999; Miller and Cronin-Golomb, 2010). A combination of unexamined factors that may significantly interact with DE exposure, such as age at time of exposure, sleep disturbances, and educational attainment, may be behind the greater tendency of women to develop the disorder.

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