Actions of Systemic Mediators Govern Air Pollutant-Induced Effects on the Brain

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Abstract

Air pollution is associated with increased risk of neurodegenerative and neuropsychiatric conditions such as Alzheimer's disease, Parkinson's disease, and depression. Despite evidence from epidemiological and toxicological studies, brain health outcomes are not included in many prominent air pollution health estimates due to several knowledge gaps, notably mechanistic support for human health effects. Air pollution perturbs systemic processes, including release of glucocorticoids through hypothalamic-pituitary-adrenal (HPA) axis activation, which may be a key driver of pollutant-induced health outcomes. Understanding how systemic mechanisms contribute to brain effects would provide key evidence supporting a causal relationship between pollutant exposure and disease. Moreover, there is a need for models that encompass systemic processes and human variability to facilitate the study of mechanisms underlying pollutant-induced brain effects identified in population studies. This thesis addressed these challenges by: 1) investigating the role of glucocorticoids in mediating air pollution-induced effects on the brain and peripheral metabolic outcomes associated with brain health; and 2) establishing an *ex vivo* approach that enables the role of systemic mediators of pollutant-induced brain effects to be investigated in a human model. Using samples from a rodent exposure study, I demonstrated that ozone leads to transcriptional changes in disease-relevant pathways (serotonergic, neurotrophic, cellular stress) in the hippocampus, alongside alterations in peripheral insulin signaling and circulating metabolites (sphingolipids, tryptophan, kynurenine). Application of the glucocorticoid synthesis inhibitor metyrapone revealed a mechanistic role of HPA axis activation, indicating that glucocorticoids may mediate air pollution-induced effects on the brain through both direct actions or indirectly through perturbation of peripheral metabolism. Treatment of hippocampal neurons with plasma samples from ozone-exposed rats reproduced gene expression changes observed in brain tissue, establishing the utility of an *ex vivo* model to

explore the role of systemic mediators. I also applied the *ex vivo* approach to the human context, showing that plasma collected from individuals exposed to diesel exhaust produced transcriptomic changes in human induced pluripotent stem cell-derived neurons. Together, this thesis demonstrated the critical role of systemic mediators in driving pollutant-induced effects on the brain and established a human sample-based model that can be applied to further study underlying mechanisms.

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Abbreviations

5-HT 5-hydroxytryptamine

Aβ amyloid β

ACTH adrenocorticotropic hormone

AD Alzheimer's disease

AKT akt serine/threonine kinase 1

BAD bcl-2-asssociated death promoter

BBB blood brain barrier

BCL2 b-cell lymphoma 2

BCL2L1 bcl2 like 1

BDNF brain derived neurotrophic factor

CEBPB CCAAT enhance binding protein beta

Cer Ceramide

CNS central nervous system

CORT corticosterone

CRF corticotrophin releasing factor

CPM counts per million

DAMPs damage associated molecular patterns

DE diesel exhaust

DEGS differentially expressed genes

DEN diesel exhaust with N-acetylcysteine

DEP diesel exhaust with placebo

DIV day in vitro

DPBS Dulbecco’s phosphate buffered saline

FAP filtered air with placebo

FBS fetal bovine serum

FC fold change

FI fluorescence intensity

FOS Fos proto-oncogene, AP-1 transcription factor subunit

GBD Global Burden of Disease

GC glucocorticoid

GFAP glial fibrillary acidic protein

GILZ glucocorticoid inducible leucine zipper

GPD1 glycerol-3-phosphate dehydrogenase 1

GR glucocorticoid receptor

GSK-3 glycogen synthase kinase 3 α/β

HexCer Hexosylceramide

HIF3α hypoxia inducible factor 3α

HK2 hexokinase 2

HPA hypothalamic-pituitary-adrenal

IBA1 ionized calcium-binding adapter molecule 1

IGF1 insulin-like growth factor-1

IL-1β interleukin 1β

iNOS inducible nitric oxide synthase

iPSC induced pluripotent stem cell

IR-β insulin receptor-β

IRS1 insulin receptor substrate 1

IRS2 insulin receptor substrate 2

KMO kynurenine monooxygenase

LacCer Lactosylceramide

LDH lactate dehydrogenase

LEP leptin

LOD level of detection

Log2FC log2-fold change

LPS lipopolysaccharide

MAOA monoamine oxidase A

MET metyrapone

MT1 metallothionein 1

MT2 metallothionein 2

mTOR mammalian target of rapamycin

MR mineralocorticoid receptor

NAC N-acetylcysteine

NAMs New Approach Methodologies

NPC neural progenitor cell

OCT4 octamer binding transcription factor 4

PAI-1 plasminogen activator inhibitor-1

PAMPs pathogen associated molecular patterns

PCA principal component analysis

PD Parkinson's Disease

PDL poly-D-lysine

PEPCK phosphoenolpyruvate carboxykinase

PGK1 phosphoglycerate kinase 1

PIK3R1 phosphoinositide-3-kinase regulatory subunit 1

PM particulate matter

PPIA peptidylprolyl isomerase A (cyclophilin A)

PSD95 post synaptic density protein 95

PTEN phosphatase and tensin homolog

RETN resistin

ROS reactive oxygen species

RNS reactive nitrogen species

SERPINE1 serpin family E member 1

SGK1 serum and glucocorticoid-regulated kinase

SLC2A1 solute carrier family 2 member 1

SM Sphingomyelin

T2D type II diabetes

TNFα tumour necrosis factor α

TRAP traffic-related air pollution

TRKB tyrosine kinase receptor B

TUJ1 β-tubulin III, clone TUJ1

VEGFa vascular endothelial growth factor a

VEH vehicle

VGLUT2 vesicular glutamate transporter 2

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Chapter 1: General Introduction

1.1 Overview

Air pollution exposure presents a significant public health risk. Emerging epidemiological evidence suggests that air pollution exposure is associated with increased risk of neurodegenerative and psychiatric diseases, such as Alzheimer's Disease (AD), Parkinson's Disease (PD), depression and anxiety (reviewed in Allen et al., 2017; Power et al., 2016). However, much is still unknown regarding the mechanisms through which air pollution affects the brain, especially with regards to human-relevant (e.g. incorporates interindividual variability, better represents human cell biology) data. This thesis aims to explore how air pollution impacts the brain, to establish biological plausibility for how air pollution confers risk of these diseases. In particular, this work illustrates the importance of systemic mechanisms, such as stress hormones known as glucocorticoids (GCs), in driving air pollution induced effects on the brain, and importantly establishes a novel approach to examine systemic effects of air pollution exposure on the brain utilising a human-relevant, *in vitro* model. This introduction serves to 1) review the components of air pollution, the health effects of air pollution in a general context, and specifically the current knowledge regarding the effects of air pollution on the brain, 2) explain the possible routes through which air pollutants may affect the brain, including the prevailing hypothesis that systemic inflammatory and oxidative stress signals drive pollution induced responses in the brain, 3) present an argument that GCs may also be central mediators governing air pollution induced effects on the brain, 4) describe current approaches for air pollution toxicology research, and 5) discuss the current challenges in the field that this thesis seeks to address, and present my research objectives, hypotheses and rationale.

1.2 What is air pollution?

Air pollution is a complex mixture of particulates and gases arising from a variety of sources. Particulate matter (PM) is composed of solid compounds suspended in the air that vary greatly in chemical composition based on factors such as location and source. The most common components include organic and elemental carbon, nitrate, ammonium, sulfate, sodium, and silicon. Biological compounds such as fungal spores and pollen, and heavy metals such as lead may also be present. PM is commonly classified by aerodynamic diameter, with $PM_{0.1}$ representing particles < 0.1 μm in mass median aerodynamic diameter, $PM_{2.5}$ representing particulates < 2.5 μm in mass median aerodynamic diameter, and PM_{10} representing particles < 10 μm in mass median aerodynamic diameter (Environment and Climate Change Canada 2025). $PM_{2.5}$ is the most routinely measured and reported air pollutant, and its health impacts are the most widely studied and well understood.

The gaseous components of air pollution include nitrogen oxides, ozone, sulfur dioxide, carbon monoxide, and volatile organic compounds. Nitrogen oxides, NO and NO_2, form from the combustion of fuel products used in vehicles and industrial processes. Sulfur oxides form primarily from the release of sulfur contained in minerals and coal, and sulfur oxide pollution is generally a result of oil and gas industries, fossil-fueled electric power plants, non-ferrous smelting and refining, and other industrial processes. Volatile organic compounds are carbon-containing gases and vapours, such as benzene and dichloromethane, that stem largely from gasoline fumes and solvents, oil and gas production, and wood burning. Carbon monoxide is a result of the incomplete combustion of hydrocarbon-based fuels and is mostly a direct result of the transportation industry but also can form from wood burning and forest fires. Finally, ozone is considered a “secondary” pollutant as it is produced due to the reaction of nitrogen oxides or volatile organic compounds with sunlight (Environment and Climate Change Canada 2025).

1.3 Air pollution exposure and human health

Air pollution exposure is the largest environmental risk factor for human health globally, and the second largest risk factor overall. The latest Global Burden of Disease (GBD) study estimates that air pollution exposure contributed to 7.9 million premature deaths worldwide in 2023, and 232 million disability adjusted life years (Health Effects Institute 2025). In Canada, approximately 17,400 deaths are attributable to air pollution exposure annually as of 2018, despite Canada having some of best air quality in the world (Health Canada 2024).

Several organ systems throughout the body are impacted by air pollution exposure, leading to a myriad of health impacts. Air pollution exposure increases risk of respiratory diseases such as chronic obstructive pulmonary disease and lung cancer, aggravates asthma, and leads to increased incidences of respiratory tract infections (reviewed in Bălă et al., 2021). There is also sufficient evidence attributing cardiovascular effects, such as increased risk of ischemic heart disease, myocardial infarction, and strokes, as well as elevated blood pressure, hypertension and heart failure to air pollution exposure (reviewed in Rajagopalan et al., 2018). As of 2023, air pollution exposure was the top risk factor for chronic respiratory disease, and PM was the second largest risk factor for stroke and ischemic heart disease (Health Effects Institute 2025). More recently, metabolic effects of air pollution have also been recognized, as air pollution exposure has been shown to increase risk of metabolic syndrome, diabetes, obesity, and non-alcoholic fatty liver disease (reviewed in Rajagopalan et al., 2024). The 2019 GBD project estimated that 20% of type 2 diabetes (T2D) cases worldwide were attributable to air pollution exposure (Burkart et al. 2022).

1.4 Air pollution and the brain

Beyond the more well-recognized effects of air pollution exposure on respiratory, cardiovascular, and metabolic diseases, there is an increasing recognition over the past two decades that air pollution exposure can impact brain health. Several epidemiological studies suggest that exposure to air pollution elevates risk of neurodegenerative diseases such as AD and PD, as well as neuropsychiatric conditions such as depression and anxiety (reviewed in Allen et al., 2017; Power et al., 2016). Given the ageing population and ubiquitous exposure to air pollutants around the globe, even minor impacts on relative risk of these diseases may have large scale population health impacts. The most recent GBD study included, for the first time, contributions to dementia in its pollution health impacts assessment; 626,000 deaths and 11.6 million disability adjusted life years were found to be the result of dementia attributable to air pollution exposure in 2023 (Health Effects Institute 2025).

In 2002, Calderón-Garcidueñas et al. found that dogs in Southwestern Metropolitan Mexico City, an area where pollution levels often exceed standards, displayed several indicators of neuropathology compared to those from Tlaxcala, an area with lower than average pollution levels (Calderón-Garcidueñas et al. 2002). Since then, controlled exposure studies with rodent models and *in vitro* models have made substantial progress in understanding air pollution's effects on the brain, as will be reviewed in the following sections.

1.4.1 Behaviour and cognitive function

Rodent behaviour and cognitive studies are largely in alignment with population cohort studies reporting increased occurrence of depression and anxiety symptoms, decreased cognitive function, and increased risk of dementia following exposure to air pollution. Rodents exposed to either PM or ozone exhibit depressive-like behaviours such as increased immobility and floating behaviour, and decreased latency to float in forced swim tests (Fonken et al. 2011; Mokoena et

al. 2015; Woodward et al. 2018; Ehsanifar et al. 2023). Both PM- and ozone-exposed mice also exhibit anxiety-like behaviours such as reduced time spent in the center in open-field tests (Fonken et al. 2011; Ehsanifar et al. 2023; Li et al. 2024; Xu et al. 2025; Dai et al. 2025), and decreased time spent in the open arm and the center area in the elevated plus maze (Mokoena et al. 2015; Ehsanifar et al. 2023; Li et al. 2024; Dai et al. 2025).

Cognitive impairments are also evident amongst PM and ozone-exposed rodents. Exposed rodents exhibit impaired learning and memory ability measured through the Morris water maze (Hajipour et al. 2020; Xu et al. 2022a; Dai et al. 2025), Barnes maze (Fonken et al. 2011) and Y-maze (Xu et al. 2025; Dai et al. 2025). Reduced recognition of novel objects (Mokoena et al. 2015; Woodward et al. 2018; Kilian et al. 2023; Oliver et al. 2024) has also been observed.

1.4.2 Structural deficits

The brain is a complex organ with a highly organized structure. Numerous cell types form distinct regions that communicate through a series of functionally and structurally connected networks to allow for high level cognitive processes. Disruptions of network connectivity is observed among patients with neurodegenerative diseases and psychiatric conditions and are associated with disease severity and phenotype (reviewed in Bullmore & Sporns, 2009; Pievani et al., 2014).

Through magnetic resonance imaging techniques, the effects of air pollution on the structure and functional connectivity of the brain have been investigated in numerous population cohorts. For instance, several studies have found that exposure to air pollutants altered volumes of cortical and subcortical brain regions in adults (Chen et al. 2015; Hedges et al. 2019; Cho et al. 2023; Ko et al. 2024; Thompson et al. 2025) and with prenatal and childhood exposures

(Lubczyńska et al. 2021; Miller et al. 2022; Bos et al. 2023; Kong et al. 2025; Kusters et al. 2025a; Sukumaran et al. 2025; Buthmann et al. 2025). Changes in resting state functional connectivity have also been observed in preadolescents (Pérez-Crespo et al. 2022; Cotter et al. 2023; Kusters et al. 2025b) and adults (Glaubitz et al. 2022; Gawryluk et al. 2023). Patterns of effects observed however, vary between studies likely due to differences in cohorts, measurements and analytic techniques, and pollution compositions and sources.

In rodents, histopathological assessments have revealed structural changes in pyramidal cells of the hippocampus and prefrontal cortex of mice exposed to ambient air pollution (Yang et al. 2025c), disorganization of neurons and a reduction of cell numbers in the olfactory bulb of ozone-exposed mice (Xu et al. 2025), and disorganization of Purkinje cells in the cerebellum, along with mild vacuolation and degeneration, in mice exposed to diesel exhaust (DE) (Liu et al. 2024). Interestingly, it has been reported that the lateral ventricle volume of female, but not male, rats decreases following traffic-related air pollution (TRAP) exposure, alongside an increase in the thickness of the granule cell layer of the hippocampus (Patten et al. 2020). Moreover, prenatal exposure of mice to DE led to increased cortical and hippocampal volumes at embryonic day 18; however, these effects switched to a decrease in cortical volume at postnatal day 30 specifically in males further supporting sex-specific effects of pollutants on hippocampal microstructure (Bolton et al. 2017).

1.4.3 Neurotransmitter systems

Neurons are highly specialized cells responsible for transductions of signals which allow for the cognitive and physiological functions of the body. To communicate, neurotransmitters are secreted at the synaptic cleft by the presynaptic neuron and bind to receptors on the postsynaptic neuron to allow for signal transduction. There are various neurotransmitter systems with distinct

roles: cholinergic, glutamatergic, GABAergic, dopaminergic, serotonergic, histaminergic and aminergic. Several processes can disrupt homeostasis of neurotransmitter systems, including neurotransmitter loss, oxidative stress, metabolic stress, mitochondrial dysfunction, inflammation, disruption of synaptic protein levels, and synapse loss. Maintenance of neurotransmitter systems is critical, and disruption of these systems is associated with various neurodegenerative diseases and psychiatric disorders (reviewed in Nimgampalle et al., 2023).

Air pollution exposure may lead to alterations of neurotransmitter levels in certain brain regions. Kinawy et al., found that acute exposure to gasoline-polluted air led to elevation of excitatory neurotransmitters glutamate and aspartate, and decreases in glycine and GABA, in the cerebral cortex, hippocampus and hypothalamus of rats. Chronic exposure led to an increase in aspartate and a decrease in GABA levels in the cortex, yet increases in aspartate and glutamate in the hippocampus and hypothalamus (Kinawy et al. 2014). Mokoena et al. observed decreased serotonin levels in the frontal cortex and hippocampus of rats chronically exposed to ozone (Mokoena et al. 2015). Both Cory-Slechta et al. and Allen et al. described several alterations to neurotransmitters in various brain regions of mice, with exposure time and sex dependent effects, following exposure to ultra-fine PM (Cory-Slechta et al. 2024) or concentrated ambient PM (Allen et al. 2014). Turnover of dopamine to its metabolites, 3-4-dihydroxyphenylacetic acid and homonvanillic acid, was reduced with DE exposure in the striatum and nucleus accumbens of mice (Yokota et al. 2009). Dopamine uptake appears to be reduced following pollution exposure as well, as has been observed in the striatal synaptosomes of rats exposed to fine and ultrafine PM (Andrade-Oliva et al. 2022), and in neuron-glia cell cultures exposed to DE (Levesque et al. 2011).

Alongside alterations in neurotransmitter levels, reduction of key neurotransmitter receptors following air pollution exposure have been noted in several studies. Exposure to PM significantly reduced glutamatergic N-methyl-D-aspartate receptor subunit levels in the cerebral cortex of adult mice (Godoy-Lugo et al. 2025), mRNA expression in the hippocampus of prenatally-exposed mice (Ehsanifar et al. 2019) and protein levels in the hippocampus of adult rats (Li et al. 2018). Hippocampal expression of GABA receptor subunits, as well as several serotonin receptors were also decreased in adult mice following DE exposure (Liu et al. 2024).

1.4.4 Neuroinflammation

Chronic neuroinflammation is a hallmark of neurodegenerative conditions. Once thought to be a consequence of disease processes, there is increasing evidence that neuroinflammation is a critical contributor to initiation of these neurodegenerative processes (reviewed in W. Zhang et al., 2023). Activation of microglia, the innate immune cells of the brain, is necessary for normal functioning of the brain allowing for processes such as synaptic pruning, maintenance of synaptic plasticity, neuronal apoptosis and immune surveillance (reviewed in Stephan et al., 2012). However, exposure to chronic low-level inflammation may shift cells from a homeostatic phenotype and lead to a "primed" state in which microglia exhibit exaggerated immune responses to stimuli which can exert pathological effects (reviewed in Cunningham, 2013).

Chronic exposure to ozone and PM increased expression and protein levels of ionized calcium-binding adapter molecule 1 (Iba1), a marker of activated microglia, in the olfactory bulb (Xu et al. 2025), hippocampus (Patten et al. 2020; Ehsanifar et al. 2023; Yang et al. 2025b), substantia nigra (Rivas-Arancibia et al. 2015), and whole-brain homogenate (Levesque et al. 2011). Short-term exposure to ozone has also been shown to increase Iba1 levels (Mumaw et al. 2016). Greve et al. found that exposure to DE for 4 weeks led to increased numbers of microglia

in the hippocampus that exhibit reduced branch length (an indicator of activation), but did not demonstrate increased Iba1 expression (Greve et al. 2020). Complementary to activation of microglia, elevation of proinflammatory cytokine expression and protein levels have been found in various brain regions following pollutant exposure as well (Campbell et al. 2005; Levesque et al. 2011; Fonken et al. 2011; Ehsanifar et al. 2022, 2023; Xu et al. 2025).

Exposure of microglial cell lines BV2 (mouse) and HMC3 (human) to high doses of PM have demonstrated elevated cytokine production (Kim et al. 2020; Zhang et al. 2024b). A tri-culture model of the brain utilizing human neurons, astrocytes and microglia found that PM-induced cytokine release in neuron and astrocyte co-cultures promoted migration of microglia cells and polarization towards a pro-inflammatory phenotype. Tricultures treated with $PM_{2.5}$ exhibited elevated cytokine production and neurotoxicity compared to the neuron-astrocyte co-culture (Kang et al. 2021). In addition to direct effects of PM exposure, treatment of both primary rat microglial cultures and a rat microglial cell line (HAPI) with serum from rats exposed to ozone led to a "primed" phenotype in which lipopolysaccharide (LPS)-induced inflammatory responses were exaggerated compared to treatment with serum collected from rats exposed to filtered air (Mumaw et al. 2016).

1.4.5 Mitochondrial dysfunction and oxidative stress

The brain is particularly vulnerable to oxidative damage, due to its high concentrations of lipids, high oxygen demands, and deficient antioxidant systems compared to other organs (reviewed in Friedman, 2011). Mitochondrial dysfunction is both a consequence and initiator of oxidative stress – dysfunction of mitochondria increases production of reactive oxygen species (ROS), which can then further damage the mitochondria, as well as other lipids, proteins, and

DNA. These outcomes can interfere with proper neuronal functioning and contribute to development of pathology (reviewed in Houldsworth, 2024).

Exposing the human neuroblastoma cell line SH-SY5Y to PM has been previously shown to disrupt mitochondrial morphology and mass, reduce ATP production and cellular respiration, and increase intracellular ROS (Wang et al. 2019; Lin et al. 2022; Li et al. 2022). The rat neuron-like pheochromocytoma cell line PC-12 similarly had increased ROS and nitrate release after exposure to DE (Kim et al. 2014). Similar effects have also been observed in microglial cell lines BV2 and HMC3 (Morris et al. 2024; Zhang et al. 2024b). Treatment with antioxidants N-acetylcysteine (NAC) or glutathione rescued some of the PM-induced effects on SH-SY5Y (Wei et al. 2017) and HMC3 (Zhang et al. 2024b) cells.

In rodents, several studies report markers of oxidative stress in several brain regions, including increased levels of lipid peroxidation, inhibitions in activity of antioxidant enzymes superoxide dismutase and glutathione peroxidase, and increased nuclear localization of the transcription factor nuclear factor κB, following exposures to ozone (Rivas-Arancibia et al. 2015, 2024) and PM (Cole et al. 2016; Hajipour et al. 2020; Ehsanifar et al. 2021; Vastegani et al. 2023).

1.4.6 Neurogenesis

Neurogenesis in the hippocampus, the process through which new neurons are developed in the adult brain, is critical for memory formation and cognitive functions (reviewed in Toda et al., 2019). Rats exposed to TRAP-$PM_{0.2}$ during prenatal and early life stages exhibited a reduction of EdU+ neurons (a proliferation marker) in the dentate gyrus, indicating impaired neurogenesis (Woodward et al. 2018). Another study found that exposure to ozone led to a decrease in the amount of doublecortin+ (a marker for immature neurons) cells in the dentate

gyrus of rats after 60 days, indicating impaired neurogenesis (Rivas-Arancibia et al. 2009). Conversely however, Patten et al. found that male rats exposed to TRAP during prenatal and early life stages had increased amounts of doublecortin+ in the subventricular zone, suggesting increased neurogenesis (Patten et al. 2020).

Brain derived neurotrophic factor (BDNF) plays a variety of roles in neuronal development, including regulation of hippocampal neurogenesis. Decreased expression of BDNF, along with its receptor, tyrosine receptor kinase B (TrkB), within the hippocampus (Liu et al. 2024) and whole brain tissue (Li et al. 2024) has been observed in rodents following exposure to PM. Beyond rodent exposure studies, *in utero* exposure to $PM_{2.5}$ decreased fetal serum concentration of BDNF (Wang et al. 2023) and placental gene expression of BDNF at birth (Saenen et al. 2015-8).

1.5 How air pollutants can exert effects on the brain

There are several potential routes through which air pollutants may interact with the brain to cause biological effects described in the previous section. “Axonal transport” refers to the direct translocation of PM to the brain via trigeminal (nasal) or vagal (respiratory) nerve pathways. “Systemic transport” refers to the transfer of PM or bioactive factors generated following pollution exposure through the systemic circulation to the brain (Figure 1.1)

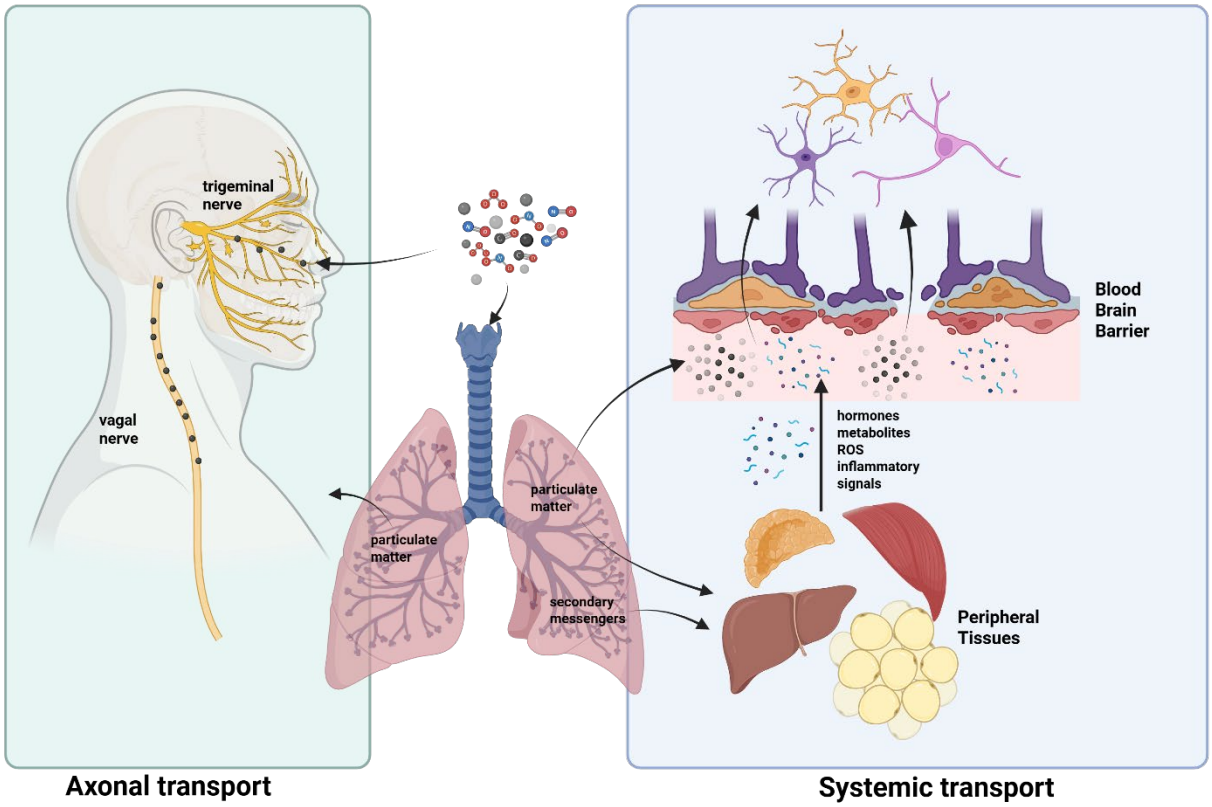


Figure 1.1 Routes through which air pollutants may impact the brain. Particulate matter may be transported via trigeminal or vagal nerve tracts to directly interact with the brain (axonal) or translocate from the lungs to the bloodstream to interact with the blood brain barrier (systemic). Alternatively, gaseous pollutants and particulates may react with the lung epithelium, leading to release of systemic responses which trigger the release of bioactive factors into circulation which may then affect the brain (systemic). Figure created using Biorender.

While initially reported that ultrafine PM could translocate from the lungs to the bloodstream and periphery in humans (Nemmar et al. 2002), this finding was controversial as this study was met with concern over technical aspects (Burch 2002) and subsequent studies were not able to replicate this exact finding (Mills et al. 2006; Wiebert et al. 2006; Möller et al. 2008). In rodents, ultrafine PM have been detected in liver tissue (which must be via circulation) (Oberdörster et al. 2002), spleen, and lymph nodes (Kim et al. 2025) and in blood samples (Kreyling 2016; Qi et al. 2022). Although notably, Qi et al. also examined PM in sera samples from human participants, and only found evidence of PM in the sera of two patients out of a cohort of 25 with various brain disorders, and in one healthy control participant (out of 26), whereas they detected PM in the cerebral spinal fluid of 8 out of the 25 patients. A recent study by Rasking et al. found evidence of black carbon particles in kidney biopsies of 25 patients, which provides additional evidence of transport through the circulatory system (Rasking et al. 2023).

While it is generally accepted that any circulating PM, if present, would not be able to pass through the blood brain barrier (BBB) in a healthy state unless bound to circulating biomolecules, it could induce damage which may increase the permeability of the BBB. This could allow particulates to pass through and interact with the brain tissue directly. For instance, exposure to TRAP-$PM_{0.2}$ in rats led to a decrease in zonula occludens-1, a tight junction marker, as well as increased iron deposits in the CA1 region of the hippocampus indicating damage to the BBB (Woodward et al. 2018). Similarly, exposure to O3, PM, or both decreased zonula occludens-1 levels in hippocampal tissue of mice (Yang et al. 2025a).

In addition to direct interaction of PM with the brain, both PM and gaseous air pollutants cause reactions in the lungs which can lead to adverse effects in peripheral organs, subsequently

triggering the release of bioactive factors into the systemic circulation. These circulating factors may then interact with both the BBB and brain tissue itself to cause effects. The brain is not separate from the rest of the body but is rather intimately connected and governed by widespread peripheral effects that influence other organ systems and vice versa. While the BBB was originally thought to be impermeable, further studies revealed that selective transport of molecules from circulation into the brain does occur. In fact, this is a critical process allowing for movement of glucose into the brain, necessary for meeting the intense energy demands of the central nervous system (CNS) (Lund-Andersen 1979; Dick et al. 1984). In addition to glucose, several other molecules can be transported across the BBB that may importantly contribute to, or disrupt, brain function (reviewed in Gonçalves & De Felice, 2021), including those which may be altered by air pollution exposure.

1.6 The prevailing hypothesis: inflammation and oxidative stress are responsible for pollutant induced health effects on the brain

Arguably, the most widely acknowledged mechanism for how air pollutants may influence extrapulmonary effects throughout the body, including the brain, is the induction of systemic inflammatory and oxidative stress responses.

Inflammation refers to a complex response to external (such as microorganisms) or internal (such as tissue damage) stimuli that may pose a danger to the host organism. Inflammation is a multi-step process involving sensing of a “danger signal” through recognition of pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs), transmission of the signal through signaling cascades leading to the release of cytokines, chemokines, and other molecules, and finally the inflammatory response carried out by effector cells to eliminate a pathogen or repair the damaged tissue. While inflammation is a necessary adaptive response for everyday survival, failure to resolve inflammatory activity can

result in low-grade chronic inflammatory conditions, that are maladaptive and lead to tissue damage and disruption of homeostasis. Several diseases, including metabolic syndrome, diabetes, heart failure, atherosclerosis, cancer, chronic kidney disease, and non alcoholic fatty liver disease, amongst others, are considered inflammation-related disorders. In fact, inflammation-related diseases comprise over 50% of global deaths (reviewed in Furman et al., 2019).

Oxidative stress refers to the imbalance of oxidants, such as ROS or reactive nitrogen species (RNS), to reductants leading to disruption of cellular redox homeostasis. Mitochondrial or ER dysfunction, or overactivation of certain enzyme systems, as a result of pathological conditions such as inflammation or hypoxia, contribute to the generation of ROS/RNS. These ROS/RNS can exert damage to biomolecules, oxidizing DNA, proteins and lipids, impacting cellular health by disrupting key signaling pathways or ultimately inducing cell death (reviewed in Lugrin et al., 2014).

Oxidative stress and inflammation are intimately related. Both chronic and acute inflammatory states lead to the generation and release of ROS/RNS and dysregulation of redox homeostasis. For instance, the proinflammatory signaling cascade, induced by toll-like receptor activation of PAMPs or DAMPs, activates NADPH oxidase enzymes, which generate ROS, as well as induce expression of inducible nitric oxide synthase (iNOS) which promotes the generation of nitrogen oxide. While this formation of oxidant species assists with pathogen elimination, it can also promote sustained proinflammatory activity. In turn, oxidative damage to tissue can elicit immune responses, including through activation of toll-like receptors, furthering inflammatory conditions (reviewed in Lugrin et al., 2014).

Chronic exposure to air pollutants is documented in several studies to elicit oxidative stress and inflammation. Both gaseous pollutants such as ozone, a powerful oxidizing agent, or PM (which may contain oxidizing compounds on its surface), can elicit oxidative tissue damage in the lung epithelium through peroxidation of lipids, protein modification, DNA damage, and generation of other radicals (Dellinger et al. 2001; Gurgueira et al. 2002-8). As a consequence, a local inflammatory response is generated, leading to cytokine release, activation of alveolar macrophages, infiltration of other immune cells, as well as further oxidative stress, inflammatory dysfunction, and tissue damage (Kennedy et al. 1998; Salvi et al. 1999; Fujii et al. 2001; Bassett et al. 2001; Costa and Dreher 1997-9). This pulmonary inflammation may then trigger widespread systemic inflammation and oxidative stress, resulting in elevated levels of circulating inflammatory mediators (Viehmann et al. 2015; Pope et al. 2016; Li et al. 2017), peroxidized lipids (Santiago-López et al. 2010) and other potentially harmful byproducts. Additionally, fine particles may be transported through the bloodstream and interact with other organ tissues directly to generate localized tissue damage or inflammatory responses.

Chronic systemic inflammation and oxidative stress are widely considered key contributors to the development of neurodegeneration and other brain health outcomes. Individuals with higher blood cytokine levels, chronic inflammatory diseases such as diabetes or atherosclerosis, or viral or bacterial infections experience increased rates of cognitive decline and risk of developing neurodegenerative diseases (reviewed in Bayraktaroglu et al., 2025). Use of long-term nonsteroid anti-inflammatory drugs on the other hand, is associated with reduced AD risk (Breitner et al. 1994; Rich et al. 1995; Beard et al. 1998). Circulating inflammatory mediators can disrupt BBB function, enter the brain and elicit a neuroinflammatory environment (reviewed in Banks, 2008). Additionally, activated peripheral immune cells can infiltrate the

brain (Fiala et al. 2002; McManus et al. 2014). This neuroinflammatory environment can lead to neuronal dysfunction through shifting phenotypes of microglia and astrocytes from homeostatic to inflammatory, or inducing oxidative stress responses which may trigger or exacerbate neuronal damage and disease-like pathology (reviewed in Cunningham, 2013).

Given that systemic inflammation and oxidative stress have clear effects on brain health, it is not surprising that the neurotoxicological effects of air pollution exposure are often attributed to these mechanisms without extensive empirical evidence. While select pharmacological intervention studies may support this notion – for instance, Vastegani et al. and Nery-Flores et al. showed that curcumin, a drug with antioxidant and anti-inflammatory properties, protected against ozone and PM-induced effects in the hippocampus and cerebral cortex, ozone-induced impairments in behaviour, and elevation of serum cytokine levels (Nery-Flores et al. 2018; Vastegani et al. 2023) – there is insufficient evidence that inflammatory and oxidative stress are solely responsible for all the effects of air pollution exposure on the brain. In several cases, proinflammatory profiles are not evident following pollution exposure. Mumaw et al. found that serum from ozone-exposed rats primed cultured microglial cells to inflammatory stimuli, in the absence of any change in circulating cytokines (CCL2, CCL11, TNFα, IL-6 and IL-1β) indicating a role for some other bioactive factor (Mumaw et al. 2016). In a similar vein, Schisler et al. found that plasma from individuals exposed to DE altered gene expression in cultured coronary artery cells, with no change in plasma levels of IL-6 or TNFα present (Schisler et al. 2015). Previous work from our laboratory has found that acute exposure to ozone or the PM EHC-93 actually led to general inhibition of proinflammatory genes in several tissues (Thomson et al. 2013, 2016), and generally little effect on circulating cytokines (Thomson et al. 2016). These lines of evidence suggest some other circulating factor may be responsible for

mediating pollutant-induced effects throughout the body, including on the brain, which may precede any proinflammatory response.

1.7 The Hypothalamic-Pituitary-Adrenal axis: a potential key mediator of air pollution health impacts

The hypothalamic-pituitary-adrenal (HPA) axis is the body's central stress response mechanism, resulting in the production of GC hormones. Activation of the HPA axis begins with neural input or other stimulus to the hypothalamic paraventricular nucleus, which signals the production and secretion of corticotropin releasing factor (CRF). CRF signals endocrine cells of the pituitary gland to produce adrenocorticotropic hormone (ACTH), which then travels through systemic circulation to stimulate melanocortin 2 receptors present in adrenal cells to initiate a series of reactions that culminates in the production of GCs via 11β-hydroxylase enzymes (Figure 1.2). While in humans (as well as dogs, hamsters, and fish) cortisol is the principal endogenous GC, in rats, mice and birds, the principal endogenous GC is corticosterone (reviewed in Spencer & Deak, 2017). At normal physiological levels, GCs primarily occupy the high affinity mineralocorticoid receptor (MR) to regulate circadian and ultradian rhythm responses. Under stress (or at peak levels during the circadian rhythm) when GC levels are elevated beyond normal physiological levels, the MRs become fully occupied allowing GCs to then bind to the lower affinity glucocorticoid receptor (GR). Both receptors, upon binding, form homodimer or heterodimer transcription factor complexes which bind to GC response elements to regulate gene expression to govern a variety of biological processes, including inflammatory responses, insulin and glucose metabolism, lipid metabolism, etc. Under circumstances of acute stress, HPA axis activation is necessary for survival to allow for physiological responses to deal with the stress, followed by a rapid resolution of the stress response by means of a negative feedback mechanism. However, chronic stress, leading to persistent elevation of GCs, leads to a

variety of detrimental effects that may contribute to the development of disease (reviewed in Agorastos & Chrousos, 2022; Cohen et al., 2012; Rohleder, 2016)

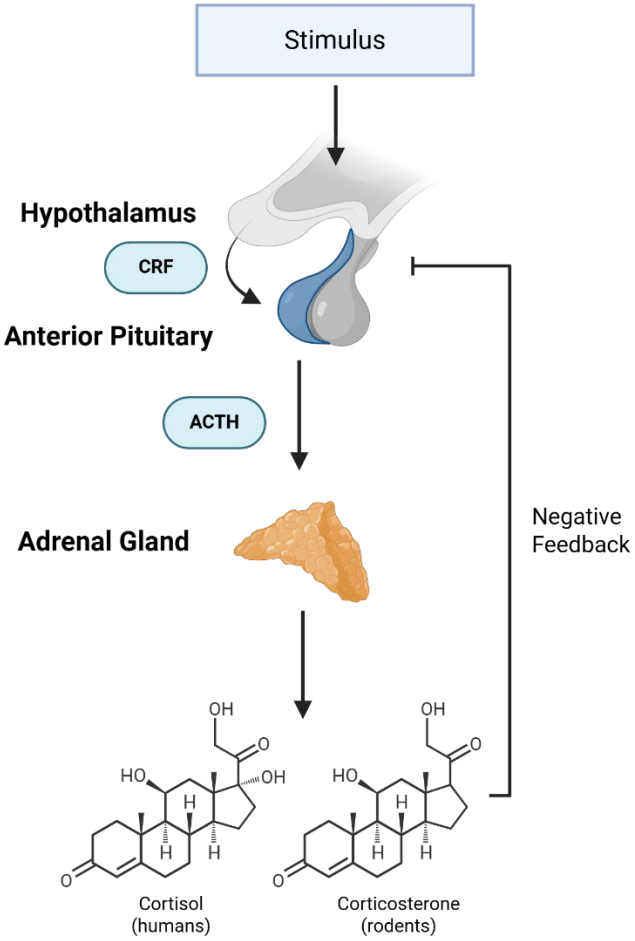


Figure 1.2. The hypothalamic-pituitary-adrenal axis. Upon nervous input following exposure to a stressor, or inflammatory signaling, the hypothalamus produces corticotropin-releasing factor (CRF), which is transported to the anterior pituitary and triggers release of adrenocorticotropic hormone (ACTH) into circulation. In response, the adrenal gland stimulates production of corticosterone (rodents), or cortisol (humans). Elevated corticosterone / cortisol triggers a negative feedback mechanism, inhibiting further activation. Figure created in Biorender.

In 2013, our laboratory demonstrated that Fischer-344 rats acutely exposed to ozone (0.8 ppm), the PM EHC-93 (50 mg/m^3), or both, had elevated levels of circulating corticosterone, as well as ACTH. Additionally, activation of the GC-responsive genes glucocorticoid inducible

leucine zipper (*GILZ*) and serum and glucocorticoid-regulated kinase 1 (*SGK1*) was evident in several tissues (Thomson et al. 2013). A subsequent study (Thomson et al. 2016) employed metyrapone, an 11β-hydroxylase antagonist, to investigate the role of GCs in mediating ozone-induced effects in rats. Metyrapone treatment prevented gene expression responses in several tissues following ozone exposure, confirming that elevated corticosterone levels following ozone exposure can impact organ systems. Moreover, gene expression responses in the liver, lung, heart and spleen of ozone-exposed rats positively correlated with those observed in an additional group of rats administered corticosterone treatment prior to exposure to clean air. Since then, findings that cortisol is elevated in participants of controlled exposure studies to ozone (Miller et al. 2016a) and DE (Thomson et al. 2021) have been published. Work by another group has similarly shown elevation of circulating corticosterone in ozone-exposed rats (Henriquez et al. 2018), and established that adrenalectomy prevented ozone-induced transcriptional responses in the brainstem and hypothalamus (Henriquez et al. 2019).

Several chapters of this thesis utilize the samples collected from the metyrapone intervention study described above (Thomson et al. 2016) to further investigate the specific role of GCs in mediating changes in circulating metabolites and biological effects on the brain following pollution exposure. In the 1950's with the popularization of GCs as a therapeutic for inflammatory diseases, the potential for GCs to influence cognition and behaviour first became recognized upon the emergence of detrimental cognitive side effects (reviewed in Lupien et al., 2007). As a lipid-soluble molecule, GCs can translocate across the BBB. In 1968, McEwen et al. critically showed that radio-labelled corticosterone is retained in the brain, especially in the hippocampus (McEwen et al., 1968). Further studies confirmed that the brain, particularly the hippocampus, expresses GRs and MRs (McEwen et al. 1986; Seckl et al. 1991). Given the

importance of the hippocampus in diseases such as dementia and depression, it is unsurprising that HPA axis dysregulation is evident amongst patients with depression, as well as AD, PD, and other dementias (Lucassen et al. 2014; Ouanes and Popp 2019). Additionally, evidence suggests that patient cortisol levels correlate with worsening disease progression (John G. Csernansky et al. 2006), and GC excess correlates with decreased brain volumes in cognitively normal participants (Geerlings et al. 2015; Echouffo-Tcheugui et al. 2018), and smaller hippocampal volumes and memory impairments in elderly subjects (Lupien et al. 1998). Subjects exposed to chronic psychosocial stress featured impaired connectivity of the frontoparietal network and reduced attention shifting ability (Liston et al. 2009).

Experimental studies have found that while acute stress or basal GC levels may exert beneficial effects on the brain such as increasing synaptic plasticity, chronic elevations in GCs can lead to dendritic atrophy and impairments in synaptic plasticity (Woolley et al. 1990; Magariños and McEwen 1995; Alfarez et al. 2009; Pedrazzoli et al. 2019), cell death (Sapolsky et al. 1985; Kurek et al. 2016), and impairments in adult neurogenesis (Gould et al. 1997; Tanapat et al. 2001; Coe et al. 2003; Anacker et al. 2013; Kino 2015) in hippocampal neurons. It is hypothesised that these GC-induced effects on the brain may disrupt the HPA axis negative feedback mechanism, leading to further dysregulation of the HPA axis. GCs may also elevate production of amyloid-β (Aβ) plaques and hyperphosphorylation of Tau, pathological features of AD, as stress and treatment with the GR agonist dexamethasone have been found to promote this phenotype, both *in vivo* and *in vitro* (Green et al. 2006; Sotiropoulos et al. 2008, 2011; Catania et al. 2009).

In addition to direct effects on neurons, GCs also play a critical role in mediating inflammatory processes, including exerting effects on microglia and astrocytes. While actions of

GCs are widely renowned for being anti-inflammatory, stress and elevated GCs may prime inflammatory cells in the hippocampus and cortex, making them more susceptible to later inflammatory challenge (Johnson et al. 2002; Sorrells et al. 2009; Munhoz et al. 2010; Frank et al. 2012; Liu et al. 2018). Moreover, the anti-inflammatory actions of GCs may be critical to reducing neuroinflammation and preventing death of dopaminergic neurons, the pathological feature of PD (Castaño et al. 2002; Ros-Bernal et al. 2011); however chronic GC elevation can induce GC resistance, which potentiates responses to inflammatory stimuli (Cohen et al. 2012).

Beyond these known effects of GCs on the brain, GCs also influence several metabolic processes in the periphery, which may be linked to brain health outcomes. Metabolic diseases are often comorbid with neurodegenerative and psychiatric diseases (reviewed in Barbagallo & Dominguez, 2014; Yue et al., 2025), of which metabolic disruptions such as insulin resistance and dysregulation of lipid metabolism are also a feature (reviewed in Kulas et al., 2020). This indicates a commonality of disease pathways between these conditions. Originally named for its role in regulating glucose metabolism, a primary action of GCs is to provide a supply of glucose to the brain. To facilitate this, GCs act to promote glucose synthesis in the liver and inhibit glucose uptake in peripheral tissues. Additionally, GCs also play a role in regulating lipid metabolism. GCs are known to increase lipolysis in the adipose tissue, generating free fatty acids and glycerol, but can also stimulate lipogenesis synergistically with insulin. Enzymes responsible for synthesis of ceramide species, which comprise of a sphingosine backbone and fatty acid chain, are also elevated by GCs. Ceramides serve as critical signaling molecules, which can antagonize insulin signaling pathways (reviewed in Magomedova & Cummins, 2016). Interestingly, evidence suggests that GCs can also regulate insulin sensitivity through CNS control, through actions on the arcuate nuclei (Yi et al. 2012). Through these actions, sustained

GC elevation can impact body composition and insulin sensitivity, elevating risk of metabolic diseases. Individuals with metabolic syndrome, type II diabetes, insulin resistance, and other metabolic diseases tend to display dysregulation of HPA axis function, although patterns of cortisol levels are not consistent (Björntorp et al. 1999; Chiodini et al. 2007). Cushing's disease patients, who experience excess cortisol secretion, tend to experience metabolic disruptions and increased risk of metabolic syndrome and type II diabetes (reviewed in Ferraù & Korbonits, 2015), in addition to increased risk of psychiatric conditions such as depression and anxiety, and neurocognitive deficits such as impaired memory and concentration (reviewed in Pivonello et al., 2015).

Given the impacts of GC excess and chronic stress on the brain and metabolic outcomes, and the current literature showing the pollutant-induced elevations of GCs impact gene expression in several organs including the brain, the HPA axis may be a central mediator driving the association between air pollution exposure and brain health effects.

1.8 Approaches to studying air pollution health effects

While epidemiological studies have provided key insights into potential health effects of air pollution exposure and disease risks, these studies are limited by several factors that contribute to uncertainty which prevent conclusive evidence needed to establish biological plausibility. For instance, humans have variable lifestyles and move throughout several locations daily which is difficult to estimate in exposure models that usually rely on residential address. Moreover, areas with high air pollution are often larger urban environments, close to major roadways, or industrial facilities. These environments may impact health outcomes in ways unrelated to air pollution (noise, heat, proximity to green or blue space, physical activity, food access) and may have different population characteristics from lower pollution environments

(Sarkar and Webster 2017). Therefore, controlled exposure studies, in which conditions can be more carefully manipulated, are necessary to better understand the biological effects of air pollution exposure. While acute controlled exposure studies with human subjects have been performed, these studies are limited to non-invasive measurements and can still be affected by confounding factors.

Traditionally, animal experiments have been the gold standard in toxicological research. This is also true of air pollution exposure, with controlled animal exposure models contributing to the bulk of our current understanding regarding air pollution health effects. However, over the last decade there has been a growing movement to shift away from animal testing in toxicology. Several government bodies, including within Canada, the United States of America, the United Kingdom, and the European Union have announced plans to phase out animal testing within their regulatory agencies (Hansell and Hartung 2025). This is due to several reasons, including concerns about the translatability of animal research to humans, as well as animal welfare.

With the need for human relevant, animal free testing strategies, has come the development of New Approach Methodologies (NAMs). NAMs include *in vitro*, *in chemico,* or *in silico* methods that can be used to improve toxicity testing and contribute to the reduction and replacement of animal experiments (Ouedraogo et al. 2025). With rapid improvements to cell culture technology – such as organoids and microfluidic "organ on a chip" models – *in vitro* systems can now better represent physiological conditions. Additionally, with improved technology *in silico* models may better predict potential toxicity.

There are still several barriers to the wide-spread integration of NAMs into regulatory testing, including concerns of reliability and relevance of NAMs for safety assessment, added financial costs, need to train additional staff and develop new analytical pipelines, legislative

constraints, and reluctance to move away from familiar, well established methodologies, amongst others (Sewell et al. 2024-3-25). Despite these barriers however, NAMs are gaining popularity. In the field of air pollution research, Air Liquid Interface models are a promising approach to study the pulmonary effects of both PM and gaseous pollutant exposure on human lung cells under more physiologically relevant conditions. However, study of extrapulmonary effects of air pollution using NAMs remains a challenge. While *in vitro* studies have examined direct effects of PM by addition of PM to the cell culture media, these studies exclude the potential critical role that systemic mechanisms, such as GCs, may play in driving extrapulmonary health effects of air pollutants.

To allow for the study of how systemic mechanisms might drive air pollution health effects, an *ex vivo* approach has been employed by some groups. This approach involves treatment of cultured cells with plasma or sera from animals or humans exposed to an air pollutant, to examine how bioactive circulating factors may cause responses in a target cell or tissue type. Sera from mice or rats exposed to ozone prevented acetylcholine-induced vasodilation in isolated coronary arteries, similar to what was observed *in vivo* (Robertson et al. 2013; Paffett et al. 2015). Sera from ozone-exposed rats also primed microglial cells to be more susceptible to inflammatory challenge (Mumaw et al. 2016). Plasma from humans exposed to DE or NO_2 led to elevation of adhesion molecules VCAM-1 and ICAM-1, and gene expression changes associated with inflammatory signaling in primary human coronary artery endothelial cells (Channell et al. 2012; Schisler et al. 2015). This *ex vivo* approach has been utilised in clinical studies as well, particularly in research about preeclampsia (Baboonian et al. 1989; Baker et al. 1995; Henson et al. 1996; Faas et al. 2008), and more recently in a study examining responses of 3D skeletal muscle tissue culture to sera from individuals with long COVID-19 or

chronic fatigue syndrome (Mughal et al. 2025). An *ex vivo* approach may therefore be a useful tool to examine how systemic mediators govern air pollution induced effects on the brain, especially given that it could be used with human cells and plasma samples.

1.9 Rationale, hypotheses and objectives

Despite the substantial progress made towards understanding the health effects of air pollution, particularly on the brain, several challenges remain in the field which are necessary to address to accurately assess risk and inform policy decisions surrounding air quality. Several prominent health estimates, such as those conducted by the Environmental Protection Agency (USA) and Health Canada, do not currently include brain health effects due to a lack of sufficient evidence (U.S Environmental Protection Agency 2020).

First, further study into the systemic metabolic effects of air pollution may provide insights into potential brain health outcomes and common disease pathways. It is well known that metabolic diseases such as obesity, T2D, and insulin resistance are associated with increased risk of neurodegenerative disease development (reviewed in Barbagallo & Dominguez, 2014; Yue et al., 2025). This may be a consequence of resulting metabolic changes due to these disease states then affecting the brain, or similar underlying mechanisms initiating both disease states. For instance, elevated cortisol levels are a commonality in both metabolic (Di Dalmazi et al. 2012) and neurodegenerative diseases, as well as psychiatric conditions (Ouanes and Popp 2019). Cortisol exhibits widespread effects, including regulation of insulin signaling processes and lipid metabolism. GCs characteristically have antagonistic actions to insulin, stimulating glucose production, lipolysis and pre-adipocyte differentiation, and inhibiting glucose uptake and glycogen synthesis (Ferris and Kahn 2012-11-1). GCs also have several known effects on the brain, as discussed in Section 1.7. Additionally, metabolic disruptions are also a hallmark of

neurodegenerative diseases, with disrupted insulin signaling in the brain being observed, as well as changes in circulating lipid metabolites (reviewed in Kulas et al., 2020). Therefore, it is possible that rather than air pollution elevating risk of these diseases through independent pathways, exposure activates common pathways which contribute to progression of metabolic, neurodegenerative and neuropsychiatric conditions alike. However, toxicological studies examining air pollution health effects are often limited in scope to one disease outcome, or analysis of a single tissue or biological pathway, and therefore do not often consider the relationships between several physiological systems and subsequent health effects. In chapters 2, 3 and 4 of this thesis, I utilized a rodent ozone-exposure model to further explore peripheral metabolic changes following pollution exposure that may associate with brain health outcomes. I hypothesized that air pollution-induced changes in metabolic outcomes are regulated by glucocorticoids.

Second, there is a need for human-relevant models that can be employed to investigate the effects of air pollution exposure on the brain. While rodent models have contributed greatly to showing a biological connection between air pollution exposure and brain effects, rodents have inherently different biology that may lead them to respond very differently to humans, as is evident from the relatively low translatability between preclinical research and clinical trials (Pound and Ritskes-Hoitinga 2018). Additionally, the human species is widely diverse, and several individual traits may influence susceptibility to pollution exposure. For instance in a controlled DE exposure study, individuals with asthma tended to have elevated cortisol levels post exposure, whereas individuals without asthma did not (Thomson et al. 2021). While effects of some traits, such as sex, diet or certain genetic predispositions may be modeled in rodents, rodent models (often inbred) cannot capture the full scale of diversity amongst humans (Pound

and Ritskes-Hoitinga 2018). *In vitro* cell culture systems can allow for study of pollutant-induced effects on human-derived brain cell cultures, however existing studies only explore the direct effects of PM on cells, often at relatively high doses. These studies also utilize cell lines, which are genetically identical. Therefore, these current models do not account for the key role systemic processes may play and cannot be used to model diverse populations. To create a model that could be used to study air pollution induced effects on human-relevant brain cells that incorporates systemic mechanisms, I hypothesized that plasma from pollutant-exposed individuals will cause transcriptional alterations in brain cell cultures which reflect actions of systemic mediators on the brain. In chapters 5 and 6 of this thesis, I examine whether this *ex vivo* approach can reproduce *in vivo* effects on the brain using a rat ozone exposure model and characterize responses of human stem-cell derived neuron cultures to plasma from individuals exposed to DE.

Third, our understanding of mechanisms which mediate pollutant-induced effects on the brain remains incomplete. As described earlier, there are several possible routes through which air pollution may impact the brain, including through activation of peripheral systems. Understanding the mechanisms that underpin the biological effects of air pollution is important for several reasons. First, for health estimate models to establish that there is sufficient evidence for a particular health effect of a toxicant such as air pollution, biological plausibility of that health effect needs to be demonstrated through development of an adverse outcome pathway (Figure 1.3). In an adverse outcome pathway, it is necessary to not only identify an adverse outcome (i.e health effect at a clinical or population level) associated with pollutant exposure (the initiating event), but to also to identify upstream effects leading to the adverse outcome. The relationship between key events within the adverse outcome pathway can be defined as causal or

predictive, depending on the current evidence provided in the literature. Providing causal evidence of these key event relationships by identifying underlying mechanisms (through pharmacological inhibition models) strengths the biological plausibility of an adverse outcome (U.S Environmental Protection Agency 2020). Second, identifying underlying mechanisms can inform risk estimates and experimental or observational studies as to whether particular subgroups may be more at risk. For example, there is a wide variety in HPA axis function within the human population. Those who have a more hyperactive or dysregulated HPA axis due to other risk factors/disease conditions may be more susceptible to pollutant-induced health effects (Thomas et al. 2018). Finally, early initiating events may help identify other potential adverse outcomes of pollutant exposure that may not have been considered previously. Throughout this thesis, I examine the contributions of underlying mechanisms in mediating observed effects of air pollution. In particular, I hypothesized that HPA axis activation is a central mediator of pollutant-induced effects on the brain. In chapters 2, 3, 4 and 5 I examined the role of the HPA axis in driving ozone-induced effects on the brain and peripheral metabolic outcomes, by employing metyrapone, an 11-β-hydroxylase inhibitor, to prevent the ozone-induced increase in GCs (Thomson et al. 2016).

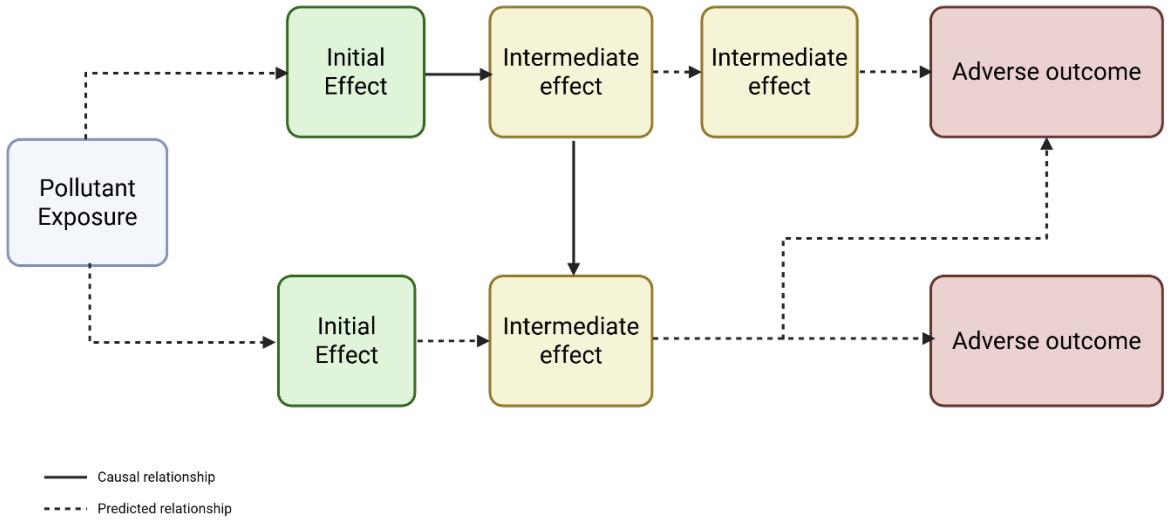


Figure 1.3. Example of an adverse outcome pathway following air pollution exposure. Represents the structure of an adverse outcome pathway used to assess biological plausibility of exposure to an air pollutant on a key clinical or population level effect (adverse outcome). Relationship between boxes is determined by strength of empirical evidence, with solid lines representing a causal relationship established through pharmacological or genetic knock out models, and dotted lines representing predicted relationships. Adapted from the 2020 Integrated Science Assessment for Ozone and Related Photochemical Oxidants (U.S Environmental Protection Agency 2020). Figure created in Biorender.

To address these current challenges and hypotheses, the objectives of this thesis were to:

1) Investigate the contribution of GCs in mediating air pollution induced effects on the brain and peripheral metabolic outcomes associated with brain health (Chapters 2-4).

2) Establish an *ex vivo* approach that enables the role of systemic mediators of pollutant-induced brain effects to be investigated in a human model (Chapters 5 and 6) .

Chapter 2: Ozone increases plasma kynurenine-tryptophan ratio and impacts hippocampal serotonin receptor and neurotrophic factor expression: Role of stress hormones

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2.1 Authorship Contributions

The overall rat ozone exposure study from which samples are leveraged for this manuscript, was conceived and led by EMT, with inhalation technical expertise provided by JG. The analyses described in the present manuscript were designed by MR and EMT. ELISAs were performed by AF. Isolation of RNA, cDNA synthesis and qPCR was performed by MR. Statistical analysis was conducted by MR and AW. MR wrote the initial manuscript draft, with review and editing of the manuscript by EMT. A subset of the work was completed by MR as an undergraduate coop student; analyses and manuscript writing/editing were completed during MR's graduate program.

2.2 Abstract

Air pollution is associated with adverse impacts on the brain, including cognitive decline and increased incidence of dementia, depression and anxiety; however, underlying mechanisms remain unclear. We have shown that both ozone and particulate matter activate the hypothalamic-pituitary-adrenal (HPA) axis, increasing plasma glucocorticoids (GCs) and altering mRNA profiles in multiple tissues including the brain. HPA axis dysregulation has been associated with central nervous system (CNS) impacts, including key effects in the hippocampus; accordingly, we hypothesized that pollutant-dependent increases in GC levels impact biological pathways relevant to brain health. Fischer-344 rats were treated with metyrapone (0 or 50 mg/kg), a GC synthesis inhibitor, and exposed to ozone (0 or 0.8 ppm) for 4 h (n=5/group) to investigate the role of GCs in ozone-dependent effects on tryptophan metabolism and expression of serotonin receptors and neurotrophic factors. Ozone increased plasma levels of the tryptophan metabolite kynurenine (~2-fold) and decreased tryptophan levels (~1.2 fold). Hippocampal expression of serotonin receptors exhibited differential regulation following exposure, and expression of key neurotrophic factors (brain-derived neurotrophic factor, vascular endothelial growth factor A, insulin-like growth factor-1, tyrosine kinase receptor B, b-cell lymphoma 2) was decreased. Some, but not all effects were abrogated by metyrapone treatment, suggesting both GC-dependent and -independent regulation. Exposure to exogenous corticosterone (CORT; 10 mg/kg) followed by clean air reproduced the ozone effects that were blocked with metyrapone, confirming the specificity of effects to GCs. These results indicate that ozone can modify pathways relevant to brain health and establish a role for the HPA axis in mediating these effects.

2.3 Introduction

Air pollution has widespread health impacts, with an estimated 4.2 million deaths attributed to ambient air pollution worldwide (Cohen et al. 2017). While these estimates are based upon the relationship between air pollution and cardiovascular and respiratory disease, a growing literature indicates a link between air pollution and central nervous system (CNS) disorders, including cognitive decline, neurodegenerative and neuropsychiatric disorders such as Alzheimer's disease (AD), dementia, depression, anxiety, bi-polar disorder and autism, as summarized by recent reviews (Power et al. 2016; Allen et al. 2017). Given the substantial and growing prevalence of these conditions (Whiteford et al. 2015), and ubiquitous exposure of the population to pollutants, even small pollutant-dependent changes in relative risk represent a significant public health burden.

While particulate matter (PM) has been the main target of investigation for neurological impacts, with exposure associated with increased inflammation (Ehsanifar et al. 2019; Bai et al. 2019), impaired neurogenesis (Woodward et al. 2018) and behavioural alterations (Fonken et al. 2011), gaseous components of air pollution can also impact the brain. Due to its highly reactive nature, ozone gas is entirely consumed in the lungs upon inhalation, but can impact tissues and processes beyond the lungs through processes such as systemic increases in oxidative stress (Kelly 2003), inflammation (González-Guevara et al. 2014) and activation of the hypothalamic-pituitary-adrenal (HPA) axis (Thomson et al. 2013, 2016). Epidemiological studies report associations between exposure to ozone and incidence of neuropsychiatric and neurodegenerative disorders (Lim et al. 2012; Kioumourtzoglou et al. 2017; Zhao et al. 2018) and decreased cognitive performance in middle aged adults (Chen and Schwartz 2009; Gatto et al. 2014). Several rodent studies demonstrate that ozone exposure is capable of causing neurophysiological changes such as decreased short and long term memory retention (Dorado-

Martínez et al. 2001; Rivas-Arancibia et al. 2009), reduced hippocampal cell numbers (Rivas-Arancibia et al. 2009), increased apoptotic indicators and endoplasmic-reticulum stress (Rodríguez-Martínez et al. 2016), increased depression and anxiety-like behaviour and decreased serotonin levels in the frontal cortex and hippocampus (Mokoena et al. 2015). However, the mechanisms through which ozone could result in such impacts on the CNS are not fully understood.

As extrapulmonary effects of ozone, unlike PM, are attributable solely to secondary effects rather than having the potential to also be caused by direct effects of the pollutant, ozone provides a useful model to study indirect mechanisms that could underlie systemic effects that may be common to other pollutants. We have shown previously that both ozone and PM activate the HPA axis and increase plasma levels of the stress hormone corticosterone (CORT), resulting in glucocorticoid (GC)-dependent effects in multiple tissues including the brain (Thomson et al. 2013, 2016, 2019). GC dysregulation is considered an underlying mechanism contributing to the development of a number of CNS disorders (reviewed in Lucassen et al., 2014). For instance, patients with depression (Sachar et al. 1973; Peeters et al. 2004; Sepehr et al. 2005) and dementia (reviewed in Ouanes & Popp, 2019) have been shown to have disrupted secretion of cortisol suggesting impaired regulation of the HPA axis. GCs can alter processes associated with CNS disorders such as tryptophan metabolism (Comings et al. 1995; Badawy 2017), serotonin signaling (reviewed in Lanfumey et al., 2008) and neurogenesis (reviewed in Kino, 2015), and chronic elevation of GC levels can lead to impairment of HPA axis regulation as well as GC resistance (Cohen et al. 2012; Zhu et al. 2014). Given that HPA axis dysregulation impacts brain processes and that exposure to air pollutants activates the HPA axis, we hypothesized that the

HPA axis may serve as an important mediator of pollutant effects on biological pathways relevant to brain health.

The hippocampus is a stress-sensitive, serotonin-receptor rich region of the brain impacted by a number of neurological disease processes (reviewed in Sala et al., 2004). Adult neurogenesis occurs primarily in the hippocampus (reviewed in Ming & Song, 2011), and hippocampal atrophy is often observed in dementia (Chow et al. 2012; Evans et al. 2018) and depression (Bremner et al. 2000; Macqueen et al. 2003). Chronic elevation of GCs has been associated with decreases in memory, a primary function of the hippocampus, and loss of hippocampal volume (Lupien et al. 1998). Here, we use an acute ozone exposure model to facilitate assessment of early impacts in the hippocampus and plasma that can be linked directly to exposure, and that are less likely to be obscured by the significant cellular and tissue changes that can arise as a result of disease progression in chronic exposure models. Specifically, we examined impacts on tryptophan metabolism and serotonin signalling, as well as effects on genes involved in neuronal cell death and hippocampal neurogenesis, mechanisms thought to contribute to neurotoxicity and brain health. Metyrapone, an 11-β-hydroxylase inhibitor that blocks the ozone-induced increase in GC synthesis (Thomson et al. 2016), and exogenous CORT were used to investigate whether observed effects occurred in a GC-dependent or independent manner.

2.4 Methods

2.4.1 Animals

Male Fischer-344 rats weighing 200-250 g (specific pathogen free; Charles River, St. Constant, Québec, Canada) maintained on a 12 h light/dark cycle were provided with food and water ad libitum. Housing consisted of HEPA-filtered individual plexiglass cages with wood-chip bedding. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

2.4.2 Inhalation exposure

Inhalation exposure methods were previously described in detail (Thomson et al. 2016). Briefly, rats were subcutaneously injected with either metyrapone (50 mg/kg; Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) or vehicle (40% propylene glycol in buffered saline) 1 h prior to nose-only inhalation exposure to either clean air or 0.8 ppm ozone (n=5/group) for 4 h. Previously conducted dosimetric calculations (Thomson et al., 2005) estimated that exposure to 0.8 ppm ozone produced a centriacinar dose on the order of 3 to 4-fold higher than the internal dose resulting from a plausible exposure scenario in humans (0.12 ppm ozone for 12 h followed by exposure to 0.06 ppm ozone for 12 h). For ethical reasons, nose-only exposures were restricted to 4 h, and so the dose rate was clearly higher in the experimental model compared to an environmental exposure spread over a 24 h period. An additional group (n=5) was subcutaneously injected with CORT (10 mg/kg; Sigma-Aldrich) 1 h prior to exposure to clean air (4 h).

2.4.3 Sample collection

Rats were anesthetised with isoflurane (5% at 1.5 L of O2/min) and euthanised via exsanguination 0 or 24 h post exposure. Blood was collected from the abdominal aorta and stored in vacutainer tubes containing EDTA at 10 mg/ml and phenyl methyl sulfonyl fluoride at 1.7 mg/ml. Blood was centrifuged at 1448 × g for 10 min to separate plasma. Brain tissue was dissected, and the hippocampus was collected and added to RNAlater (Sigma-Aldrich Canada Co.). All samples were snap-frozen in liquid nitrogen and stored at -80 °C.

2.4.4 Tryptophan and kynurenine concentrations

Plasma concentrations of tryptophan and kynurenine were measured using ELISA kits (Immusol, France) according to manufacturer's directions.

2.4.5 RNA isolation

Hippocampal samples (frozen) were ground using a mortar and pestle over dry ice and sonicated briefly in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) for homogenization. Total RNA was isolated following manufacturer's instructions. Quantification of RNA was performed using RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon) and RNA quality was assessed using Experion^{TM} RNA STdSens Analysis Kit (Bio-Rad, Mississauga, Ontario, Canada). cDNA was generated according to manufacturer's directions with MuLV reverse transcriptase and random hexamers (Applied Biosystems, Mississauga, Ontario, Canada). Additionally, negative control samples were created by incubating RNA samples with all reagent components except reverse transcriptase to assess genomic DNA contamination.

2.4.6 RT-qPCR

Universal Probe Library design software (Roche Diagnostics Canada, Laval, Québec, Canada) and Primer-BLAST software (National Center for Biotechnology Information, Bethesda, Maryland) were used to generate primers (Appendix A) with an optimal annealing temperature of 60 °C. A serial dilution of rat brain cDNA was used to validate primers and confirm high-efficiency reactions. Duplicate reactions containing BRIGHTGreen 2x qPCR Mastermix (Bio-Rad Laboratories Ltd., Mississauga, Ontario, Canada), primers (200 nM) and cDNA were incubated (10 µl/well; 384-well plate) in a spectrofluorometric thermal cycler (Lightcycler 480, Roche Diagnostic Canada). The following PCR cycle was utilized: 95 °C for 3 min to activate the iTAQ polymerase followed by 50 cycles of denaturation at 95 °C, annealing at 60 °C, and elongation at 72 °C, each for 10 s. Fluorescence was monitored at every cycle during the elongation step. Melt curves were conducted for every run to confirm unique product. Expression calculations were conducted relative to β-actin (verified to be invariant with treatment) using the delta-delta Ct method (Livak and Schmittgen 2001).

2.4.7 Statistical analyses

Data were assessed by two-way ANOVA using factors *Ozone* (0, 0.8 ppm) and *Metyrapone* (0, 50 mg/kg), followed by Holm-Sidak multiple comparison procedure to determine significant effects ($\alpha = .05$). Pearson correlations were conducted to compare responses to ozone and CORT. All statistical analyses were performed using Sigma-Plot 13.0 (Systat Software Inc., San Jose, California). For simplicity, only factor interactions and main effects that included *Ozone* are presented in text and figures.

2.5 Results

2.5.1 Kynurenine and tryptophan concentration in plasma

Increases in the ratio of kynurenine/tryptophan are associated with a number of conditions, including various CNS disorders (reviewed in Lovelace et al., 2017). To determine whether ozone could affect this ratio, plasma concentrations of kynurenine and tryptophan were measured immediately after exposure (Figure 2.1). Ozone increased plasma kynurenine (approximately 2-fold; *Ozone* main effect, $p < 0.001$) and decreased tryptophan (*Ozone* main effect, $p = 0.023$). As a result, the ratio of kynurenine to tryptophan increased significantly with ozone exposure (*Ozone* main effect, $p < 0.001$). Metyrapone treatment did not significantly modify these effects.

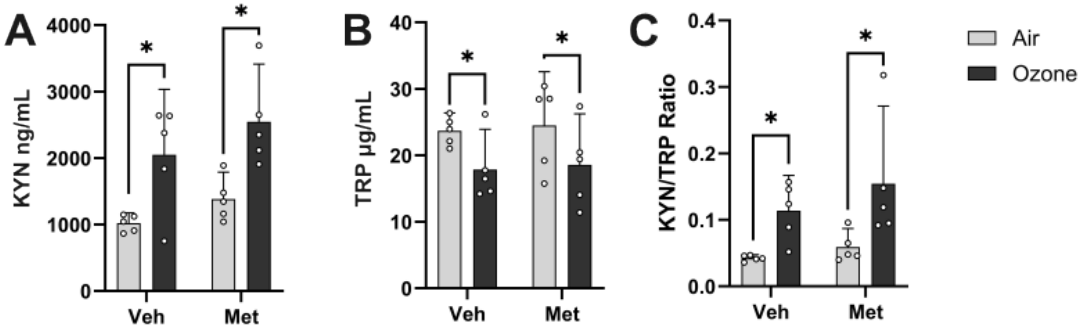


Figure 2.1. Plasma kynurenine-tryptophan ratio following ozone exposure. Plasma concentrations of kynurenine and tryptophan were measured in Fischer-344 rats exposed to ozone (0.8 ppm) or air for 4 hours following treatment with metyrapone (MET) or vehicle (VEH). Two-way ANOVA was used to evaluate significant *Ozone* (ozone vs. air) and *Metyrapone* (50mg/kg metyrapone vs. VEH) effects, followed by Holm-Sidak procedure to elucidate pattern of significant effects. **A)** Kynurenine (KYN); **B)** Tryptophan (TRP); **C)** Kynurenine-Tryptophan ratio. Bar graphs represent geomean ± SD. * Ozone vs. air within VEH or MET, $p < 0.05$; n = 5/group.

2.5.2 MAO-A and KMO expression

Monoamine oxidase A (*MAOA*) and kynurenine monooxygenase (*KMO*) are both enzymes which can alter concentrations of different end products of tryptophan metabolism. *MAOA* oxidizes serotonin, reducing serotonin availability. *KMO* catalyzes the conversion of kynurenine into 3-hydroxykynurenine, a precursor for quinolinic acid which is thought to have neurotoxic effects (reviewed in Lugo-Huitrón et al., 2013). Ozone decreased hippocampal *MAOA* mRNA expression immediately after exposure, an effect prevented by metyrapone (*Ozone* x *Metyrapone* interaction, $p = 0.018$; Figure 2.2A). *KMO* mRNA was also decreased by ozone, and this effect was prevented with metyrapone treatment (*Ozone* x *Metyrapone* interaction, $p < 0.001$; Figure 2.2B).

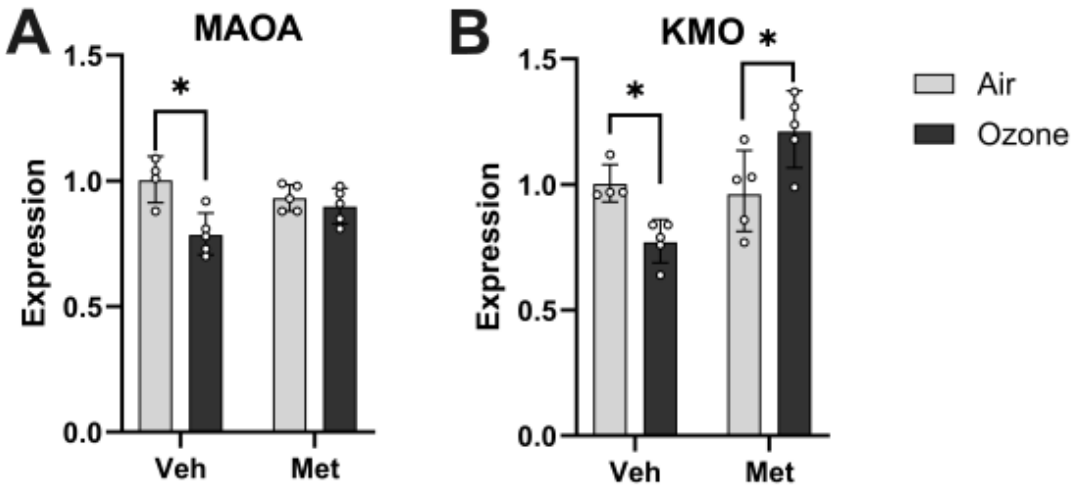


Figure 2.2. Hippocampal gene expression of MAOA and KMO following ozone exposure. Hippocampal expression of *MAOA* and *KMO* enzymes was measured in Fischer-344 rats pre-treated with metyrapone (MET) or vehicle (VEH) and exposed to ozone (0.8ppm) or air for 4 h. Expression represented as fold change (FC) relative to VEH/air group. Statistical analysis conducted using two-way ANOVA (factors: *Ozone* and *Metyrapone)* followed by Holm-Sidak multiple comparison procedure to elucidate pattern of significant effects. **A)** *MAOA*; **B)** *KMO*. Bar graphs represent geomean ± SD. *Ozone vs air within VEH or MET, $p < 0.05$; $n = 4$ or 5/group.

2.5.3 Serotonin receptor expression in the hippocampus

Serotonin (5-HT) receptors mediate many serotonergic functions within the brain. Analysis of hippocampal serotonin receptor mRNA expression immediately after exposure revealed that ozone exposure resulted in differential expression of many of these receptors (Figure 2.3). Significant decreases in *5-HT1A*, *5-HT1B* and *5-HT4* following ozone exposure were all metyrapone independent (for all: *Ozone* main effect, $p < 0.001$). In contrast, metyrapone abrogated ozone-dependent decreases of *5-HT2A* (*Ozone* x *Metyrapone* interaction, $p < 0.001$) and *5-HT7* (*Ozone* x *Metyrapone* interaction, $p = 0.02$), as well as the ozone-dependent increase in *5-HT2C* (*Ozone* x *Metyrapone* interaction, $p = 0.009$). No significant change was observed in *5-HT6* expression.

2.5.4 mRNA expression of neurotrophic genes

Both serotonin and GCs regulate expression of many genes involved in neurogenesis and survival within the hippocampus (Banasr et al. 2004; Odaka et al. 2017). Accordingly, we investigated whether ozone affected the expression of genes involved in neurogenic and neurotrophic processes in a CORT-dependent manner (Figure 2.4). Ozone decreased brain-derived neurotrophic factor *(BDNF*; *Ozone* main effect, $p = 0.015$) and vascular endothelial growth factor A (*Vegfa*; *Ozone* main effect, $p < 0.001$), effects that were both metyrapone-independent. In contrast, ozone-dependent decreases in tyrosine kinase receptor B (*trkB*; *Ozone* x *Metyrapone* interaction, $p = 0.011$), B-cell lymphoma 2 (*Bcl2*; *Ozone* x *Metyrapone* interaction, $p < 0.001$) and insulin-like growth factor-1 (*Igf1*; *Ozone* x *Metyrapone* interaction, $p = 0.051$) tended to be sensitive to metyrapone.

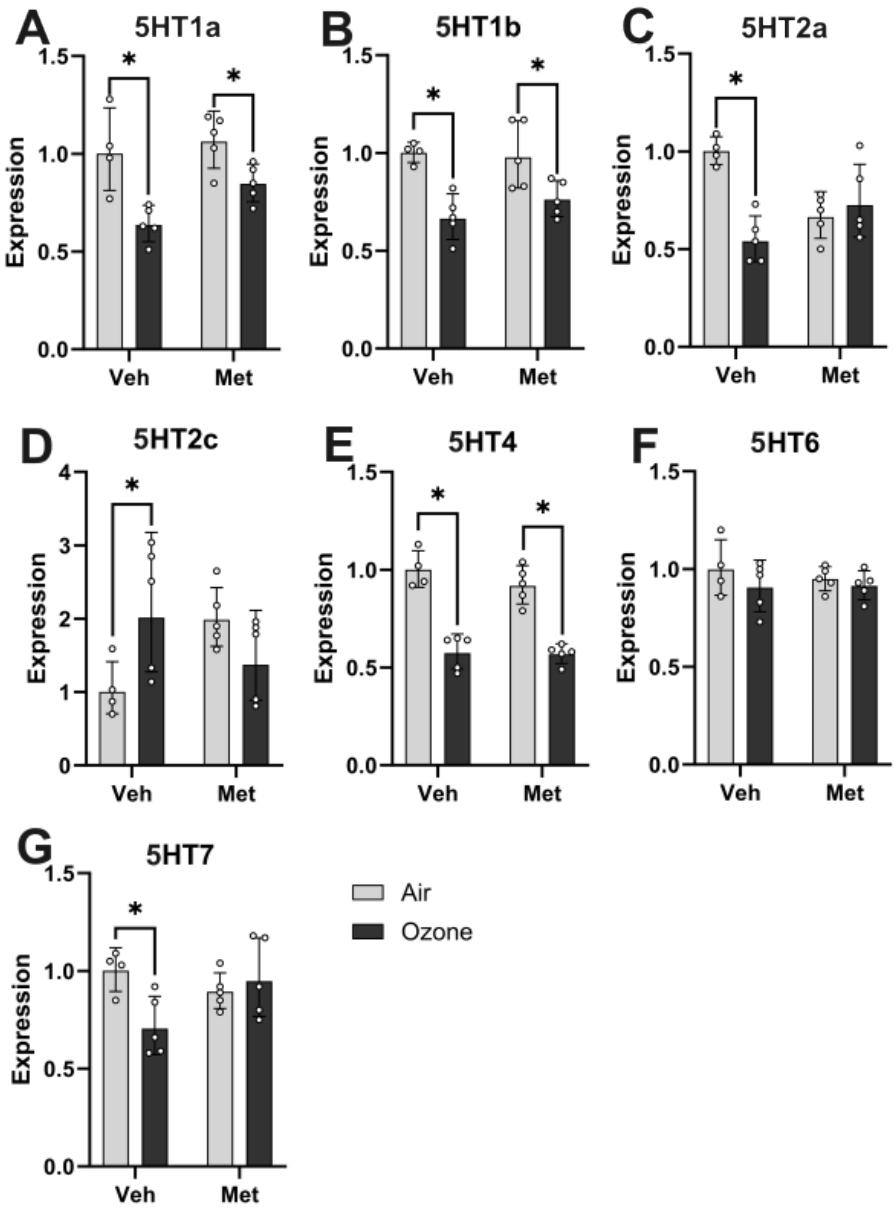


Figure 2.3. Ozone-induced gene expression changes of serotonin receptors within the hippocampus. Hippocampal mRNA of serotonin receptors was measured in Fischer-344 rats treated with metyrapone (MET) or vehicle (VEH) prior to exposure to ozone (0.8 ppm) vs. air for 4 h. Expression is represented as fold change (FC) relative to VEH/air exposure group. Significant *Ozone* (air vs. ozone) and *Metyrapone* (50 mg/kg metyrapone vs. VEH) effects were determined using two-way ANOVA and Holm-Sidak procedure to elucidate pattern of significant effects. **A)** *5-HT1A*; **B)** *5-HT1B*; **C)** *5-HT2A*; **D)** *5-HT2C*; **E)** *5-HT4*; **F)** *5-HT6*; **G)** *5-HT7*. Bar graphs represent geomean ± SD. *Ozone vs air within VEH or MET, $p < 0.05$; n = 4 or 5/group.

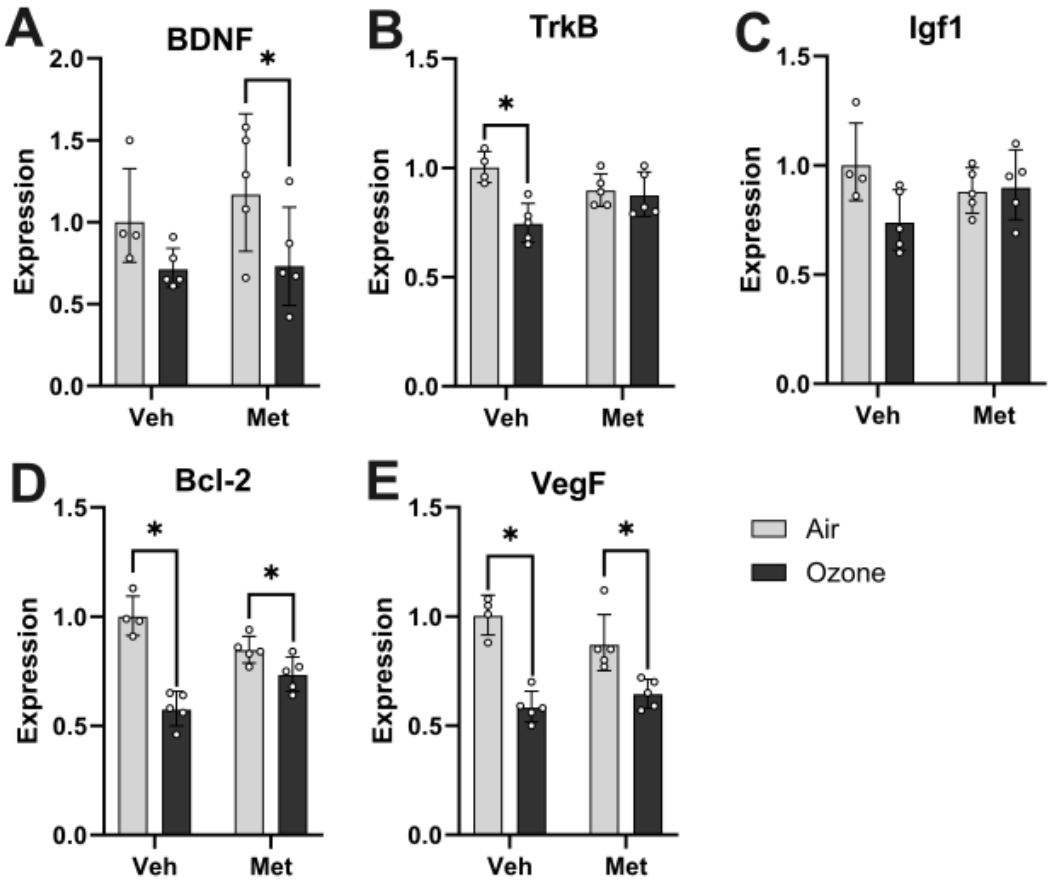


Figure 2.4. Hippocampal expression of neurotrophic genes following ozone exposure. Fischer-344 rats were treated with metyrapone (MET) or vehicle (VEH) before 4 h exposure to either ozone (0.8 ppm) or air. Hippocampal expression of genes measured with RT-qPCR and represented as fold change (FC) relative to VEH/air exposure group. Two-way ANOVA with Holm-Sidak multiple comparisons procedure was used to evaluate significant effects (factors: *Ozone, Metyrapone*). **A)** *BDNF*; **B)** *trkB*; **C)** *Bcl2*; **D)** *Vegfa*; **E)** *Igf1*. Bar graphs represent geomean ± SD. *Ozone vs air within VEH or MET, $p < 0.05$; n = 4 or 5/group.

2.5.5 mRNA Expression after 24 hours

To determine whether effects of short-term exposure to ozone were transient or sustained, mRNA expression was measured 24 h after ozone exposure and compared to effects detected immediately after exposure. For all genes, mRNA expression returned to or approached control levels 24 h after exposure (Figure 2.5).

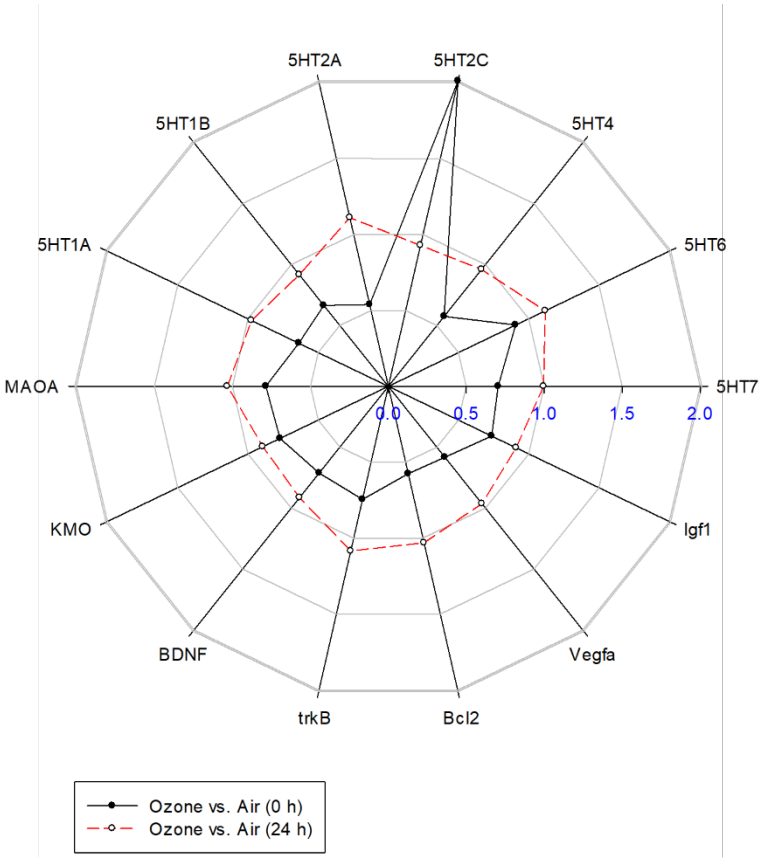


Figure 2.5. Gene expression changes 0 and 24 h post-ozone exposure. Data from Fisher-344 rats treated with vehicle (VEH) and exposed to ozone (0.8 ppm) for 4 h. Samples collected 0 h or 24 h after exposure. Gene expression is represented as fold change relative to VEH/air exposure group corresponding to appropriate time point. n = 5/group.

2.5.6 Reproduction of ozone effects with exogenous CORT treatment

To independently confirm the GC-specificity of ozone effects, rats were administered CORT and effects were compared for genes that had exhibited a significant *Ozone* x *Metyrapone* interaction. CORT treatment reproduced effects of ozone exposure (r=0.83, p= 0.01; Figure 2.6).

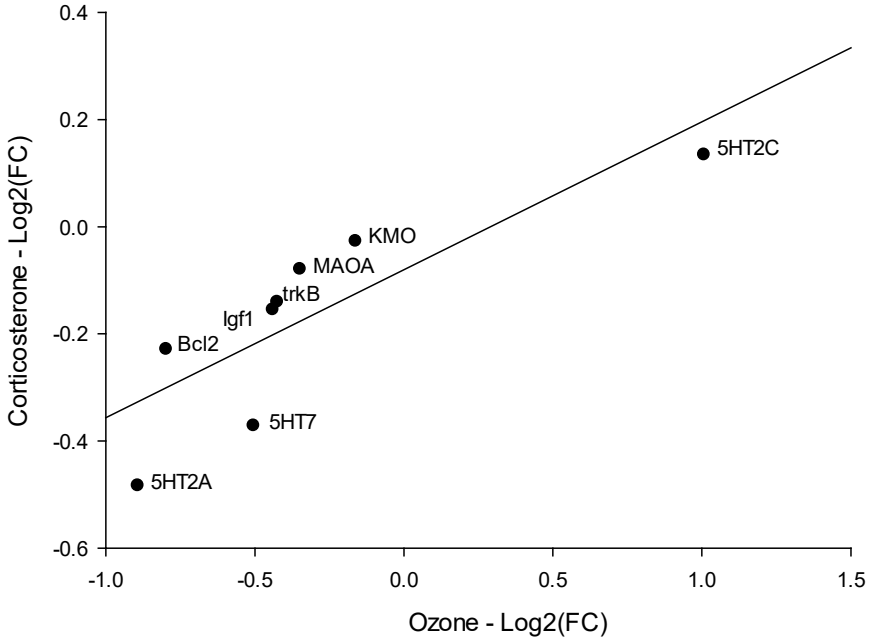


Figure 2.6. Reproducing ozone-induced changes of stress-regulated genes by treatment with exogenous CORT. Data from Fischer-344 rats treated with CORT (CORT) and exposed to clean air for 4 h, or treated with vehicle (VEH) and exposed to ozone (0.8 ppm) for 4 h. Hippocampal gene expression is represented as fold change (FC; Log2) relative to VEH/air. Expression of genes with a significant *Ozone* x *Metyrapone* interaction (Two-way ANOVA) was compared to expression of genes from VEH/ozone group using Pearson correlation (r = 0.83; p = 0.01). n = 5/group.

2.6 Discussion

To gain insight into mechanisms that could underlie associations between air pollutants and CNS impacts, we measured key factors in plasma and the hippocampus relevant to serotonergic/neurotrophic pathways, and evaluated the role of HPA axis activation as a potential mediator. Our findings reveal that 1) acute ozone exposure resulted in a significant increase in the plasma kynurenine/tryptophan ratio, 2) ozone exposure resulted in differential expression of MAOA and KMO, many serotonin receptors, and neurotrophic genes, 3) blockade of ozone-induced GC release abrogated some but not all of these effects, and 4) treatment with exogenous CORT reproduced ozone-induced effects that had been prevented by metyrapone, confirming the specificity of effects to GCs. Our data show that exposure to ozone can affect serotonin and neurotrophic pathways in both GC-dependent and independent manners.

Altered metabolism of tryptophan, a serotonin and kynurenine precursor, has implications in brain health. It has been hypothesized that a shift in tryptophan metabolism from the production of serotonin towards the production of kynurenine may lead to reduced serotonin availability (Lapin and Oxenkrug 1969) as well as to increases in neurotoxic kynurenine metabolites (Saito et al. 1993), both of which have been associated with CNS disorders (Lugo-Huitrón et al. 2013). Additionally, increases in circulating kynurenine/tryptophan ratios are associated with a variety of diseases, including lung cancer (Suzuki et al. 2010), coronary disease (Sulo et al. 2013), glioma pathophysiology (Adams et al. 2014), AD (Widner et al. 2000), chronic kidney disease (Schefold et al. 2009), relapses of multiple sclerosis (Mancuso et al. 2015), and suicide (Sublette et al. 2011; Bradley et al. 2015; Messaoud et al. 2018). Elevated GCs activate tryptophan-2,3-dioxygenase, one of two main enzymes responsible for kynurenine metabolism, and treatment with dexamethasone and hydrocortisone can decrease plasma tryptophan levels and may alter availability of tryptophan in the brain (reviewed in Miura et al.,

2008). The increase in the kynurenine/tryptophan ratio we observed following ozone exposure was not diminished with metyrapone treatment, suggesting involvement of a GC-independent pathway. Indoleamine 2,3-dioxygenase, the other enzyme responsible for kynurenine metabolism, is activated by cytokines (reviewed in Miura et al., 2009); however, there is little evidence of increased plasma cytokines immediately after ozone exposure in this model (Thomson et al., 2016).

In addition to altered tryptophan metabolism, ozone exerted differential GC-dependent and independent effects on the expression of genes implicated in serotonin and kynurenine metabolism, and of serotonin receptors, in the hippocampus. Decreases in *MAOA*, *KMO*, *5-HT1A*, *5-HT1B*, *5-HT2A*, *5-HT4*, *5-HT7* and an increase in *5-HT2C* were found following ozone exposure, with *MAOA*, *KMO*, *5-HT2A*, *5-HT2C* and *5-HT7* regulated via GC-dependent mechanisms. The GC-dependency of these effects is supported by previous studies showing GC regulation of these genes (Holmes et al. 1997; Le Corre et al. 1997; Dwivedi et al. 2005; Lee et al. 2009). While GCs have been shown to downregulate *5-HT1A* (Meijer and de Kloet 1994; Le Corre et al. 1997; Neumaier et al. 2000; Vázquez et al. 2012), the lack of *Ozone* x *Metyrapone* interactions in *5-HT1A*, *5-HT1B*, and *5-HT4* suggest that these genes are regulated primarily via GC-independent mechanisms in our ozone model. The physiological consequences of altered serotonin receptor activity and expression have been the focus of many studies seeking to understand the role of these receptors in neuropsychiatric disorders, a number of which show that alterations in receptor activity are associated with and can lead to either anti-depressant or pro-depressant effects (Sibille et al. 1997; Hedlund et al. 2005; Wesołowska et al. 2006; Bonaventure et al. 2007; Lucas et al. 2007; Licht et al. 2009; Martin et al. 2014; Nautiyal et al. 2016; Nautiyal and Hen 2017). Our results are broadly consistent with prior studies that have shown alterations

in serotonin content in the brain after ozone exposure (Huitrón-Reséndiz et al. 1994; Paz and Huitrón-Reséndiz 1996; González-Piña and Paz 1997; Mokoena et al. 2015).

In addition to tryptophan metabolism and serotonin system dysregulation, interference with hippocampal neurogenesis as well as neuronal survival has been suggested to contribute to a variety of CNS disorders (reviewed in Toda et al., 2019). Stress has been shown to play a significant role in neurogenesis, as both acute and chronic stress, as well as exogenous CORT, have been demonstrated to impair neurogenic processes (Gould et al. 1997; Tanapat et al. 2001; Coe et al. 2003; Kino 2015). Additionally, it has been suggested that adult hippocampal neurogenesis is critical in modulating the ability of the hippocampus to exert negative feedback control over the HPA axis, and thus decreases in neurogenesis could put the individual more at risk of stress-induced neurotoxicity (Mirescu et al. 2004; Toda et al. 2019). In the present study, the role of both GC-dependent and independent mechanisms in mediating ozone effects is evident in the reduced expression of key neurotrophic and neuroprotective genes *BDNF*, *Igf1*, *Vegfa*, *Bcl2* and *trkB*. Ozone-dependent decreases of *Igf1*, *Bcl2* and *trkB* were blocked by metyrapone, consistent with known regulation of these genes by GCs (Lembessis et al. 2004; Basta-Kaim et al. 2014). In contrast, while GC regulation of *BDNF* and *Vegfa* has been previously demonstrated (Barbany and Persson 1992; Smith et al. 1995), our results suggest regulation by other factors. Decreases in the expression of these neurotrophic factors could reduce their neuroprotective effect, leading to a decrease in neurogenesis and increase in neuronal cell death (Anilkumar and Prehn 2014; Bathina and Das 2015; Lange et al. 2016). Thus, we provide evidence that air pollution may influence neurogenesis and neuronal cell death pathways, a hypothesis established by past studies (Rivas-Arancibia et al. 2015; Rodríguez-

Martínez et al. 2016; Woodward et al. 2018; Hedges et al. 2019), and that this can occur through GC-dependent and independent pathways.

The physiological ramifications of the observed effects likely depend upon innate differences in sensitivity, duration of exposure, and the context in which the stress hormone-dependent effects occur. Ozone-exposure can activate many systemic pathways in addition to the HPA axis, such as oxidative stress and inflammation which have been suggested to mediate air-pollution induced neurological effects previously (Rivas-Arancibia et al. 2009; Mokoena et al. 2015). While these various pathways may act independently from each other, it is important to recognize that these pathways can also interact (Thomson 2019). For instance, inflammatory processes can activate the HPA axis (reviewed in Bellavance & Rivest, 2014), and the HPA axis can also increase sensitivity to the immune response through priming of microglia (Frank et al. 2012). GCs may also increase oxidative stress (You et al. 2009), and these pathways may act to regulate the same downstream targets. Additionally, humans are exposed to variety of different pollutants and other environmental factors, which may act on these pathways to induce synergistic or additive effects that can impact health outcomes (reviewed in Mauderly & Samet, 2009; E. M. Thomson, 2019). Genetic and epigenetic factors as well may also influence how pollutant-induced changes occur and manifest among the human population.

Several factors warrant consideration when interpreting these results. Despite training in nose-only exposure tubes, nose-only exposure itself is stressful (Thomson et al. 2009), and thus ozone effects are occurring in the context of existing stress. As equivalent conditions were used for all treatment groups, this should not confound interpretation of results. Secondly, this experiment was conducted using Fischer rats, known to have a hyper-responsive stress axis (Dhabhar et al. 1993). We have shown that rats that differ in HPA axis function have differential

sensitivity to impacts of ozone on lung injury and inflammation, providing insight into a potential susceptibility factor (Thomas et al. 2018); such innate differences may also be relevant to CNS effects of pollutant exposure. Third, metyrapone could impose off-target effects independent of GC-synthesis blockade. However, reproduction of ozone-induced effects with exogenous CORT independently confirmed that effects observed are GC-dependent. Finally, the present study was based on analyses conducted in the whole hippocampus, and was therefore insensitive to potential regional specific effects of GCs on gene expression (e.g. Le Corre et al., 1997). Previous work examining ozone-dependent GC signaling demonstrated widespread actions across brain regions, with some evidence of regional differences (Thomson et al. 2019).

In conclusion, we show for the first time that exposure to the common air pollutant ozone alters the ratio of tryptophan and kynurenine in blood, an effect associated with several disease states. We also provide evidence supporting a role for GCs in mediating the link between pollutant exposure and neurotoxicity by demonstrating that exposure to ozone can impact serotonin and neurogenic systems through GC-dependent and -independent mechanisms. Our results, alongside results from previous studies showing how ozone exposure activated stress-responsive regions of the brain (Gackière et al. 2011) and can induce stress hormone-dependent transcriptional responses within the brain (Thomson et al. 2013, 2019; Henriquez et al. 2019), are consistent with a role for the HPA axis in contributing to the neurophysiological abnormalities instigated by air pollution exposure. Elucidation of underlying mechanisms through which air pollutants can impact the CNS should provide support for epidemiological associations and insight into those subpopulations that may be most at risk.

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2.8 Funding sources

Funding for this study was provided by the Clean Air Regulatory Agenda and the Addressing Air Pollution Horizontal Initiative.

2.9 Ethics statement

All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

2.10 Supplemental Figures

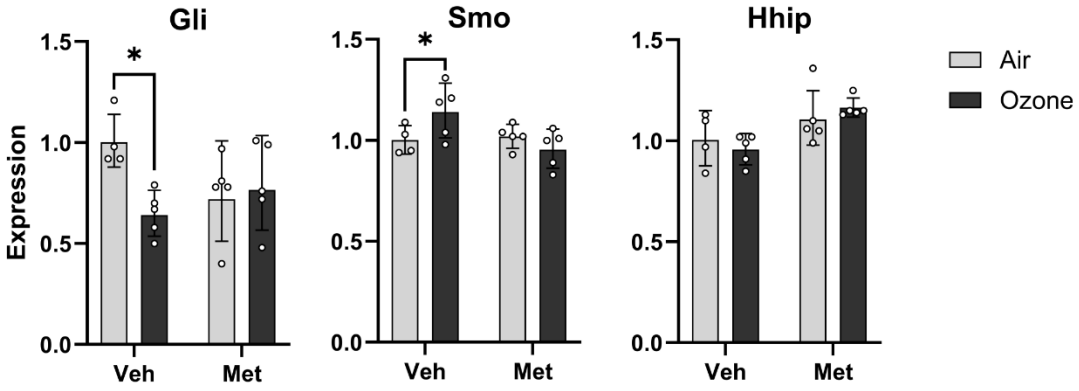


Figure 2.S1. Effects of ozone exposure on hedgehog signaling pathway genes. Fischer-344 rats were treated with metyrapone (MET) or vehicle (VEH) before 4 h exposure to either ozone (0.8 ppm) or air. Hippocampal expression of genes measured with RT-qPCR and represented as fold change (FC) relative to VEH/air exposure group. Two-way ANOVA with Holm-Sidak multiple comparisons procedure was used to evaluate significant effects (factors: *Ozone, Metyrapone*). Bar graphs represent geomean ± SD. *Ozone vs air within VEH or MET, $p < 0.05$; n = 4 or 5/group

Chapter 3: Modulation of insulin signaling pathway genes by ozone inhalation and the role of glucocorticoids: A multi-tissue analysis

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3.1 Authorship contributions

The overall rat ozone exposure study, from which samples are leveraged for this manuscript, was conceived and led by EMT. The analyses described in the present manuscript was designed by MR and EMT. RNA isolation of tissues was performed by previous undergraduate co-op students. cDNA synthesis of all tissues (besides liver) was performed by MR. qPCR was performed by AF and MR. Protein analysis was performed by AF. Statistical analysis was conducted by MR and AW. The initial manuscript draft was written by MR, with review and editing by EMT.

3.2 Abstract

Air pollution is associated with increased risk of metabolic diseases including type 2 diabetes (T2D), of which dysregulation of the insulin-signaling pathway is a feature. While studies suggest pollutant exposure alters insulin signaling in certain tissues, there is a lack of comparison across multiple tissues needed for a holistic assessment of metabolic effects, and underlying mechanisms remain unclear. Air pollution increases plasma levels of glucocorticoids (GCs), systemic regulators of metabolic function. The objectives of this study were to 1) determine effects of ozone on insulin-signaling genes in major metabolic tissues, and 2) elucidate the role of GCs. Male Fischer-344 rats were treated with metyrapone, a GC synthesis inhibitor, and exposed to 0.8 ppm ozone or clean air for 4 h, with tissue collected immediately or 24 h post exposure. Ozone inhalation resulted in distinct mRNA profiles in the liver, brown adipose, white adipose and skeletal muscle tissues, including effects on insulin-signaling cascade genes (*Pik3r1*, *Irs1*, *Irs2*) and targets involved in glucose metabolism (*Hk2*, *Pgk1*, *Slc2a1*), cell survival (*Bcl2l1*), and genes associated with diabetes and obesity (*Serpine1*, *Retn*, *Lep*). GC-dependent regulation was observed in the liver and brown and white adipose tissues, while effects in skeletal muscle were largely unaffected by metyrapone treatment. Gene expression changes were accompanied by altered phosphorylation states of insulin-signaling proteins (BAD, GSK, IR-β, IRS-1) in the liver. The results show that systemic effects of ozone inhalation include tissue-specific regulation of insulin-signaling pathway genes via both GC-dependent and independent mechanisms, providing insight into mechanisms underlying adverse effects of pollutants.

3.3 Introduction

Air pollution is the largest environmental risk factor to human health according to the World Health Organization (GBD 2015 Risk Factors Collaborators 2016) with current estimates attributing 4.2 million premature deaths worldwide annually to outdoor air pollution exposure (World Health Organization 2022). While these estimates are based on the relationship between air pollution and respiratory and cardiovascular diseases, a link between air pollution exposure and other diseases, such as type 2 diabetes (T2D) and insulin resistance, is becoming increasingly apparent.(Li et al. 2019; Liu et al. 2019). T2D affects approximately 6.28% of the global population, with prevalence projected to rise (Khan et al. 2019). Thus identifying modifiable factors which could contribute to development of this disease is crucial to prevention. T2D is often characterized by insulin resistance, of which disruption of the insulin-signaling pathway is a major contributor (Withers and White 2000; Fröjdö et al. 2009).

Ozone is a highly reactive gas that is completely consumed in the lungs upon inhalation (Pryor 1992). Despite this, ozone affects multiple tissues within the body through systemic responses such as oxidative stress (Kelly 2003), inflammation (González-Guevara et al. 2014) and hypothalamic-pituitary-adrenal axis (HPA) activation (Thomson et al. 2013, 2016). A positive association between ozone exposure and T2D risk has been demonstrated in the epidemiological literature (Li et al. 2021). Experimental studies have found that ozone exposure increases blood glucose levels (Vella et al. 2015; Miller et al. 2015, 2016a), induces glucose intolerance (Bass et al. 2013; Thomson et al. 2018), and triggers insulin resistance (Vella et al. 2015). Notably, multiple studies found that ozone alters the insulin-signaling pathway in different tissues; effects include induction of glycolytic and gluconeogenic genes in the liver (Miller et al. 2015), and impairment of PKB/AKT phosphorylation in the muscle (Vella et al. 2015; Ying et al. 2016) and liver (Ying et al. 2016). Clearly, metabolic control is dictated by a

series of signals and actions from multiple tissues within the body in response to both supply and demand needs and homeostatic regulation (Ye and Medzhitov 2019). Accordingly, the influence of pollutant exposure on metabolic pathways within one tissue may influence the response of another, and vice versa. However, despite evidence that ozone exposure can alter insulin signaling in certain tissues, identifying how the tissues may work in concert to produce a phenotype of metabolic dysregulation remains unclear, as comparison between studies is complicated due to use of different models and assessment of different endpoints across tissues. Therefore, examining in a single model responses to pollutant exposure across key tissues involved in metabolism is needed to gain a more complete understanding of the effects of pollutants on metabolism and potential disease states.

In addition to the lack of multi-tissue comparisons, the underlying mechanisms driving pollution-induced effects on insulin signaling, development of insulin resistance, and T2D remain unclear. Understanding the underlying mechanisms not only establishes further confidence in the biological plausibility for a link between exposures and disease, but also gives insight into susceptible subpopulations. In particular, determining early initiating events is important in order to distinguish between direct effects of the pollutant needed to identify key mediators as opposed to changes that manifest as the disease progresses. Air pollution acts on the HPA axis and increases levels of circulating glucocorticoids (GCs), the body's central stress hormone (Thomson et al. 2016; Miller et al. 2016a). This rise in GCs can drive numerous pollutant-induced effects, as demonstrated through studies employing pharmacological or surgical intervention (Thomson et al. 2016; Henriquez et al. 2019, 2022; Rose et al. 2020). GCs are important regulators of glucose homeostasis, and GC excess can lead to insulin resistance and development of T2D (Di Dalmazi et al. 2012). For instance, diabetes mellitus is often a

complication that arises in patients with Cushing's syndrome, which is characterized by excess GCs (Pivonello et al. 2010). The insulin-signaling pathway is regulated by GCs in multiple tissues, with GCs often acting antagonistically to the actions of insulin, such as by stimulating an increase in glucose production, promoting lipolysis and pre-adipocyte differentiation, and inhibiting glucose uptake and glycogen synthesis (Ferris and Kahn 2012-11-1). Therefore, we hypothesized that air pollution exposure leads to early molecular events associated with insulin-signaling pathway dysregulation that are GC-dependent and tissue-specific.

To address these knowledge gaps, we examined early events following ozone exposure, minimizing the potential influence of cellular and tissue changes that may arise with disease progression. We further assessed the role of GCs in mediating ozone-induced effects through treatment with metyrapone, an 11-β-hydroxylase inhibitor that blocks the ozone-induced increase in GCs (Thomson et al. 2016). The objectives of this study were to 1) determine the impacts of ozone exposure on the insulin-signaling pathway in major metabolic tissues, including the liver, skeletal muscle, brown adipose and white adipose, and 2) elucidate whether effects occur in a GC-dependent or -independent manner.

3.4 Methods

3.4.1 Animal handling

Male Fischer-344 rats between 200-250 g (specific pathogen free; Charles River, St. Constant, Quebec, Canada) were kept on a 12 h light/dark cycle, with food and water provided ad libitum. Animals were housed in individual plexiglass cages with HEPA-filtration and wood-chip bedding. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada.

3.4.2 Exposure

Inhalation exposure methods were previously described in detail (Thomson et al. 2016). To summarize, Fischer-344 rats treated with metyrapone (50 mg/kg; Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) or vehicle (40% propylene glycol in buffered saline) via subcutaneous injection 1 h prior to exposure to either clean air or 0.8 ppm ozone for 4 h (nose-only inhalation; n = 5/group). Exposure to 0.8 ppm ozone for 4 h represents a centriacinar dose 3 to 4 fold higher than an estimated single day exposure scenario in humans.(Thomson et al. 2005) Nose-only exposures were restricted to 4 h for ethical reasons, resulting in a higher dose rate within our experimental model than would be found with environmental exposure over 24 h.

3.4.3 Sample collection

Rats were anesthetised with isoflurane (5% at 1.5 L of O2/min) and euthanised via exsanguination 0 or 24 h post exposure. Tissue samples, including the liver, skeletal muscle (gracilis) and brown and white adipose tissue, were collected, snap-frozen in liquid nitrogen and stored at −80 °C.

3.4.4 RNA isolation and cDNA synthesis

Frozen tissue samples were ground with mortar and pestle over dry ice followed by sonication in TRIzol reagent (Invitrogen Canada Inc., Burlington, Ontario, Canada) to

homogenize. Isolation of total RNA was performed following manufacturer's instructions. RNA was quantified using RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon) and quality was evaluated using Experion RNA STdSens Analysis Kit (Bio-Rad, Mississauga, Ontario, Canada). cDNA synthesis (500 ng RNA per 40 µL reaction for skeletal muscle, brown adipose and white adipose, 1 µg RNA per 20 µL reaction for liver) was performed using MuLV reverse transcriptase and random hexamers (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Mississauga, Ontario, Canada).

3.4.5 qPCR

qPCR was performed using QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems) and TaqMan Rat Insulin Signaling Pathway Arrays (384-well cards; Applied Biosystems). Array cards were prepared and ran according to manufacturer's directions, with 4 samples and 96 targets per plate. cDNA was added to the plate fill reservoirs (0.45 ng/µL for all tissues except liver 0 h, 10 ng/µL). The PCR cycle used was as follows: 50 ºC for 2 min for UNG incubation, 92 ºC for 10 min to activate AmpliTaq Fast DNA Polymerase, and 40 cycles of 95 ºC for 1 s to denature followed by 60 ºC for 20 s to Anneal/Extend. Expression calculations were conducted relative to the geometric mean of the most stable housekeeping genes for each tissue (Liver – Protein Phosphatase 1 Catalytic Subunit Alpha and Beta-2-Microglobulin; Skeletal Muscle - Protein Phosphatase 1 Catalytic Subunit Alpha and Hydroxymethylbilane Synthase; Brown Adipose – β-actin and Beta-2-Microglobulin; White Adipose –Protein Phosphatase 1 Catalytic Subunit Alpha and Hydroxymethylbilane Synthase) determined using RefFinder (Xie et al. 2012), via the delta-delta Ct method (Livak and Schmittgen 2001). qPCR of *Pck1* was run separately, using the Lightcycler 480 system (Roche Diagnostics Canada, Laval, Québec, Canada). cDNA (10 ng/µL), BRIGHTGreen 2x qPCR Mastermix (Bio-Rad) and primers (200 nM; sense primer: ACTGGTTCCGGAAAGACAAA, antisense primer: GCGGGAGTTCTCACCAAAT) were

incubated using the following PCR cycle: 95 ºC for 3 min, 50 cycles of denaturation at 95 ºC, annealing at 60 ºC and elongation at 72 ºC, each for 10 s. Expression calculations were conducted relative to β-actin using the delta-delta Ct method (Livak and Schmittgen 2001).

3.4.6 Protein analysis

Frozen liver tissue was ground using a mortar and pestle. Approximately 25 mg of ground tissue was added to 500 µL of lysis buffer (Bio-Rad), 2 mM phenylmethylsulfonyl fluouride, and lysis factor QG (Bio-Rad). To analyze concentrations of phosphorylated proteins, the Bio-Plex Pro™ Cell Signaling Akt Panel (Bio-Rad) was used according to manufacturer's instructions. 200 µg/mL of protein was used per sample (10 µg per assay well), and fluorescence intensity was normalized to β-actin.

3.4.7 Statistical analysis and data visualization

Data were assessed using two-way ANOVA (factors *Ozone* (0, 0.8 ppm) and *Metyrapone* (0, 50 mg/kg)) followed by post-hoc Holm-Sidak multiple comparisons procedure ($\alpha = 0.05$). Statistical analyses were performed using SigmaPlot v13.0 (Systat Software, San Jose, CA) or R 4.1.2 (R Core Team 2025) and the *multcomp* (Hothorn et al. 2008)*, MASS* (Venables and Ripley 2002)*, nortest* (Gross and Ligges 2015)*, lawstat* (Gastwirth et al. 2020)*,* and *doBy* (Højsgaard and Halekoh 2021) packages. Heat maps were generated using the R package *pheatmap* (Kolde 2019). Hierarchal clustering based on Pearson correlation distance and average linkage. Principal component analysis (PCA) was performed using GraphPad Prism v.9.3.1. (GraphPad Software, San Diego, CA), with standardized scaling applied. To simplify, only significant main effects and factor interactions that included *Ozone* are presented in figures.

3.5 Results

3.5.1 Impacts of ozone exposure on insulin signaling pathway genes within major metabolic tissues

We examined expression of genes within the insulin-signaling pathway across various metabolic tissues (liver, skeletal muscle, and brown and white adipose) following ozone exposure. Insulin signaling pathway genes were altered immediately following 4 h exposure to ozone (0 h post exposure) in all tissues examined, with gene expression profiles clearly differing across tissues (Figure 3.1A). Profiles tended to return to control levels by 24 h. Most differentially expressed genes were unique to a given tissue; the liver exhibited the greatest number of significant differentially expressed genes, whereas the white adipose tissue and muscle showed few changes (Figure 3.1B).

Ozone exposure led to differential expression of genes involved in the insulin-signaling cascade (Figure 3.1C), including downregulation of insulin receptor substrate 1 (*Irs1*) in brown adipose and insulin receptor substrate 2 (*Irs2*) in brown and white adipose tissue, and alterations of phosphoinositide-3-kinase regulatory subunit 1 (*Pik3r1*) in the liver and brown adipose tissue.

Key target genes of the insulin-signaling pathway were also differentially expressed following exposure (Figure 3.1C). Serpin family E member 1 (*Serpine1*), a gene associated with diabetes and obesity (Alessi et al. 2000; Taeye et al. 2005), was increased in all tissues with ozone exposure. Other genes that act as markers of these conditions, leptin (*Lep*) (Sader et al. 2003) and resistin (*Retn*) (Steppan et al. 2001), were upregulated in the brown adipose. Bcl2 like 1 (*Bcl2l1*), an anti-apoptotic gene and target of insulin signaling, was increased in both adipose tissues. Fos proto-oncogene, AP-1 transcription factor subunit (*Fos*), a key component of the AP-1 transcription factor responsible for regulation of a variety of cell functions, was upregulated in the liver and muscle and downregulated in brown adipose tissue.

The glucose metabolism pathway was also impacted (Figure 3.1C), especially in the liver, with downregulation of the hexokinase 2 (*Hk2*) and phosphoenolpyruvate carboxykinase (PEPCK) gene *Pck1*, and increased expression of the glycolytic enzyme phosphoglycerate kinase 1 (*Pgk1*), and solute carrier family 2 member 1 (*Slc2a1*), which encodes the GLUT1 glucose transporter. In contrast to the liver, the brown adipose demonstrated an increase in *Hk2* and *Pck2*, as well as a decrease in *Slc2a1* following ozone exposure. Similar to the liver, ozone exposure led to a reduction of *Pck2* in white adipose tissue (Figure 3.1A,C).

3.5.2 Tissue specificity of ozone-induced effects on insulin signaling gene expression

As the pattern of expression following ozone exposure appeared to differ across tissues, we assessed sources of variability and commonality in responses to ozone through hierarchical clustering (Figure 3.2A) and PCA (Figure 3.2B) . Samples clustered together by tissue via hierarchal clustering, and tended to separate by tissue via PCA, indicating that expression within tissue types was more similar than expression patterns among tissues within individual animals, confirming the tissue-specificity of ozone effects. The gene expression profiles in brown adipose and white adipose tissues showed the greatest correlation following ozone inhalation ($r = 0.62$, $p < 0.001$). The liver was positively correlated with the white adipose ($r = 0.48$, $p < 0.001$), brown adipose ($r = 0.34$, $p = 0.004$), and muscle ($r = 0.31$, $p = 0.01$) tissues. The muscle response was positively correlated with white adipose tissue ($r = 0.33$, $p = 0.006$) and was not correlated with brown adipose tissue ($r = 0.08$, $p = 0.5$).

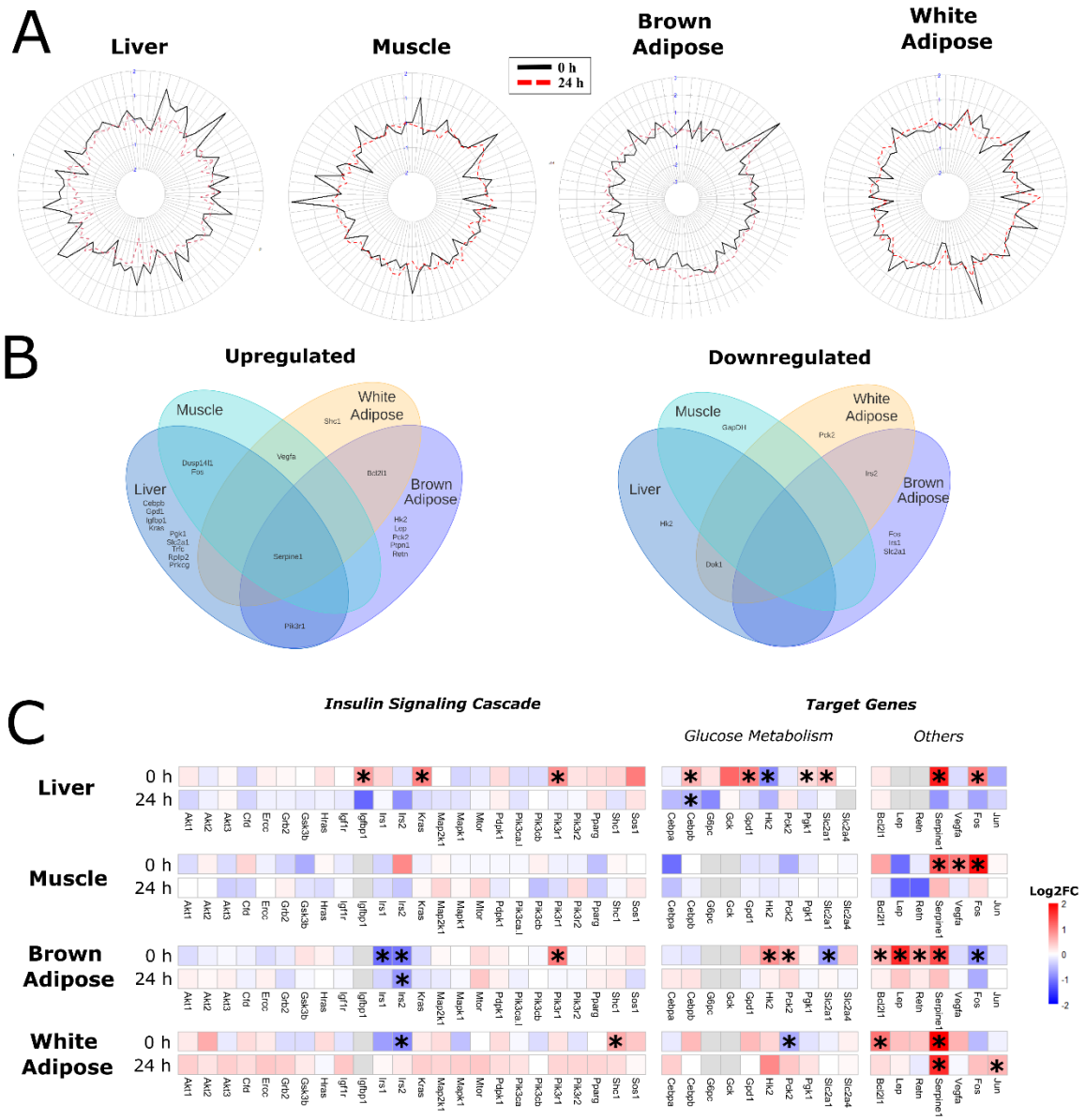


Figure 3.1. Ozone exposure effects on insulin signaling pathway genes in various metabolic tissues. Fischer-344 rats were exposed to clean air (Veh/Air) or 0.8 ppm ozone (Veh/Ozone) for 4 h. Tissue was collected immediately (0 h) or 24 h post-exposure. Gene expression was assayed using insulin signaling qPCR arrays (Taqman) in the liver, skeletal muscle, and brown and white adipose. Expression is represented as Log2-Fold Change (Log2FC) and normalized to Vehicle/Air. A) Radar charts of Log2FC of Veh/Ozone at 0 h (black solid line) and 24 h (red dotted line). B) Venn diagrams summarizing significantly upregulated and downregulated genes in the Veh/Ozone group compared to Veh/Air. C) Heat maps depicting Log2FC of Veh/Ozone at 0 h and 24 h. Two-way ANOVA (Ozone, Metyrapone) with post-hoc holm-sidak was used for statistical analysis. VEH Air vs. Ozone *$p < 0.05$ (time-matched) $n = 5$.

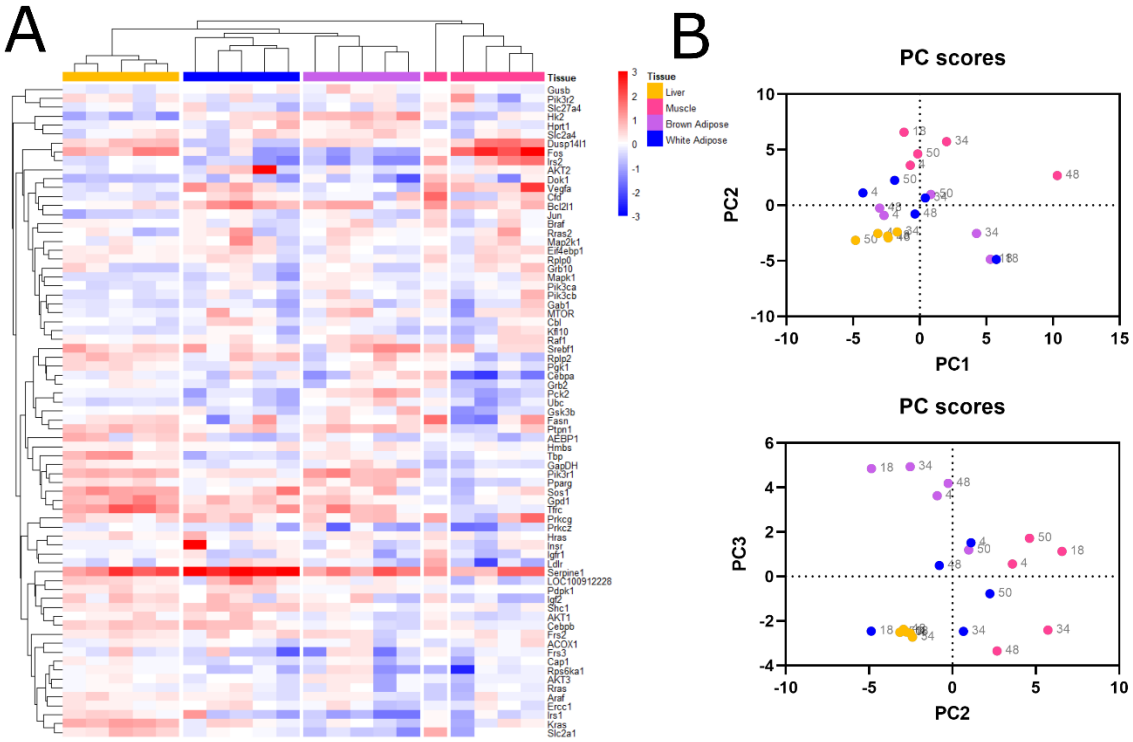


Figure 3.2. Tissue-specificity of ozone-induced effects on insulin-signaling pathway genes. Expression of insulin-signaling pathway genes was measured in Fischer-344 rats following exposure to clean air or 0.8 ppm ozone for 4 hours in the liver (yellow), skeletal muscle (pink), brown adipose (purple) and white adipose (blue). Tissue-specific patterns of gene expression profiles following ozone exposure were evaluated through hierarchal clustering (A) or PCA (B) of samples. Expression represented as Log2-Fold Change (Log2FC) relative to the Veh/Air group. Numbers in (B) represent sample IDs.

3.5.3 The role of GCs in mediating ozone-induced effects

As GCs are critical modulators of metabolism, and especially the actions of insulin, we sought to examine the role of GCs in mediating the ozone-induced effects. Treatment with metyrapone, a GC synthesis inhibitor, prevented or diminished (*Ozone* × *Metyrapone* interaction, $p < 0.05$; Vehicle vs. Metyrapone within Air, $p > 0.05$) a subset of ozone-induced effects in the liver and white and brown adipose tissues (Figure 3.3). In particular, metyrapone treatment reduced the ozone-induced upregulation of *Pik3r1* and *Serpine1* in the liver (interaction $p = 0.009$, $p = 0.02$, respectively) and brown adipose tissue (interaction $p = 0.005$, $p = 0.04$, respectively), as well as *Retn* (interaction $p = 0.01$) and *Pck2* (interaction $p = 0.03$) in brown adipose tissue exclusively, and *Bcl2l1* (interaction $p = 0.04$) in white adipose tissue. Ozone-induced down-regulation of *Irs1* (interaction $p = 0.043$) and *Fos* (interaction $p = 0.02$) was prevented by metyrapone treatment in brown adipose tissue. Additionally, while the *Ozone* × *Metyrapone* interaction for *Hk2* in the liver ($p = 0.07$), as well as *Irs2* in the brown adipose ($p = 0.06$) or white adipose ($p = 0.1$) tissues did not reach significance, there was no statistical difference between Air vs. Ozone exposure within the Metyrapone group for these genes (*Hk2* Liver, $p = 0.6$; *Irs2* Brown Adipose; $p = 0.39$; *Irs2* White Adipose, $p = 0.33$), suggesting a blunting of ozone effects by metyrapone. In contrast to effects observed in the liver, brown adipose and white adipose tissues, metyrapone treatment did not affect ozone-induced effects in skeletal muscle.

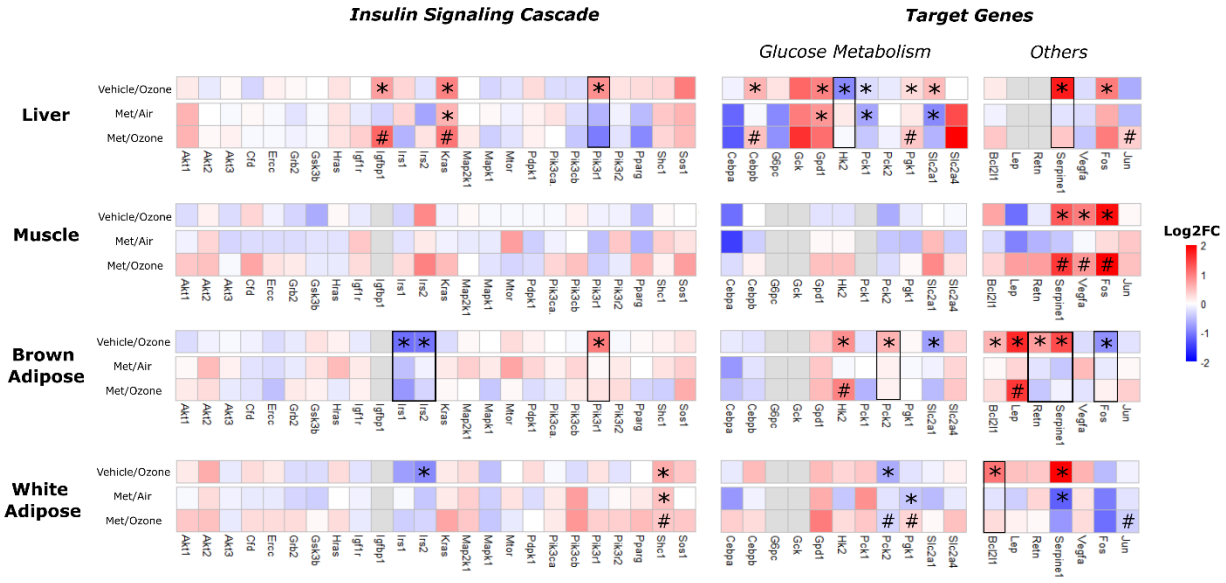


Figure 3.3. Role of glucocorticoids in mediating ozone-induced effects in key insulin-signaling pathway genes. Fischer-344 rats were treated with metyrapone, a glucocorticoid synthesis inhibitor, 1 h prior to exposure to clean air or 0.8 ppm ozone for 4 h. Expression of key insulin-signaling cascade and target genes in the Veh/Ozone, Met/Air and Met/Ozone groups (0 h) are displayed by tissue and represented as Log2FC relative to Veh/Air. $p < 0.05$ vs. Veh/Air* or Met/Air# (Two-way ANOVA with post-hoc Holm Sidak); n = 5/group. Grey boxes indicate that expression was not detected. Black outlines indicate genes significantly altered by ozone exposure (VEH Air vs. Ozone $p < 0.05$) with a significant ($p < 0.05$) or borderline significant ($p < 0.07$) Ozone × Metyrapone interaction when metyrapone does not significantly alter expression in the absence of ozone (Veh/Air vs. Met/Air, $p > 0.05$).

3.5.4 Insulin signaling phosphoprotein levels in the liver

In addition to regulation at the transcriptional level, post-translational changes in phosphorylation states of key proteins involved in insulin-signaling can lead to dysregulation and impairment of glucose homeostasis. As most of the significant gene expression changes observed were within the liver, and given the liver's key role in regulating glucose homeostasis, we analyzed phosphorylation levels of proteins associated with the insulin-signaling pathway in liver tissue immediately following the 4 h exposure (Figure 3.4). Ozone exposure increased relative phosphorylation levels of specific residues in Bcl-2-associated death promoter (BAD; Ser^{136}; *Ozone* Main Effect, p = 0.0049; Figure 3.4B), glycogen synthase kinase 3 α/β (GSK-3 α/β; Ser^{21}/Ser^{9}; *Ozone* Main Effect, p = 0.0081; Figure 3.4C), Insulin Receptor-β (IR-β; Tyr^{1146}; *Ozone* Main Effect, p = 0.0035; Figure 3.4D), and IRS1 (Ser^{636}/Ser^{639}; *Ozone* Main Effect, p = 0.0006; Figure 3.4I) with no significant changes found in levels of Akt Serine/Threonine Kinase 1 (Akt; Ser^{473}; Figure 3.4A), Mammalian target of rapamycin (mTOR; Ser^{2248}; Figure 3.4E), p70 S6 Kinase (Thr^{389}; Figure 3.4F), Phosphatase and tensin homolog (PTEN; Ser^{380}; Figure 3.4G), or S6 Ribosomal Protein (Ser^{235}/Ser^{236}; Figure 3.4H). Ozone effects tended to be maintained in the presence of metyrapone, although possibly of reduced magnitude.

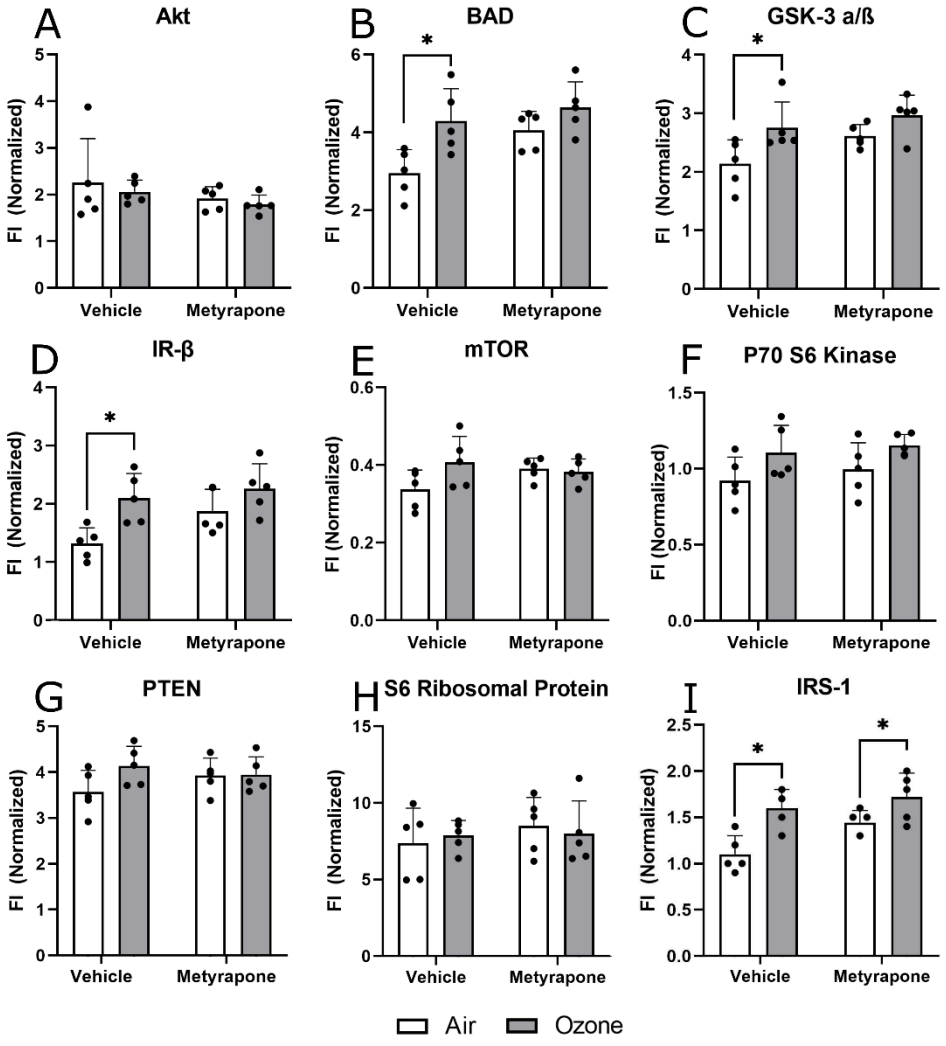


Figure 3.4. Ozone exposure effects on insulin-signaling phosphorylation levels in the liver. Levels of key phosphorylated proteins in the insulin-signaling pathway were measured in the liver of Fischer-344 rats exposed to clean air or 0.8 ppm ozone for 4 h following metyrapone treatment (0 h time point). A) Akt (Ser^{473}), B) BAD (Ser^{136}), C) GSK-3 α/β ($Ser^{21/9}$), D) IR-β (Tyr^{1146}), E) mTOR (Ser^{2248}), F) P70 S6 Kinase (Thr^{389}), G) PTEN (Ser^{380}), H) S6 Ribosomal Protein ($Ser^{235/236}$), I) IRS-1 ($Ser^{636/639}$). Bars represent mean ± SD, n = 5/group. Fluorescence Intensity (FI) normalized to β-actin. Two-way ANOVA (Ozone, Metyrapone) with post-hoc Holm Sidak was used for statistical analysis; *$p < 0.05$, Air vs. Ozone.

3.6 Discussion

Exposure to air pollution has been associated with increased incidence of metabolic diseases such as T2D and insulin resistance, which may be due in part to disruption of the insulin-signaling pathway. However, a lack of studies examining impacts on the insulin-signaling pathway across tissues, and the underlying mechanisms involved, has hampered the more holistic characterization of systemic effects of air pollution exposure needed to understand how these effects could contribute to disease development. Using pharmacological intervention and a multi-tissue analytical approach, our study revealed that exposure to ozone differentially altered expression of insulin-signaling genes across all metabolic tissues examined (liver, skeletal muscle, white and brown adipose tissues) in both a GC-dependent and independent manner. While alterations were transient in this acute exposure model, observed changes were consistent with alterations linked to metabolic disease processes.

The insulin-signaling pathway is characterised by the interplay of various receptors, enzymes, and modulatory proteins. The most well known actions of this pathway are promoting glucose uptake through facilitating translocation of GLUT4 to the cell membrane via induction of the PI3K pathway following phosphorylation of IRS1 (Chang et al. 2004). However, the insulin-signaling pathway also acts through IRS1-dependent and -independent pathways on many other transcription factors to modulate other critical functions such as cell growth and survival, protein synthesis, glycogen synthesis and lipogenesis (Haeusler et al. 2018). Our findings that exposure to ozone modifies expression of genes implicated in the insulin-signaling pathway across multiple metabolic tissues builds upon previous studies demonstrating insulin-signaling changes in specific metabolic tissues following exposure to pollutants (Vella et al. 2015; Miller et al. 2015; Thomson et al. 2016; Miller et al. 2016a; Liu et al. 2017; Thomson et al. 2018).

Alterations in expression of insulin-signaling pathway components, such as upregulation of *Pi3kr1* (which can be inhibitory) (Giorgino et al. 1997; Barbour et al. 2004; McCurdy et al. 2012) in the liver and brown adipose tissue, inhibitory serine-phosphorylation of IRS1 within the liver, as well as decreases in *Irs1* and *Irs2* within the adipose tissues suggest potential impairment of the insulin signaling cascade in these tissues following ozone exposure. IRS1 and 2 are key intermediate signaling proteins that transmit signals to intracellular pathways in response to binding of insulin to the insulin receptor. Downregulation of IRS1 and IRS2 gene expression is associated with insulin resistance in multiple cell types including adipocytes (Carvalho et al. 1999), and elevated serine phosphorylation of IRS1 can promote degradation (Shah et al. 2004), reduce activity (Aguirre et al. 2002) and enhance insulin resistance (Qiao et al. 1999, 2002; Shah et al. 2004).

Ozone exposure also led to alterations in transcription factors targeted by the insulin-signaling cascade including *Fos* and CCAAT enhancer binding protein beta (*Cebpb*) within various tissues, and increased mRNA levels of certain genes associated with obesity and T2D incidence such as *Serpine1*, *Lep*, and *Retn*. *Serpine1*, which encodes plasminogen activator inhibitor-1 (PAI-1), was highly upregulated in all tissues examined following ozone exposure, aligning with previous studies that have found elevated PAI-1 levels in circulation (Thomson et al. 2016; Mirowsky et al. 2017; Davis et al. 2020; Ghosh et al. 2021), as well as lung and adipose tissues (Budinger et al. 2011-4-11) following exposure to pollutants such as ozone and $PM_{2.5}$. Treatment with a PAI-1 inhibitor has been shown to prevent induction of inflammatory markers and vascular thrombosis following $PM_{2.5}$ exposure (Ghosh et al. 2021). Elevations in circulating PAI-1 are commonly found in patients with T2D (Yarmolinsky et al. 2016), as well as patients with coronary artery disease (Francis et al. 1988), another condition associated air pollution

exposure (Franklin et al. 2015). Pollutant-induced increases in *Serpine1* expression thus may be a potential mechanism through which air pollution contributes to adverse health effects. Similarly, air pollution has also been associated with increases in circulating leptin (Miller et al. 2015), in line with the increase in leptin mRNA levels in the brown adipose observed in this study.

Ozone also may alter cell death pathways, as increased expression of *Bcl2l1,* which encodes the anti-apoptotic protein Bcl-xL, was observed in the brown and white adipose following exposure. Similarly, while no change in expression of *Bcl2l1* was observed in the liver, we observed increased serine phosphorylation of BAD, which inhibits its pro-apoptotic ability to sequester Bcl-xL (Yang et al. 1995; Zha et al. 1996).

Glucose metabolism genes seemed especially impacted in the liver, a major regulator of glucose homeostasis. PEPCK is a key rate-limiting enzyme in gluconeogenesis, and transcriptional regulation of this gene dictates activity levels (Hanson and Reshef 1997). Expression of PEPCK genes were downregulated in the liver, suggesting impairment of gluconeogenesis and promotion of glycolysis in this tissue, corresponding to upregulation of the glycolytic enzyme PGK1. These effects, alongside inhibition of GSK-3 through serine phosphorylation, which allows for activation of glycogen synthase, are consistent with activation of the insulin-signaling pathway (Hatting et al. 2018). Far fewer effects on glucose metabolism genes were observed in the adipose tissues. Consistent with ozone-dependent effects in the liver, white adipose tissue showed a decrease in expression of *Pck2*, whereas in brown adipose tissue *Pck2* as well as *Hk2*, which encodes an enzyme responsible for the conversion of glucose to glucose-6-phosphate, were upregulated. This difference in regulation of *Pck2* between the liver and brown adipose is consistent with previous reports of tissue-specific regulation, such as

adipose-specific regulation of *Pck2* via PPARγ-2, an adipocyte-specific transcription factor (Devine et al. 1999). Additionally, PEPCK appears to play a role in glyceroneogenesis, particularly within adipose tissue (Reshef et al. 1970; Kalhan et al. 2001; Olswang et al. 2002; Franckhauser et al. 2006). Upregulation of *Pck2* within the brown adipose, as well as elevation of glycerol-3-phosphate dehydrogenase 1 (*Gpd1*) in the liver, of which increased mRNA levels following ethanol administration leads to triglyceride accumulation in mice (Sato et al. 2014), are broadly consistent with increases in glycerol and triglyceride levels found following ozone exposure in previous studies (Miller et al. 2015; Thomson et al. 2018).

A notable observation in this study was that patterns of effects were tissue-specific rather than broadly consistent across tissues. GC regulation may explain some of these differences. Despite GCs being systemic mediators, their actions depend also upon the local context. GCs are known to induce lipolysis, decrease glucose uptake and induce insulin resistance in adipose tissue, increase glucose production and reduce insulin receptor signaling, and promote lipid accumulation in the liver, reduce glycogen synthesis and glucose uptake and promote protein catabolism and degradation in the skeletal muscle (Vegiopoulos and Herzig 2007). These cell-type and tissue-specific actions are conferred by interactions of GC-receptor complexes with additional accessory factors such as co-activator/co-repressor complexes in the control of gene expression within the cell (Vegiopoulos and Herzig 2007; Patel et al. 2014).

In the present study, GC-dependent effects were observed in the liver, brown adipose and white adipose tissues whereas none were observed in the muscle. Ozone-dependent regulation of *Serpine1* expression appears to be mediated by GC action in the liver and adipose tissues, consistent with studies demonstrating upregulation of this gene by dexamethasone, a synthetic GC, in these tissues (Barbara et al. 1992; Morange et al. 1999). GC-dependent effects were

especially prevalent in the brown adipose tissue, regulating expression of genes such as *Irs1* and *Irs2*, *Pik3r1*, *Pck2*, *Retn* and *Fos*, consistent with previous studies examining effects in adipose tissue (Shojima et al. 2002; Caperuto et al. 2006; Yu et al. 2010; Sefton et al. 2019). Interestingly, we did not observe any evidence of GC-dependent effects in the muscle. For instance, *Serpine1* exhibited GC-dependent patterns of regulation in the liver, brown adipose and white adipose tissues, but not in the muscle despite also being upregulated in this tissue. While GCs have been shown to exert effects on the muscle within the literature (Ruzzin et al. 2005; Hu et al. 2009), these results suggest that in the present model some other mediator is involved in the regulation of these genes within the muscle. One possibility may be factors associated with oxidative stress, which has been previously demonstrated to mediate ozone-induced changes in insulin-sensitivity and protein carbonyl content within the muscle (Vella et al. 2015). Additionally, exposure to pollutants can activate the sympathetic-adreno-medullar axis leading to increased release of catecholamines (Miller et al. 2016b; Wang et al. 2022). Catecholamines can regulate a variety of genes associated with metabolism processes within muscle (Viguerie et al. 2004) and thus may represent another mechanism through which air pollution exerts GC-independent effects.

Changes in insulin-signaling genes observed in this model occur in the context of a transient decrease in glucose tolerance (demonstrated with Fischer-344 rats under the same exposure conditions as this study) (Thomson et al. 2018) but a lack of ozone effects on levels of circulating insulin (Thomson et al. 2016) or glucose (Supplementary Figure 3.S2). Given this lack of change in circulating insulin in this model, our study suggests that a possible route through which pollutants such as ozone may cause effects consistent with disease, such as changes in glucose tolerance, may be through changes in expression of insulin-signaling pathway related genes in an insulin-independent context. Glucose tolerance is regulated by insulin

signaling (Chang et al. 2004), and thus results from this study suggesting impairment of the insulin-signaling cascade, such as downregulation of *Irs1* and *Irs2*, are consistent with this finding. We also found patterns of insulin-signaling target genes (including those involved in glucose metabolism) consistent with activation of the insulin pathway. These may occur in an insulin-independent manner; indeed, several mechanisms, including GC action, can contribute to the regulation of these genes. For instance, obesity and diabetes-associated genes *Lep* and *Serpine1* (Alessi et al. 2000; Sader et al. 2003; Taeye et al. 2005) are subject to GC-dependent regulation (Barbara et al. 1992; Slieker et al. 1996; De Vos et al. 1998; Morange et al. 1999). Additional explanations for the effects observed in this study include temporal differences leading to changes in insulin or activation of the pathway earlier in exposure, which aligns with the increase in tyrosine phosphorylation of IR-β observed suggesting receptor activation. Alterations of these key functional targets, in addition to impairments in insulin-signaling cascade genes that could confer insulin resistance is a potential route through which pollution may lead to increased risk of developing metabolic diseases.

Various factors should be considered when interpreting results of this study. First, exposures were conducted using a nose-only exposure system, which may be stressful despite progressive training prior to exposure (Thomson et al. 2009). Secondly, metyrapone may exert ozone-independent effects; comparison of metyrapone and vehicle-treatment within the air-exposed group was used to identify any such effects. A significant strength of the present study was the comparison of ozone effects across tissues within a single model, enabling attribution of differential effects across tissues to differential regulation rather than to model and experimental differences across studies. Assessment of site- or cell type-specific changes in gene expression was beyond the scope of the present work, but could provide additional insight. This study was

exclusively conducted with male rats and thus is unable to identify possible sex differences that may arise following ozone exposure. As metabolic regulation and stress responses can vary by sex, it is possible that the ozone-induced effects observed in this study may differ in females. Finally, while use of two post-exposure time points (immediately and 24 h post exposure) provided some insight into the temporal nature of these effects, further examination of effects between the 0 and 24 h periods following exposure might allow additional understanding of pollutant effects within these tissues.

In conclusion, the present study provides insight into how air pollutants may lead to metabolic dysfunction and diseases by demonstrating that exposure to ozone, a common urban air pollutant, differentially alters expression of key insulin signaling pathway genes across tissues. Additionally, we provide novel evidence supporting a role for GCs in mediating these pollutant effects. These findings add to the growing body of literature associating exposure to air pollutants with metabolic dysfunction and highlight a need for more holistic approaches when examining effects of pollutant exposures on disease pathways. As exposure to air pollution is ubiquitous, impairments of these key pathways could lead to significant population health impacts, particularly in individuals with existing risk factors such as impairments in insulin sensitivity or neuroendocrine control.

3.7 Acknowledgments

We wish to acknowledge the technical assistance of Kevin Curtin, Kevin Kittle, Karine Chamberland, and Julie Todd. Funding for this study was provided by Health Canada through the Clean Air Regulatory Agenda and the Addressing Air Pollution Horizontal Initiative.

3.8 Supplemental Figures

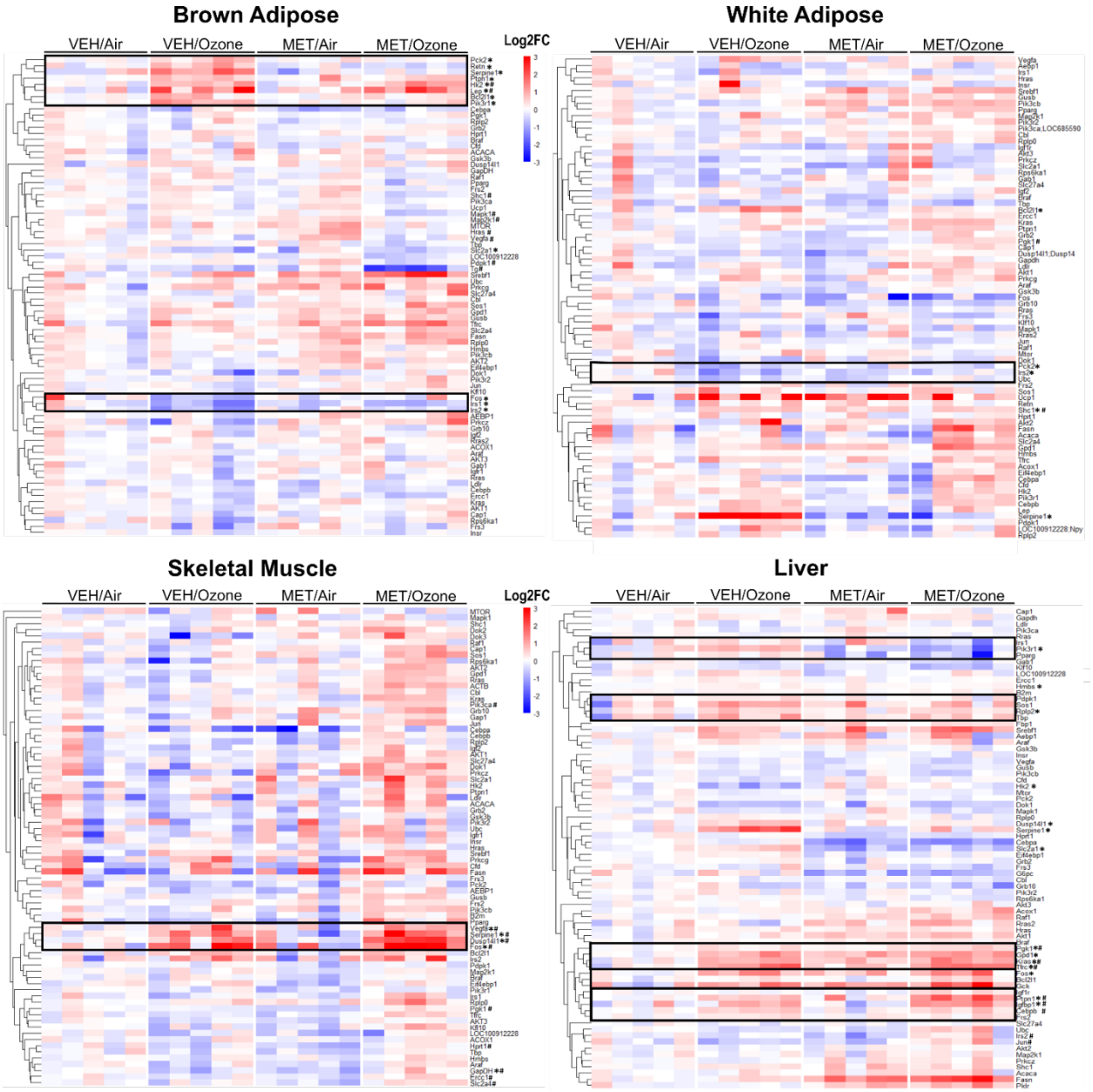


Figure 3.S1. Effects of ozone exposure and metyrapone treatment on expression of insulin-signaling pathway genes in metabolic tissues. Fischer-344 rats were treated with metyrapone or vehicle, and exposed to clean air or 0.8 ppm ozone for 4 h. Gene expression was assayed using insulin signaling qPCR arrays (Taqman) in the liver, skeletal muscle, and brown and white adipose. Expression is represented as Log2-fold chance (Log2FC) and normalized to Vehicle/Air. Two-way ANOVA (*Ozone, Metyrapone)* with post-hoc Holm-Sidak was used for statistical analysis. VEH Air vs. Ozone *$p < 0.05$; MET Air vs. Ozone $^{\#}p < 0.05$. Clustering based on Pearson correlation distance and average linkage. Black outlines represent clusters of interest.

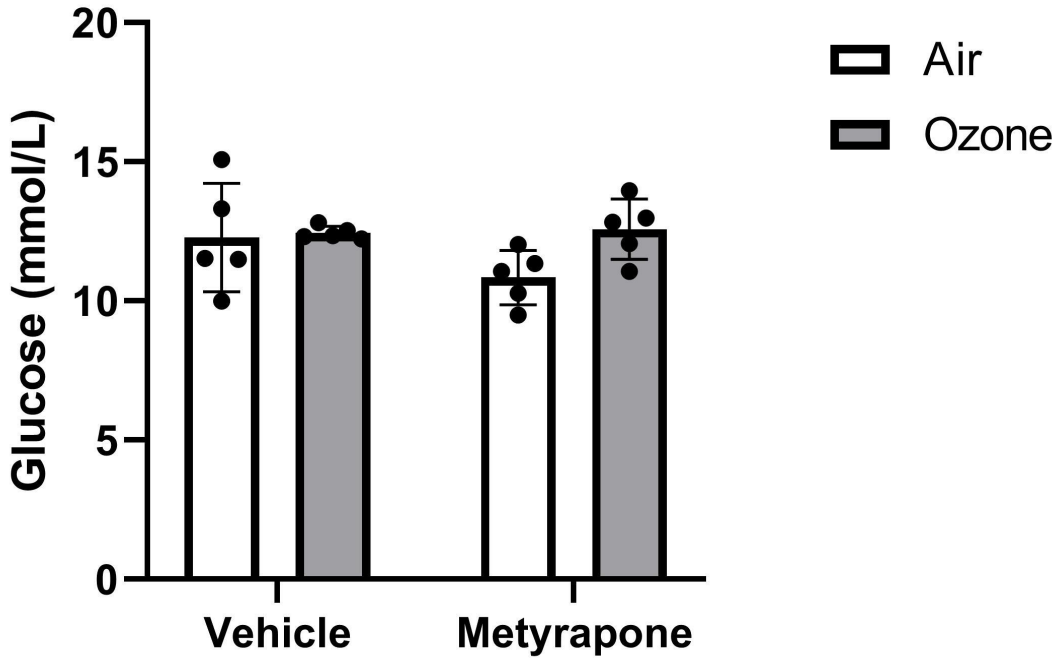


Figure 3.S2. Glucose levels in ozone-exposed rats. Glucose was measured in Fisher-344 rats exposed to ozone or clean air for 4 hours following treatment with metyrapone or vehicle-control. Two-way ANOVA (factors: *Ozone, Metyrapone*) with post-hoc Holm-Sidak was used for statistical analysis. Bars represent mean ± SD, n = 5/group.

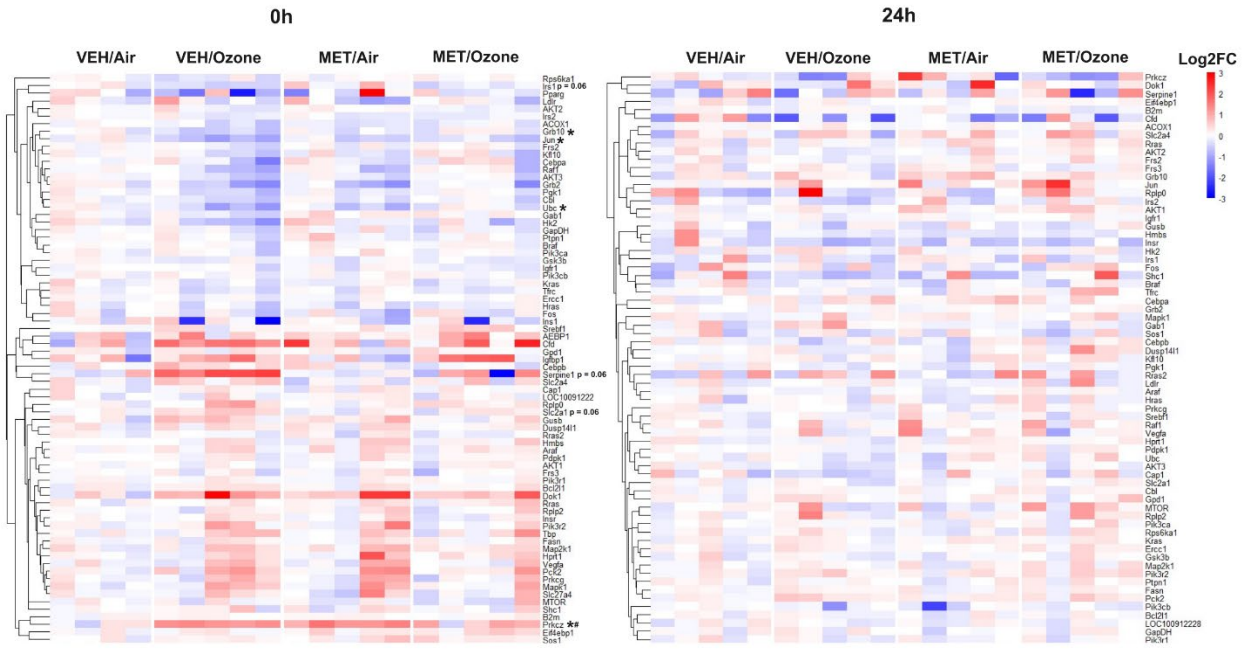


Figure 3.S3. Effects of ozone exposure and metyrapone treatment on expression of insulin-signaling pathway genes in the hippocampus. Fischer-344 rats were treated with metyrapone or vehicle, and exposed to clean air or 0.8 ppm ozone for 4 h. Tissue was collected immediately (0h) or 24h post exposure. Gene expression was assayed using insulin signaling qPCR arrays (Taqman) in the hippocampus. Expression is represented as Log2-fold chance (Log2FC) and normalized to Vehicle/Air. Two-way ANOVA (*Ozone, Metyrapone)* with post-hoc Holm-Sidak was used for statistical analysis. VEH Air vs. Ozone *$p < 0.05$; MET Air vs. Ozone $^{\#}p < 0.05$. Clustering based on Pearson correlation distance and average linkage.

Chapter 4: Ozone inhalation modifies sphingolipid metabolic pathways through glucocorticoid-dependent actions

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Manuscript in preparation

4.1 Authorship Contributions

The overall rat ozone exposure study conducted, from which samples are leveraged from for this manuscript, was conceived and led by EMT. Plasma samples from the TgF344-AD rodent model were obtained from JM. The project described in the present study was designed by MR, EMT and SALB. Lipid extractions and MS analysis were conducted by MR (ozone study samples) and IA (TgF344-AD samples). SALB performed absolute quantification of lipids. MR performed statistical analysis, prepared figures, and wrote the manuscript. Review and editing of the manuscript by EMT and SALB.

4.2 Abstract

Air pollution is a significant environmental risk for metabolic and neurodegenerative diseases such as Alzheimer's disease (AD), of which alterations to lipid metabolism are a common feature. Sphingolipids, a class of lipids that regulate critical cellular processes, play a key role in disease pathogenesis. However few studies have examined how air pollution broadly affects sphingolipid metabolism, and potential underlying mechanisms remain unclear. Air pollution increases circulating glucocorticoids, known regulators of sphingolipid metabolism. Here, we 1) assessed the effects of acute ozone inhalation on circulating sphingolipids role, 2) evaluated the role of glucocorticoids as a potential mediator, and 3) compared sphingolipid profiles following ozone exposure to those associated with an aging/AD phenotype. Male Fischer-344 rats were exposed to 0.8 ppm ozone or clean air for 4 h, ± metyrapone, a glucocorticoid synthesis inhibitor. Plasma samples were collected immediately or 24 h post exposure. Plasma lipids were quantified using LC-ESI-MS/MS and compared to the TgF344-AD rat model. Acute ozone exposure altered levels of plasma sphingolipids, reducing levels of hexosylceramide species 24 h post-exposure. Metyrapone prevented several effects, and exogenous corticosterone administration reproduced effects observed 24 h post-ozone exposure, confirming a role for glucocorticoids. Ozone-induced effects showed no correlation with the transgenic AD-phenotype, but negatively correlated with lipidomic changes associated with aging. These results establish a novel role for glucocorticoids in mediating ozone-induced dysregulation of sphingolipid metabolism, which may be a mechanism through which air pollution contributes to elevated risk of neurodegenerative and metabolic disease.

4.3 Introduction

Air pollution exposure is responsible for an estimated 7.9 million premature deaths worldwide, annually, as well as 232 million disability adjusted life years as of 2023 (Health Effects Institute 2025). While the respiratory and cardiovascular impacts of air pollution are well studied and understood, more recent evidence also indicates that air pollution increases risk of metabolic and neurological diseases such as type 2 diabetes (T2D), metabolic syndrome, Alzheimer's disease (AD), and Parkinson's disease (PD), amongst others (Power et al. 2016; Allen et al. 2017; Rajagopalan et al. 2024). These diseases are leading contributors to disability, and the number of disability-adjusted life-years resulting from these diseases has doubled since 2000 (Ferrari et al. 2024). Given the rising prevalence of these diseases (Nichols et al. 2022; Zhou et al. 2024; Noubiap et al. 2025), and ubiquitous exposure of air pollution to the global population, air pollution may be a critical modifiable risk factor. However, further understanding of the mechanisms through which air pollution modifies disease risk is necessary to improve health estimates and inform policy decisions.

Metabolic and neurological diseases share several affected biological pathways and are often comorbid. For instance, diabetes increases risk of cognitive decline and AD development (Barbagallo and Dominguez 2014). Disruption of lipid metabolism is a hallmark of both metabolic and neurodegenerative diseases (Kulas et al. 2020). Sphingolipids, a class of lipids that are active components of cell membranes as well as crucial signaling molecules, may play a key role in disease pathogenesis. Sphingolipids regulate several cellular processes including apoptosis, cell growth, cellular stress responses, and inflammation, amongst others (Hannun and Obeid 2018), and are one of the most abundant lipid classes in the brain. AD patients have been reported to have higher circulating levels of ceramides (Cers), which are central to sphingolipid metabolism, can induce apoptosis, and are synthesized in response to inflammatory signals, as

well as decreased levels of certain sphingomyelin (SM) species (Wong et al. 2017). Brains of AD patients postmortem display region-specific alterations of sphingolipid metabolism (Sooderberg et al. 1992; Han et al. 2002; Cutler et al. 2004; He et al. 2010).

A few panel studies have shown that exposure to air pollutants is associated with changes in circulating sphingolipids (Miller et al. 2016a; Xu et al. 2022b; Zhu et al. 2023; Zhang et al. 2024a). Toxicological studies conducted using rodent models support epidemiological findings that air pollution exposure affects sphingolipid metabolism. Chronic exposure to both PM2.5 (Lee et al. 2020; Li et al. 2020a; Qin et al. 2023) and ozone (Liu et al. 2022) led to changes in sphingolipid species, in both blood as well as brain and lung tissue samples. Modulation of sphingolipid metabolism may therefore contribute to the increased risk of these diseases in those exposed to higher air pollution levels.

However, knowledge regarding the pathways involved that initiate lipidomic changes following exposure to air pollutants remains incomplete. Air pollution acts on several systemic mechanisms that are known to affect lipid metabolism, such as inflammation and oxidative stress. Exposure to air pollutants also activates the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased levels of circulating glucocorticoids (GCs) (Thomson et al. 2013, 2016; Miller et al. 2016a), which have been previously reported to mediate several systemic effects of air pollution exposure including effects on insulin signaling pathways (Rose et al. 2023) and the brain (Henriquez et al. 2019; Thomson et al. 2019; Rose et al. 2020). GCs are key metabolic regulators, acting on several pathways that contribute to lipid metabolism. For instance, GCs induce lipogenesis and lipolysis in adipose tissue (Peckett et al. 2011), and contribute to lipid accumulation in the liver (Woods et al. 2015). Dexamethasone, a GC receptor agonist, has been shown to control expression of enzymes involved in fatty acid synthesis such as Acetyl-CoA

carboxylases (Zhao et al. 2010), as well as those involved in ceramide synthesis (Cinque et al. 2008; Chen et al. 2017) and sphingosine-1-phosphate formation (Nieuwenhuis et al. 2009). Therefore, GCs may contribute to air pollution-induced effects on lipid metabolism. Moreover, elevated GC levels are a commonality amongst patients with AD (Ouanes and Popp 2019), as well as metabolic diseases (Di Dalmazi et al. 2012).

To gain further understanding of how air pollution affects lipid metabolism and whether those changes may explain the association between air pollution and diseases such as AD, we measured circulating levels of sphingolipid species in rats following acute exposure to ozone, a highly reactive, gaseous urban air pollutant that is completely consumed in the lungs upon inhalation. To address whether GCs mediate any pollutant-induced changes in lipid metabolism, we employed a pharmacological inhibition model in which metyrapone was administered to rats prior to exposure to ozone, which blocks the ozone-induced increase in circulating GCs (Thomson et al. 2016). Finally, we compared circulating lipid profiles of ozone-exposed rats with those found in a rat transgenic AD model, TgF344-AD to establish whether ozone-induced changes in lipids are similar to those found in transgenic and/or aged rats.

4.4 Methods

4.4.1 Ozone exposure model

Male Fischer-344 rats between 200-250 g (specific pathogen free; Charles River, St. Constant, Quebec, Canada) were housed in individual plexiglass cages with HEPA filtration and wood chip bedding, with food and water provided ad libitum. Animals were kept on a 12 h light/dark cycle. Inhalation exposure methods have been described previously in detail (Thomson et al. 2016). Briefly, rats were treated with metyrapone (50 mg/kg; Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) or vehicle (40% propylene glycol in buffered saline) via subcutaneous injection 1 h prior to exposure to either 0.8 ppm ozone or clean air for 4 h (nose only inhalation; n = 5/group). Exposure to 0.8 ppm ozone for 4 h represents a centriacinar dose of 3 to 4 fold higher than an estimated exposure scenario in humans (Thomson et al. 2005). An additional group of rats was administered exogenous corticosterone (CORT; 10 mg/kg; Sigma-Aldrich) 1 h prior to exposure to clean air. Either immediately (0 h) or 24 h following exposure, blood was collected in vacutainer tubes containing EDTA at 10 mg/ml and phenyl methyl sulfonyl fluoride at 1.7 mg/ml. Blood was centrifuged at 1448 x g for 10 min to separate plasma. Plasma samples were stored at – 80 ˚C. All experimental protocols for the ozone model were evaluated and reviewed by the Animal Care Committee of Health Canada.

4.4.2 TgF344-AD model

The TgF344-AD model, originally developed by R. M. Cohen et al., 2013, is a rat transgenic AD model which expresses mutant human amyloid precursor protein (APP_{SW}) and presenilin 1 (*PS1ΔE9*) on an F344 background. TgF344-AD rats accumulate cerebral Aβ in an age-dependent manner, which precedes tauopathy, cognitive decline, neuroinflammation and neuronal loss. In the present study, archived plasma samples were utilised from male TgF344-AD rats [transgenic (Tg; 2-8 month n = 4, 13-22 month n = 11) and non-transgenic (NonTg; 2-8

month *n* = 9, 13-22 month *n* = 2)]]. Animals were housed on a 12 h light/dark cycle with food and water provided ab libitum. All experimental protocols for the TgF344-AD model were approved by The Animal Care Committee of the Sunnybrook Health Sciences Center.

4.4.3 Lipid extraction

Lipid extractions were performed using 50 µL of plasma samples following a modified Bligh and Dyer Method (Bligh and Dyer 1959). Briefly, plasma samples were combined with 3.2 mL of 0.1M sodium acetate and 4 mL acidified methanol [2% acetic acid], along with the following lipid standards: 5000 ng PC(12:0/13:0) (Avanti LM1000), 2000 ng PE(12:0/13:0) (Avanti LM1100), 5000 ng PS(12:0/13:0) (Avanti LM1300), 10 µM PC(13:0/0:0) (Avanti LM1600), 10 µM Glucosyl(β) Ceramide(d18:1/8:0) (Avanti 860540), 10 µM Galactosyl(β) Ceramide(d18:1/8:0) (Avanti 860538), 5 µM C16-D31 Ceramide(d18:1/16:0) (Avanti 868516), 1.25 µg/mL SM(d18:1/18:1-d9) (Avanti 791649). 3.8 mL of chloroform was added, samples were incubated 15 min on ice, and then centrifuged at 600 x g for 5 min at 4 ˚C. The organic phase was then retained, and the aqueous phase was back-extracted with chloroform thrice. Chloroform was evaporated under nitrogen gas until dry. Lipid extracts were resuspended in 300 µL of absolute ethanol [100%] and were stored at – 80 ˚C.

4.4.4 Lipid quantification

LC was performed using an Agilent Infinity II High-Speed Binary LC Pump with an Agilent Infinity II system at a flow rate of 10 µl/min. The aqueous mobile phase (Solvent A) was 0.1% formic acid and 10 mM ammonium acetate. The organic mobile phase (Solvent B) was acetonitrile/isopropanol (5:2 v/v) with 0.1% formic acid and 10 mM ammonium acetate. 5 µl of sample in EtOH, 2.5 µl EtOH, and 16 µl Solvent A were mixed and loaded into the autosampler at 4°C. 5 µl of this mix was injected onto an in-house packed ReproSil-Pur 120 C8 capillary column (particle size of 3 µm and pore size of 120 Å, Dr. A. Maisch, Ammerbruch, Germany)

measuring 100 mm×250 μm (inner diameter). The LC gradient began at 30% Solvent B, ramped from 30% to 100% Solvent B over 5 min, was maintained at 100% Solvent B for 30 min, returned to 30% Solvent B over a 1 min period, and was re-equilibrated at 30% Solvent B for 10 min prior to the next sample or blank injection (45 min method). Each duty cycle was 0.85 s. Data acquisition was performed in positive ion MRM mode. Absolute quantities were determined based on standard curves using standards for each lipid species purchased from Avanti or customed synthesized with the exception of the hydroxylated species. Species with *N*-acyl chains of 22:0 or smaller were quantified using standard curves of 16:0(2S-OH) Ceramide. Longer hydroxylated *N*-acyl chains were quantified using 24:1(2S-OH) Ceramide. Hexosylceramides were quantified using matched b-Glucosylceramide standards. Lipid quantities were expressed as pmol/mL of plasma.

4.4.5 Lipid nomenclature

Lipid nomenclature used is according to Fahy et al., 2011, which describes the structure of individual species. For example, Cer(d18:1/16:0) describes a ceramide with a dihydroxy (d) 18-carbon sphingoid backbone with one degree of unsaturation (18:1) bonded to a 16-carbon fatty acid chain with 0 degrees of unsaturation (16:0). The term “Sph” refers to sphingosine, “S1P” refers to shingosine-1-phosphate, “Cer” refers to ceramides, “LacCer” refers to lactosylceramides, and “SM” refers to sphingomyelins. As glucosylceramides and galactosylceramides cannot be distinguished with the quantification methods used in this study, the term “HexCer” collectively refers to ceramide species that have either a glucosyl or galactosyl headgroup.

4.4.6 Statistical analysis and visualisation

Statistical analysis was conducted via Two-Way ANOVA (factors: *Ozone, Metyrapone*) followed by post-hoc Holm Sidak multiple comparisons procedure ($\alpha = 0.05$). Statistical analysis

and Pearson correlations were performed with Graphpad Prism v11.0.2 (GraphPad Software, San Diego, CA). Heatmaps were generated using the R package *pheatmap* (Kolde 2019) via R v.4.4.3 (R Core Team 2025).

4.5.0 Results

4.5.1 Ozone exposure alters circulating sphingolipid levels in glucocorticoid-dependent and independent manners

We identified and measured 79 sphingolipid species present in plasma samples from the ozone exposure model animals. Immediately post exposure, circulating levels of Cer(d18:1/16:0), Cer(d18:1/22:1), HexCer(d18:1/16:0), LacCer(d18:1/16:0), and SM(d18:1/16:0) were all decreased in animals exposed to ozone (*Ozone* main effect or *Ozone* × *Metyrapone* interaction, $p < 0.05$; Figure 4.1A). SM(d18:1/18:2(COOH)) was increased in the ozone-exposed group (*Ozone* main effect, $p < 0.05$). Circulating levels of these lipid species returned to control values after 24 h recovery period. However, numerous other lipid species were decreased in plasma of ozone exposed rats relative to those exposed to clean air 24 h post exposure (Figure 4.1B), including: Cer(18:1/26:0), HexCer(d18:1/14:0), HexCer(d18:1/18:0), HexCer(d18:1/21:0), HexCer(d18:1/22:0(OH)), HexCer(d18:1/22:0), HexCer(d18:1/23:0(OH)), HexCer(d18:1/23:0), HexCer(d18:1/24:1), HexCer(d18:1/26:1), HexCer(d18:2/24:1), LacCer(d18:1/24:0), and Sph(d17:1).

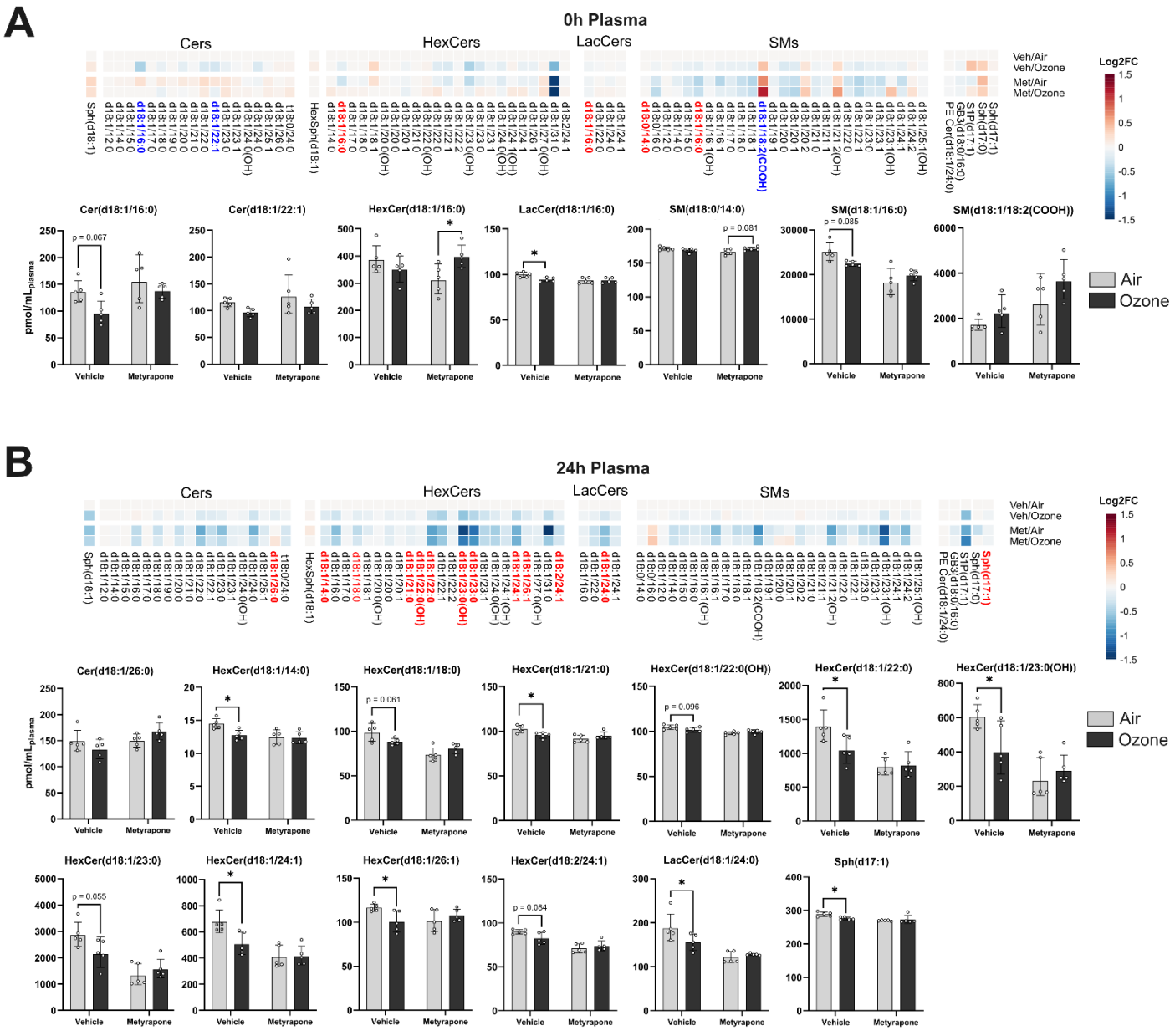


Figure 4.1. Impacts of ozone exposure on circulating sphingolipids. Male F344 rats were exposed to clean air or 0.8 ppm ozone for 4 hours, following administration of Met; 0 or 50 mg/kg]. Plasma was collected 0 h (A) or 24 h (B) following exposure, and lipids were quantified. Heatmaps represent log_2FC of lipid species relative to Veh/Air. Two-Way ANOVA (factors: *Ozone*, *Metyrapone*) with post-hoc Holm-Sidak was used for statistical analysis. Blue text represent species with a significant *Ozone* main effect, red text represents species with a significant interaction. Bar graphs represent geomean ± SD, *p < 0.05. n = 5/group. Cer: ceramide; HexCer: hexosylceramide; LacCer: lactosylceramide; SM: sphingomyelin; PE: phopshoethanolamine; S1P: shingosine-1-phosphate; Sph: shingosine; GB3: globotriaosylceramide; Veh: Vehicle; Met; metyrapone.

To determine whether ozone-induced effects on lipid metabolism were due to elevated GCs following exposure, we examined lipid profiles in rats administered metyrapone, a GC synthesis inhibitor. The ozone-induced effects observed 24 h post exposure were prevented in the metyrapone treatment group (*Ozone* x *Metyrapone* interaction, $p < 0.05$; Figure 4.1) suggesting GC-dependent regulation. As additional confirmation that ozone-induced regulation of these lipid species was due to elevated GCs, we compared lipid profiles of ozone exposed rats to an additional group of rats administered exogenous CORT (10 mg/kg). Sphingolipid levels in plasma collected from ozone-exposed rats 24 h post-exposure were significantly correlated (Pearson $r = 0.86$, $p < 0.0001$) with levels in plasma from rats administered exogenous CORT (Figure 4.2). No correlation was observed between the groups in plasma collected immediately post-exposure. Together, these data indicate that ozone modification of sphingolipid metabolism is primarily GC-dependent, which was especially evident in plasma collected 24 h post exposure.

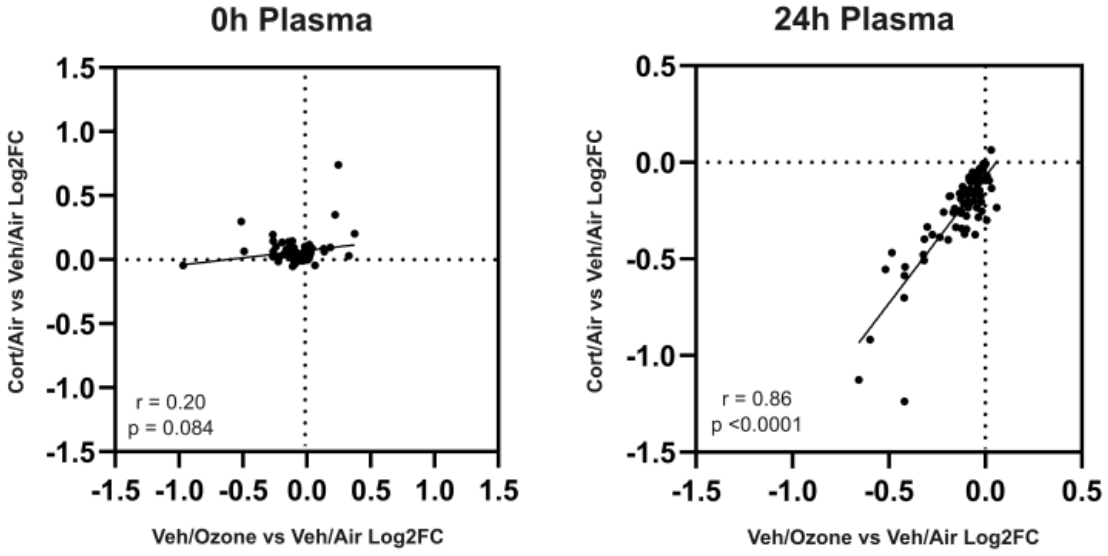


Figure 4.2. Correlation of exogenous corticosterone and ozone inhalation effects on plasma sphingolipids. Male F344 rats were exposed to clean or 0.8 ppm ozone for 4 hours. A group of rats exposed to clean air were administered corticosterone (10 mg/kg) 1 h prior to exposure. Plasma samples were collected 0 h or 24 h following exposure, and lipids were quantified. Pearson correlation of Log2FC between CORT/Air and Veh/Ozone, relative to Veh/Air, was performed. n = 5/group. Veh: vehicle; Cort: corticosterone.

4.5.2 Comparison of ozone-induced changes in sphingolipid metabolism to AD and aging phenotypes

To establish whether ozone-induced changes in lipid metabolism are similar to those observed in an AD model, we compared lipidomic profiles following ozone exposure to those observed in young (2 – 8 month) or old (13 – 22 month) male TgF344-AD rats relative to age-matched non-Tg control animals using Pearson correlation. We did not find any significant (all $p > 0.05$) correlation between lipid changes induced by ozone and those found in either young or old TgF344-AD rats (Figure 4.3). We also assessed whether ozone correlated with lipidomic changes associated with aging (Figure 4.4). Changes in circulating lipids 24 h post ozone exposure were negatively correlated with those found in old relative to young rats, both Tg (r = -

0.48, p = 0.0002) and Non-Tg (r = -0.47, p = 0.0005). Sphingolipid profiles immediately post exposure did not correlate with aging.

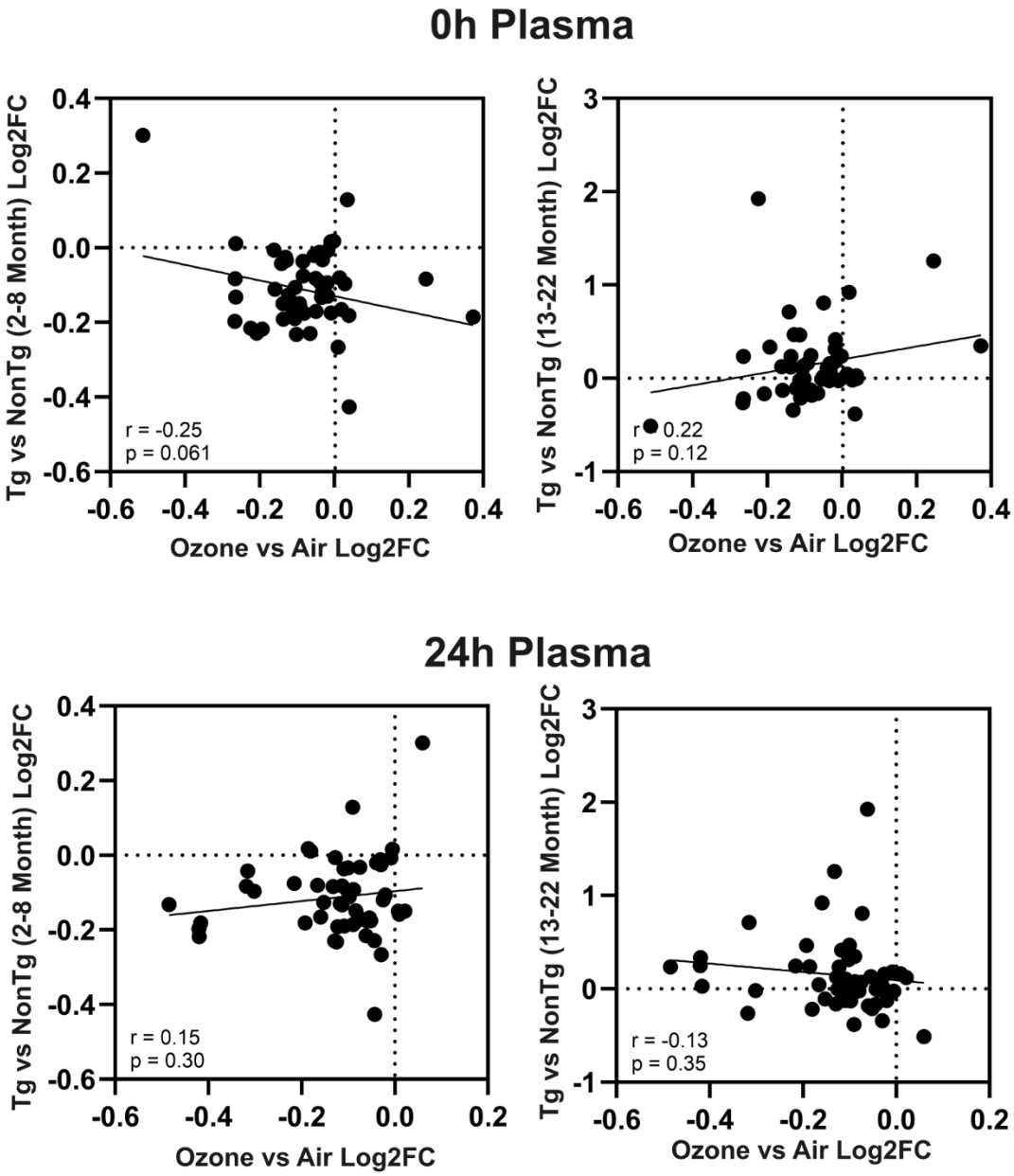


Figure 4.3 Correlations between ozone-induced and AD-induced changes in circulating sphingolipid profiles. Pearson correlation of circulating sphingolipid profiles between male F344 rats exposed to 0.8 ppm ozone (Log2FC relative to clean air) and male TgF344-AD rats (Log2FC relative to aged-matched nonTg controls). Plasma from ozone-exposed rats was collected 0 or 24 h post exposure. Tg: transgenic.

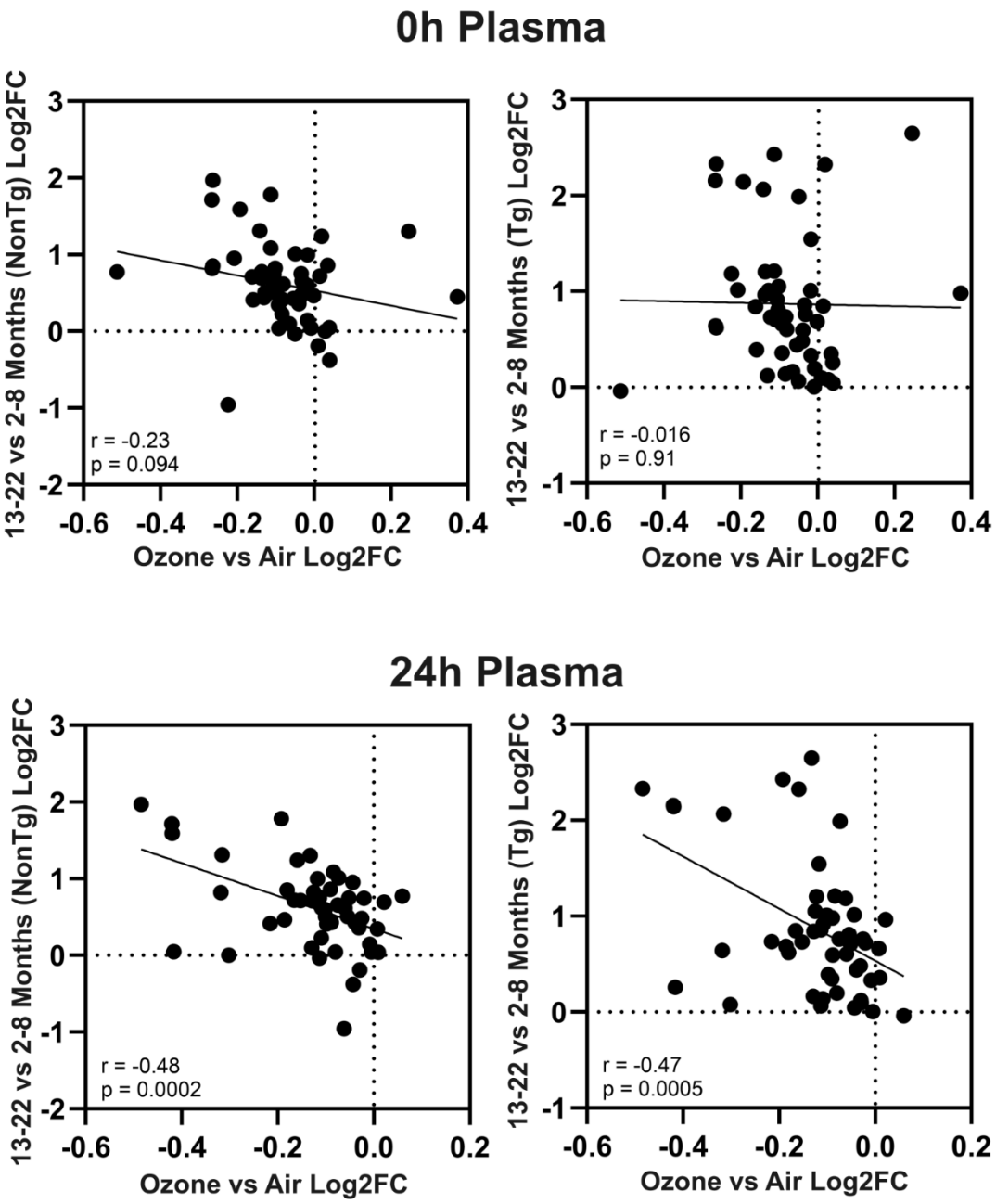


Figure 4.4 Correlations between ozone-induced and aging-induced changes in circulating sphingolipid profiles. Pearson correlation of circulating sphingolipid profiles between male F344 rats exposed to 0.8 ppm ozone (Log2FC relative to clean air) and 13-22 month old male rats (Log2FC relative to Tg-matched 2 – 8 month old rats). Plasma from ozone-exposed rats was collected 0 or 24 h post exposure. Tg: transgenic.

4.6 Discussion

Air pollution exposure is associated with elevated risk of neurodegenerative and metabolic diseases, of which dysregulation of sphingolipid metabolism is a common feature. To investigate whether air pollution exposure may modify sphingolipid metabolism as a potential disease-related pathway, we measured changes in circulating sphingolipid species and evaluated the role of the HPA axis as a potential mediator through pharmacological inhibition and exogenous CORT treatment. We then compared lipid profiles following exposure to those associated with an AD-phenotype and aging, in the TgF344-AD rat model. Here, we demonstrate that 1) acute ozone exposure modifies levels of circulating sphingolipids, 2) GCs mediate several of the ozone-induced changes on lipid metabolism, and 3) ozone-induced changes in sphingolipid levels negatively correlate with those associated with aging.

Acute ozone exposure generally reduced circulating levels of sphingolipid species, with decreases in hexosylceramides being particularly evident after a 24 h recovery period post exposure. The term hexosylceramide collectively refers to ceramides with either a single glucose (glucosylceramide) or galactose (galactosylceramide) headgroup. Modification of hexosylceramide metabolism is associated with neurodegenerative and metabolic conditions, although the pattern of effects seems to vary, compared to ceramides which are consistently elevated with disease state. Elevated hexosylceramide levels have been found in plasma of individuals with PD and brain samples of a PD mouse model (Franck et al. 2025), and mutation of the *GBA1* gene, which encodes an enzyme responsible for breakdown of glucosylceramides, is the most important genetic risk factor for PD (Migdalska-Richards and Schapira 2016). However, patients with frontaltemporal dementia are reported to have decreased hexosylceramide levels (Marian et al. 2025-6-04), hexosylceramides are downregulated in white matter of a transgenic AD mouse model (Li et al. 2026), and hexosylceramide levels negatively

correlate with indices of metabolic dysfunction including BMI and insulin resistance (Chew et al. 2019). Modification of hexosylceramide species may therefore be a mechanism through which air pollution exposure contributes to metabolic dysfunction and disease pathogenesis.

In contrast to the reduction in hexosylceramide species found in certain diseases, ceramides species are consistently increased with neurodegenerative (Wang and Bieberich 2018) and metabolic diseases (Chew et al. 2019; Berkowitz et al. 2024). In this acute exposure model, we found no evidence of increased ceramides in plasma samples. Rather, only three ceramide species were altered following ozone exposure, all of which were decreased. This is in contrast with population-based studies (Xu et al. 2022b; Zhu et al. 2023; Zhang et al. 2024a) and chronic exposure studies (Li et al. 2020a; Liu et al. 2022) that have generally shown elevated levels of ceramide and sphingomyelins with air pollution exposure, as well as select hexosylceramides species.

The ozone-induced effects on circulating sphingolipids, particularly after 24 h recovery, were largely mediated by GCs. Administration of metyrapone prevented several changes, and treatment with exogenous CORT reproduced ozone-induced effects observed following a 24 h recovery period. GCs are well characterized to induce *de novo* synthesis of ceramides (Holland et al. 2007), and indeed rats treated with exogenous corticosterone in this study prior to exposure to clean air have elevated levels of certain ceramide species immediately following exposure (Figure 4.S1). How precisely GCs may affect hexosylceramide metabolism however is unclear. One study found that treatment with dexamethasone (a GC receptor agonist) led to sustained reductions in hexosylceramides (Zhang et al. 2025), however little research has been conducted into the mechanisms through which GCs may impact hexosylceramide metabolism. It is possible that GCs may impact expression of enzymes associated with hexosylceramide synthesis,

although this would be expected to correspond to a subsequent increase in ceramides, hexosylceramide precursors, which was not the case. Alternatively, it is possible that trafficking of hexosylceramides may have been affected by GC-dependent pathways, through either reduced secretion from tissue into the bloodstream, or enhanced uptake of circulating lipids into tissues. The majority of ozone-induced effects on sphingolipid metabolism, particularly hexosylceramide changes, were observed only after a 24 h recovery period following exposure. These effects were therefore not a rapid response, as has been observed for gene expression changes and other metabolic effects in this model (Thomson et al. 2013, 2016; Rose et al. 2020, 2023), but may be a consequence of downstream metabolic changes, requiring coordination of several biological events.

While we found no correlation between ozone-induced changes in sphingolipids and those found in a transgenic AD model, ozone-induced changes 24 h post exposure did negatively correlate with those associated with aging, regardless of whether rats were transgenic or wild type.The immunosuppressive function of GCs may contribute to the lack of change in ceramide species and generally anti-aging phenotype we observed following acute ozone exposure, which aligns with a general lack of pro-inflammatory responses observed in this model (Thomson et al. 2016). Inflammation promotes ceramide synthesis and ceramides are commonly elevated during inflammatory insult (Gomez-Larrauri et al. 2025). Chronic low-grade inflammation is characteristic of aging; elevated levels of circulating inflammatory markers have been reported in older individuals (Franceschi et al. 2017). While acute exposure to ozone leads to a rise in GCs and anti-inflammatory phenotype (Thomson et al. 2016), sustained elevation of GCs can cause GC resistance, which corresponds to low grade chronic inflammation and HPA axis dysregulation (Stark et al. 2001; Cohen et al. 2012). As a result, chronic GC excess accelerates

aging-like phenotypes such as metabolic dysfunction, chronic inflammation, and cellular senescence (Lambertucci et al. 2026). Therefore, while our data show that the rise in GCs following acute ozone exposure in young rats generally led to modifications of sphingolipid metabolism that are opposite to those characteristic of aging, chronic exposure may culminate in a general proinflammatory/aging phenotype as has been noted by others.

Several strengths and limitations should be considered in the interpretation of this work. Metyrapone, the GC synthesis inhibitor employed in this study, has ozone-independent effects which could complicate interpretation of GC dependency. However, comparison of ozone-induced effects to an additional group of rats treated with exogenous CORT prior to clean air confirmed the role of GCs in mediating ozone-induced effects. We only examined effects of ozone on lipid species in plasma. While plasma level changes in lipids are frequently detected in several diseases and may serve as potential disease biomarkers, they do not necessarily represent levels of lipids in metabolic tissues. Further inquiry into sphingolipid changes in tissues of interest following ozone exposure would be of interest. Examination of lipid responses over several time points in this study allowed us to identify the temporal nature of responses. However, effects that may occur between the 0 and 24 h time point were not captured and could potentially be of interest. Only male rats were considered in this study, and so we were unable to examine whether sex differences in response to ozone may occur. Finally, as our interest was to identify early effects of pollutants, we utilised an acute exposure model in this study so that interpretation of results would not be complicated by subsequent disease-related changes in a chronic exposure model.

In conclusion, this study establishes that ozone exposure modifies circulating sphingolipid metabolism, and provides evidence supporting GCs as central mediators of these

effects. This provides insight into the relationship between air pollution and disease, adding to the existing literature implicating sphingolipid metabolism as a potential pathway through which air pollution alters metabolic function and confers risk of neurodegenerative and metabolic diseases. As the population health burden of these diseases is significant, and prevalence is projected to rise, understanding how environmental risk factors like air pollution affect health outcomes is increasingly important to improving health.

4.7 Acknowledgments

This project was funded by Health Canada through the Air Quality Program.

4.8 Supplemental Figures

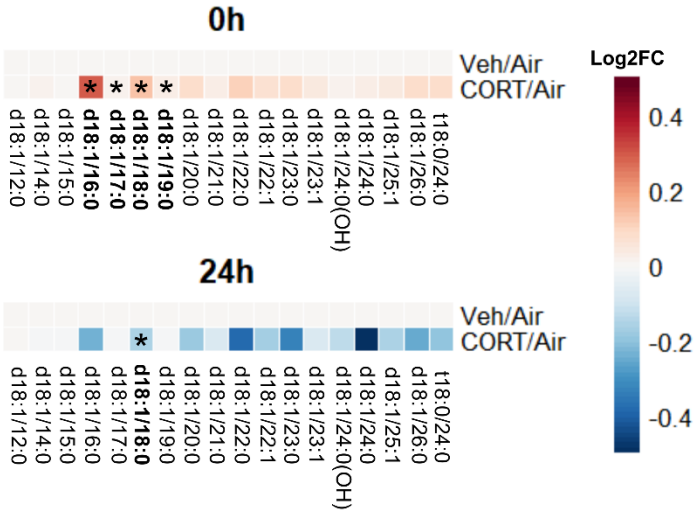


Figure 4.S1. Effects of exogenous corticosterone administration on circulating ceramide levels. Male F344 rats were administered corticosterone [0 or 10 mg/kg] 1 h prior to exposure to clean air for 4 h. Plasma samples were collected immediately (0 h) or 24 h post exposure. Heatmaps represent Log2FC of circulating ceramide species relative to Veh/Air. Statistical analysis conducted via T-test, * $p < 0.05$. n = 5 / group. Veh: vehicle; CORT: corticosterone.

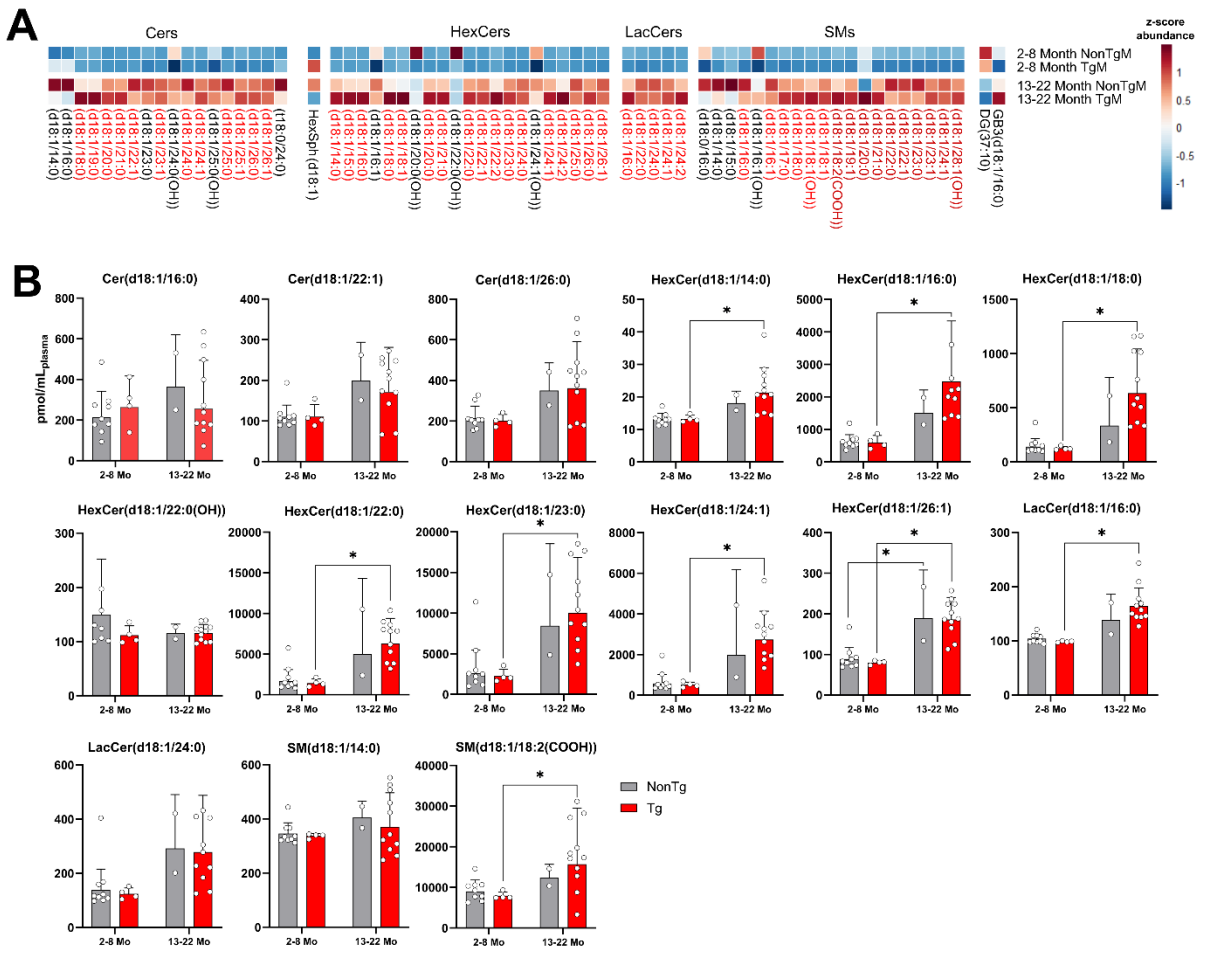


Figure 4.S2. Lipid abundances in TgF344-AD rats. A) Heatmap of z-score abundance for plasma lipid species detected in male 2-8 or 13-22 month Tg or NonTg rats. B) Bargraphs of plasma lipid abundances in male TgF344-AD rats, in select lipid species which were effected by ozone. Graphs depict geomean ± SD. Statistical analyses was conducted via Two-Way ANOVA (factors: *Age, Genotype*) with post-hoc Holm-Sidak for multiple comparisons. Red text indicates significant ($p < 0.05$) *Age* main effect. No significant *Genotype* or *Age* x *Genotype* interactions were observed. *$p < 0.05$.

Chapter 5: An *ex vivo* model of systemically-mediated effects of ozone inhalation on the brain

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5.1 Authorship Contributions

The study described in the present manuscript was designed by MR and EMT. All cell culture work, *ex vivo* exposures, RNA isolation, cDNA synthesis, qPCR and statistical analysis was conducted by MR. MR wrote the initial draft of the manuscript, and review and editing was performed by EMT.

5.2 Abstract

Air pollution is associated with increased risk of neurodegenerative and neuropsychiatric conditions. While animal models have increased our understanding of how air pollution contributes to brain pathologies – including through oxidative stress, inflammatory, and stress hormone pathways – investigation of underlying mechanisms remains limited due to a lack of human-relevant models that incorporate systemic processes. Our objective was to establish an *ex vivo* approach that enables assessment of the roles of plasma mediators in mediating pollutant-induced effects in the brain. As a proof-of-concept for application in the human context, we assessed whether such effects reproduced *in vivo* responses to pollutant exposure. Primary rat hippocampal neurons and microglia were each treated with plasma collected from rats immediately or 24 h after ozone inhalation (0 or 0.8 ppm) ± pre-treatment with the glucocorticoid (GC) synthesis inhibitor metyrapone. Microglia were further challenged with lipopolysaccharide (LPS) to evaluate modification of inflammatory responses. Plasma from the ozone-exposed group produced transcriptional changes (inflammatory, antioxidant, GC-responsive) in neurons, some of which were GC-dependent. *Ex vivo* and hippocampal responses were strongly correlated, establishing the *in vivo* relevance of the model. Plasma from the ozone-exposed group modified inflammatory responses to LPS challenge in microglia, demonstrating the model's utility to assess functional changes resulting from pollutant exposure. This study establishes that an *ex vivo* approach can reproduce ozone-induced effects in the brain. The model was sensitive to specific plasma mediators and temporal effects, and enabled assessment of functional responses. This approach may serve to investigate mechanisms underlying effects of pollutants on the human brain.

5.3 Introduction

Air pollution imposes a significant public health burden as the greatest environmental risk factor to mortality worldwide, estimated to contribute to millions of premature deaths annually (Fuller et al. 2022). The contribution of air pollution to respiratory and cardiovascular diseases is widely recognized and used as the basis of air pollution health estimates. In addition, the growing epidemiological literature linking exposure to air pollution and central nervous system (CNS) disorders – including neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (Xu et al. 2016; Power et al. 2016), neuropsychiatric conditions such as depression and anxiety (Zundel et al. 2022), and neurodevelopmental outcomes (Xu et al. 2016; Herting et al. 2024) – supports the notion that health impacts of air pollutants may be substantially greater than previously estimated. Moreover, with an aging population, growing prevalence of depression and dementia (Nichols et al. 2022), and ubiquitous exposure to pollutants, even small pollutant-dependent changes in relative risk carry significant public health implications. Accordingly, there is significant interest in gaining a better understanding of how exposure to air pollution impacts brain health, both to support the biological plausibility of epidemiological observations, and to gain insight into factors that may influence susceptibility.

Toxicological studies support a causal relationship between air pollution exposure and impacts on brain health. For instance, exposure to pollutants such as particulate matter (PM) and ozone have been shown to produce cognitive impairments such as impaired spatial learning (Fonken et al. 2011) and memory retention (Dorado-Martínez et al. 2001; Rivas-Arancibia et al. 2009) as well as depressive and anxiety like behaviours (Fonken et al. 2011; Mokoena et al. 2015; Ehsanifar et al. 2023) in rodents. Pathological effects include increased neuroinflammation, as revealed by elevated cytokine expression (Fonken et al. 2011; Hajipour et

al. 2020; Ehsanifar et al. 2023) and activation of microglia (Mumaw et al. 2016; Woodward et al. 2018; Ehsanifar et al. 2023), decreased neurogenesis (Woodward et al. 2018), and impacts on neurotransmitter systems (Mokoena et al. 2015; Rose et al. 2020).

Despite compelling evidence from epidemiological and rodent-based studies, several knowledge gaps remain as to how air pollutant exposure leads to adverse brain health outcomes, particularly within the human population. There are multiple routes through which air pollution may impact the brain; these include translocation of fine particles (Oberdörster et al. 2008; Kreyling 2016; Qi et al. 2022), nervous system activation (Gackière et al. 2011), and release of bioactive factors into circulation from peripheral organs (Miller et al. 2016a; Pope et al. 2016; Thomson et al. 2019; Rose et al. 2020). While important intervention experiments have shown involvement of specific mediators or processes (such as those cited above), it remains challenging to disentangle their relative contribution. The understanding of how such mechanisms intersect to cause effects in humans in particular is limited due to the challenges of effectively reproducing the human exposure context in the laboratory. *In vitro* studies have been employed to investigate the direct effect of PM on human brain cell models; for example, $PM_{2.5}$ treatment decreases cell viability, increases inflammatory responses and oxidative stress, and impacts energy metabolism and mitochondrial bioenergetics in the human neuroblastoma cell line SH-SY5Y (Wei et al. 2017; Lin et al. 2022; Li et al. 2022). However such studies are unable to capture the contribution of systemic mechanisms that may be significant contributors to disease development. The heterogeneity of the human population – and interindividual differences in response to pollutant exposure – is also an important consideration that remains understudied due to poor representation in animal exposures. As a result, while there have been

important advances in our understanding of mechanisms and susceptibility, there is uncertainty in the translation of results from rodent studies to the human context.

To address these challenges, we hypothesise that exposure of brain cells to plasma from pollutant-exposed individuals will provide a means to model pollutant effects in the human brain, and enable assessment of hazard, mechanism, and susceptibility in human-relevant models. Because it transports both bioactive factors (including hormones, oxidative stress products, inflammatory signals, etc.) generated following pollution exposure, as well as fine particles themselves, the circulatory system is an important route linking peripheral and CNS effects of pollutants (Thomson 2019). For instance, we have previously demonstrated through pharmacological inhibition that increases in circulating glucocorticoids (GCs) mediate ozone-induced effects on gene expression within multiple brain regions (Thomson et al. 2019), including effects on neurotrophic and serotonin systems in the hippocampus (Rose et al. 2020). Dysregulated GC levels are a hallmark of multiple neurological diseases, including depression, anxiety and dementia (Lucassen et al. 2014; Ouanes and Popp 2019). Given the relative accessibility of plasma and its importance in transporting peripheral factors to the brain, development of an *ex vivo* model that uses plasma samples to investigate effects in brain cells could be a valuable tool.

To establish the utility of such a model, it is first necessary to determine whether *ex vivo* responses to plasma from pollutant-exposed individuals could recapitulate responses in the brain following exposure. To do so, we leveraged samples from a rodent ozone inhalation study in which both brain and plasma samples were collected (Thomson et al. 2016; Rose et al. 2020). Ozone was intentionally selected as a model pollutant as it is entirely consumed in the lungs due to its reactive nature. Systemic effects of ozone exposure must occur through secondary

mediators, in contrast with ultrafine particles and metals which could produce effects in the brain through both direct and indirect mechanisms. Therefore, ozone serves as a useful model to assess the role of systemic mediators in driving pollutant effects on the brain. In particular we focus on transcriptional responses, as perturbation of gene expression offers an early, sensitive, and responsive measure that can be used to track the dynamics of response following short-term exposure to pollutants (Thomson et al. 2013; Joseph 2017). Our objectives were to 1) assess whether responses in brain cell cultures (primary rat microglia and hippocampal neurons) are sensitive to circulating mediators of pollutant effects such as GCs; 2) compare pollutant-induced transcriptional responses in the *ex vivo* model (primary hippocampal neurons) to those observed *in vivo* (hippocampal tissue); and 3) apply the *ex vivo* approach to microglia cells to evaluate utility of this model for the study of pollutant effects in brain cells beyond neurons and for assessing modification of functional responses.

5.4 Methods

5.4.1 *In vivo* exposures and sample collection

Archived samples of plasma and hippocampal tissue from a previous inhalation study (Thomson et al. 2016) were used. All experimental protocols were reviewed and approved by the Animal Care Committee of Health Canada, and are described in detail in Thomson et al., 2016. Briefly, male Fischer-344 rats between 200-250 g (specific pathogen free; Charles River, St. Constant, Quebec, Canada) were kept on a 12 h light/dark cycle, with food and water provided ad libitum. Animals were housed in individual plexiglass cages with HEPA-filtration and wood-chip bedding. Rats were treated with metyrapone (50 mg/kg; Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) or vehicle (40% propylene glycol in buffered saline) via subcutaneous injection 1 h prior to exposure to either clean air or 0.8 ppm ozone for 4 h (nose-only inhalation; n = 5/group). A 4 h exposure to 0.8 ppm ozone in rats represents a centriacinar dose 3-4 fold higher than a relevant single day exposure in humans (Thomson et al. 2005). At this dose, slight decreases in cells recovered by bronchoalveolar lavage, and some cytokine and anti-oxidant transcriptional responses, are observed, with most effects returning to baseline by 24 h post-exposure (Thomson et al. 2013, 2016). An additional group of rats were treated with corticosterone (CORT; 10 mg/kg in the same vehicle; Sigma-Aldrich) 1 h prior to exposure to clean air (n = 5). Sample size was selected on the basis of prior studies using a similiar exposure paradigm (Thomson et al. 2013). Animals were randomly assigned to treatment groups, and there were no exclusions. Researchers were not blind to group allocation. Rats were anesthetised with isoflurane (5% at 1.5 L of O2/min) and euthanised via exsanguination 0 or 24 h post exposure. Blood was collected from the abdominal aorta in vacutainer tubes containing EDTA at 10 mg/mL and phenyl methyl sulfonyl fluoride at 1.7 mg/mL. Plasma was isolated by centrifugation (1448 x g for 10 min), aliquoted and frozen at -80 ºC. Plasma samples were thawed, pooled based on

treatment group and time of plasma collection (n = 4 or 5/group), and re-frozen. Brain tissue was dissected and the hippocampus was collected and added to RNAlater (Sigma-Aldrich), before being snap-frozen in liquid nitrogen and stored at -80 ºC.

5.4.2 Primary rat hippocampal neuron cell culture

Primary rat hippocampal neurons (QBM Cell Science, Ottawa ,Ontario, Canada; Catalogue #R-Hi-501) were seeded on 96-well plates coated with poly-D lysine (PDL; 30 µg/mL, Gibco, Burlington, Ontario, Canada) and laminin (2 µg/mL, Sigma-Aldrich) at a density of 40,000 cells/well in Neurobasal Plus Media (Gibco) supplemented with B27 supplement (2%, Gibco), GlutaMAX (0.25%, Gibco) and Fetal Bovine Serum (FBS; 5%, Cytiva, Vancouver, British Columbia, Canada). Cells were incubated at 37 ºC + 5% CO_2 for 4 h, after which 50% of the media was removed and replaced with Neurobasal Plus Media + 2% B27 supplement + 0.25% GlutaMAX. Media was replaced again at day in vitro 5 (DIV5). At DIV7, 80% of the media was replaced with media containing pooled plasma samples [1, 5, 10% final concentration] for 4 or 24 h. Plasma-containing media was filtered using 0.2 µm nylon syringe filters (Corning, Tewksbury, Massachusetts). A subset of cells were treated with CORT [0, 0.1, 200 µM; Sigma-Aldrich] in 0.1% dimethyl sulfoxide (Sigma-Aldrich) for 4 or 24 h. Media-only controls (Neurobasal Plus Media + 2% B27 supplement + 0.25% GlutaMAX) were also included, as well as no-cell controls. At each time point, cell supernatant was collected and frozen at -80 ºC, and cells were lysed with lysis buffer (RNeasy Microkit, Qiagen, Toronto, Ontario, Canada) + 1% β-mercaptoethanol (Sigma-Aldrich). Lysates from two replicate wells per independent experiment were combined and frozen at -80 ºC. Four independent experiments were conducted.

4.5.3 Primary rat microglia cell culture

Primary rat microglia cultures (QBM Cell Science; Catalogue #R-G-535) were seeded on PDL-coated (50 µg/mL) 96-well plates at a seeding density of 50,000 cells/well in Dulbecco's Modified Eagle's Medium (DMEM; Cytiva) + 10% FBS + 1% D-(+) glucose and incubated at 37 ºC + 5% CO2 for 24 h. 50% of media was replaced with DMEM + 1% FBS + 1% D-(+)-glucose solution (Sigma-Aldrich) and left to incubate overnight. 80% of media was then removed and replaced with DMEM + 1% D-(+)-glucose solution containing pooled plasma samples [5% final concentration]. Plasma-containing media was filtered using 0.2 µm nylon syringe filters (Corning). After 24 h incubation, 80% media was replaced with media containing plasma samples (matching treatment groups) and lipopolysaccharide (LPS; 0 or 0.5 ng/mL; Sigma Aldrich; Catalogue #L2262) for 4 or 24 h. A subset of cells were treated with CORT [0, 0.1, 200 µM; Sigma-Aldrich] in 0.1% dimethyl sulfoxide (Sigma-Aldrich) with LPS (0 or 0.5 ng/mL) for 4 or 24 h. Media-only controls (DMEM+ 1% FBS + 1% D-(+)-glucose) and no-cell controls were also included. At each time point, cell supernatant was removed and frozen at -80 ºC and cells were lysed with lysis buffer + 1% β-mercaptoethanol (Sigma-Aldrich). Lysates from two replicate wells were combined per independent experiment and frozen at -80 ºC. Four independent experiments were conducted.

5.4.4 RNA extraction and cDNA synthesis

RNA was extracted from cell lysates using the RNeasy Microkit (Qiagen) according to manufacturer's protocol. RNA quality was assessed using the Bioanalyzer 6000 Nano kit (Agilent, Mississauga, Ontario, Canada) for RNA obtained from neurons, and 6000 Pico kit (Agilent) for RNA obtained from microglia. All RIN numbers were > 6 with majority between 8-10. RNA from neuron samples was quantified using RiboGreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon). RNA from microglia was not quantified as RNA

quantity (in comparable samples) was too low to evaluate via the RiboGreen kit while preserving enough RNA for downstream cDNA synthesis. cDNA synthesis (100-150 ng RNA per 20 µL reaction for neurons, 13.2 µL total volume for microglia) was performed using MuLV reverse transcriptase and random hexamers (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Mississauga, Ontario, Canada). cDNA was diluted 10X in Dnase-free H2O following synthesis.

5.4.5 qPCR

Primers with an optimal annealing temperature of 60 ºC were designed using Roche Universal Probe Library or Primer-BLAST. Primer sequences can be found Appendix A. Duplicate reactions containing BLAST-Taq 2X qPCR MasterMix (Applied Biological Materials, Richmond, British Columbia, Canada), primers (200 nM; Life Technologies, Burlington, Ontario, Canada) and cDNA were incubated (10 µL/well; 384 well plate) in a spectro-fluorometric thermal cycler (Lightcycler 480, Roche Diagnostics, Laval, Quebec, Canada). The following PCR cycle was utilised: 95 ºC for 3 min enzyme activation and denaturation, followed by 40 cycles of 95 ºC for 15 seconds to allow for denaturation and 60 ºC for 1 min each for annealing/extension. Expression calculations were conducted relative to PPIA (verified to be invariant with treatment) using the delta-delta Ct method (Livak and Schmittgen 2001).

5.4.6 Lactate dehydrogenase Assay

Lactate dehydrogenase (LDH) release was assessed on cell culture supernatant (50 µL combined from two replicate wells, never frozen) using the Cytotox 96 Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin) according to manufacturer protocol. Absorbance was read at 492 nm. Absorbance values were normalized to no-cell controls.

5.4.7 Statistical analyses and data visualisation

Two-way or three-way ANOVAs with post-hoc Holm-Sidak tests ($\alpha = 0.05$) were used to statistically analyze results. Data were assessed for normality and equal variance, and transformed according to 1og10 or rank as required to meet underlying assumptions. Factors considered in the ANOVA are specified in corresponding figure legends. Statistical analyses were performed, and bar graphs and correlation plots were created, using Graphpad Prism 10.2.3 (Graphpad Software, Boston, Massachusetts). Heatmaps were generated using the R package *pheatmap* (Kolde 2019). Bar graphs represent geometric mean ± geometric standard deviation.

5.5 Results

5.5.1 Plasma-relevant concentrations of corticosterone modify gene expression in primary hippocampal neurons and microglia

To first establish whether the model could respond to circulating factors of interest, we treated primary hippocampal neurons (Figure 5.1) and primary microglia (Figure 5.2) with CORT at both a low dose [0.1 μM] similar to the amount of CORT that would be present if we treated cells with plasma from ozone-exposed rats (Thomson et al. 2016), and a higher dose [200 μM].

In primary hippocampal neurons, glucocorticoid responsive genes *GILZ* (glucocorticoid-inducible leucine zipper) and *SGK1* (serum and glucocorticoid inducible kinase) both responded to CORT treatment in a dose dependent manner, as did cellular stress genes *HIF3α* (hypoxia inducible factor 3α), and *MT1* and *2* (metallothionein 1 and 2) (Figure 5.1). CORT inhibited expression of pro-inflammatory genes *TNFα* (tumour necrosis factor α) and *IL-1β* (interleukin 1β) even at the lowest dose, as expected given the anti-inflammatory nature of corticosteroids (Figure 5.1).

Primary microglia were additionally treated with LPS (0, 0.5 ng/mL) alongside CORT to evaluate responses to CORT in the context of a pro-inflammatory stimulus. CORT increased expression of *GILZ* and *MT1* and inhibited expression of pro-inflammatory genes *TNFα*, *IL-1β*, and *iNOS* (inducible nitric oxide synthase) in dose-dependent manners (Figure 5.2). The magnitude of LPS response tended to be modified by CORT as well (*LPS* x *CORT* interaction, $p < 0.05$), with the exception of iNOS which could not be detected in the absence of LPS, but the general pattern of responses were consistent whether or not LPS was present.

Together, these data indicates that primary hippocampal neurons and primary microglia are sensitive to doses of CORT relevant to levels observed *in vivo* after ozone inhalation.

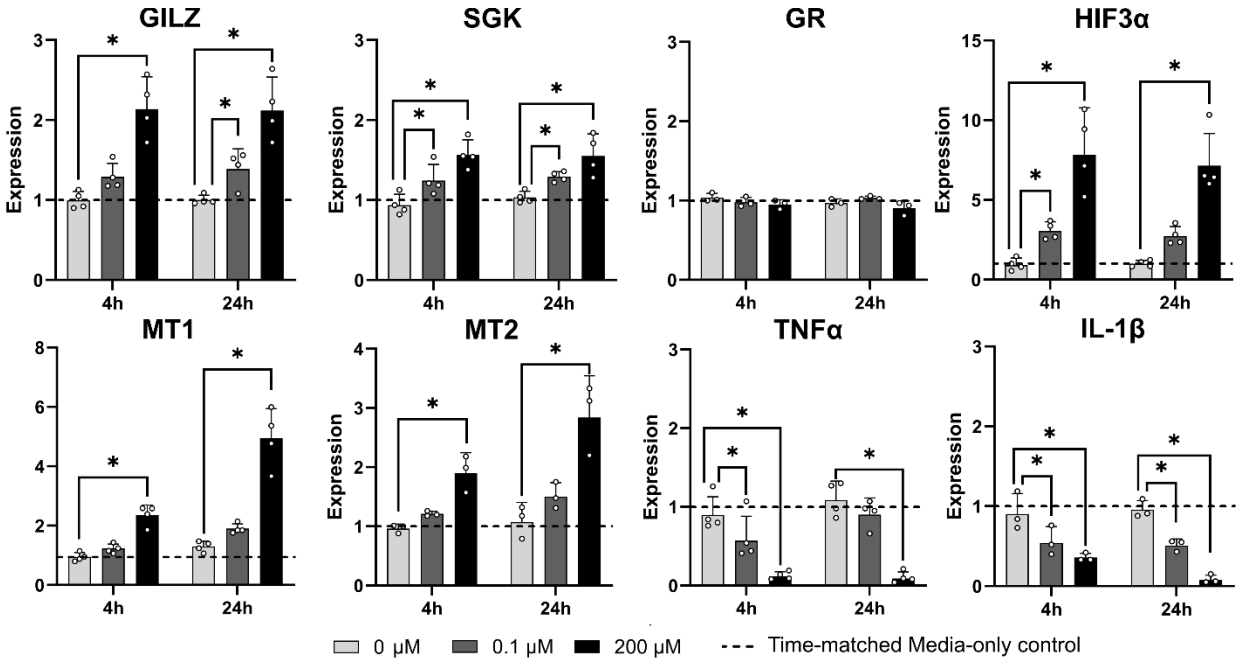


Figure 5.1 Effects of corticosterone on neuronal gene expression. Primary rat hippocampal neurons were treated with corticosterone [0, 0.1 and 200 µM] for 4 or 24 h. mRNA expression was measured with RT-qPCR. Expression is relative to time-matched media only controls (dotted line). Statistical analysis conducted via Two-Way ANOVA (factors: *Corticosterone, Time*) with post-hoc Holm-Sidak. *$p < 0.05$, $n = 4$. Bars represent geomean ± GSD

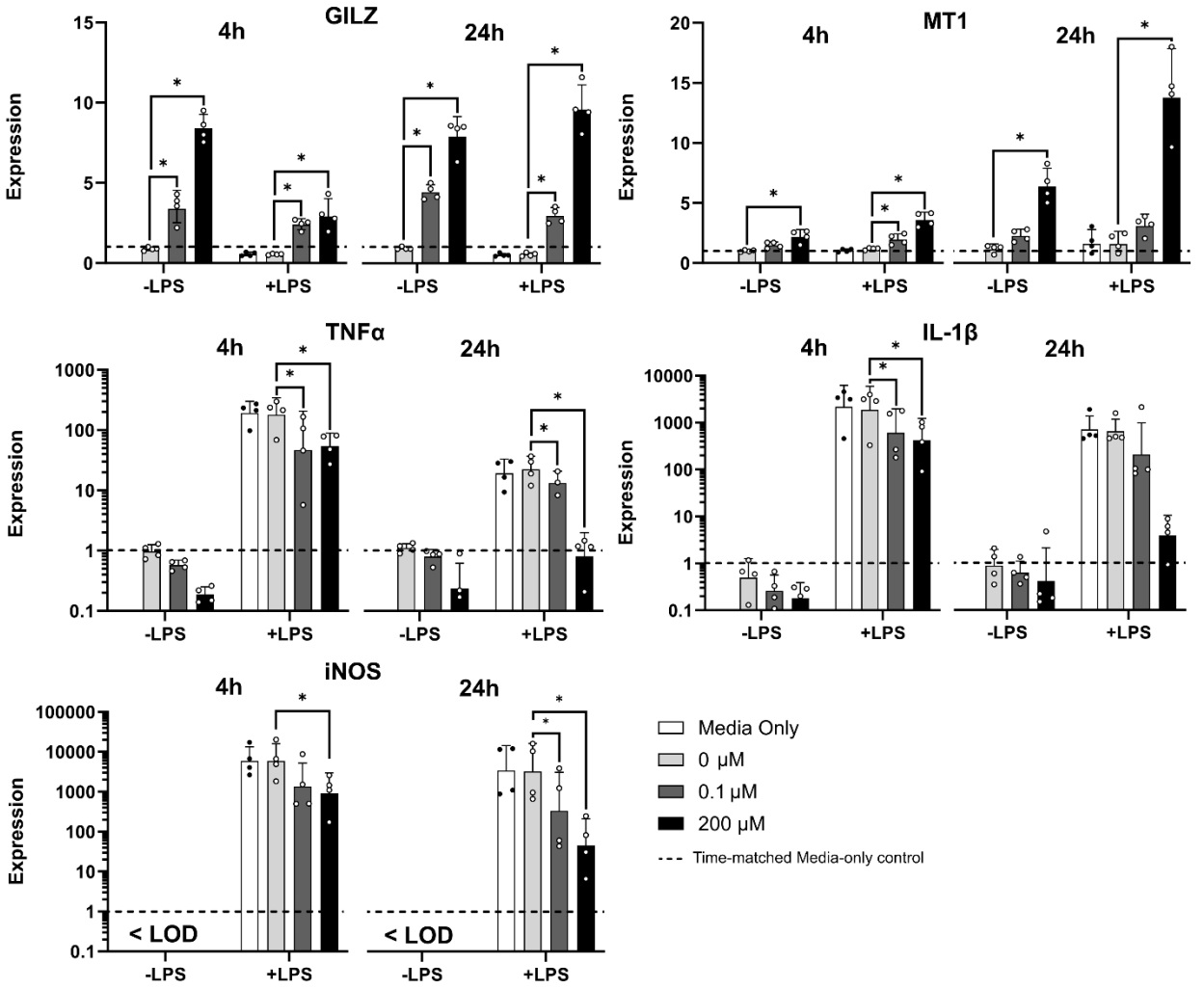


Figure 5.2 Effects of corticosterone on microglial gene expression. Primary rat microglia were treated with corticosterone [0, 0.1 and 200 μM] for 4 or 24 h alongside lipopolysaccharide (LPS; 0, 0.5 ng/mL). mRNA expression was measured with RT-qPCR. Expression is relative to time-matched media only controls (dotted line). For iNOS, expression in LPS-treated cells normalized to Cp value cut off. Statistical analysis conducted via Two-Way ANOVA (factors: *Corticosterone, LPS*) with post-hoc Holm-Sidak for each time point. *$p < 0.05$, n = 4. LOD; level of detection. Bars represent geomean ± GSD

5.5.2 Plasma from ozone-exposed rats alters hippocampal neuronal gene expression through glucocorticoid-dependent actions

The hippocampus is a particularly stress-sensitive region of the brain that is critical for long-term memory formation, and is a key brain region affected in neurodegenerative and neuropsychiatric diseases (Anand and Dhikav 2012). Given that we have previously demonstrated that ozone-exposure leads to transcriptional changes in the hippocampus in both GC-dependent and independent manners (Rose et al. 2020), we sought to isolate the impact of pollutant-induced changes in circulating factors on hippocampal neurons. Plasma collected from ozone-exposed rats immediately after exposure (0 h) significantly elevated expression of *GILZ*, *SGK*, *HIF3α*, *MT1* and *MT2* in primary rat hippocampal neurons compared to treatment with plasma from clean air-exposed rats (Figure 5.3a). *Ex vivo* ozone-induced effects were similar at 5 and 10% plasma concentrations with significant effects occurring at 5%, but were not observed at a concentration of 1% (Supplementary Figure 5.S1). Gene expression in neurons treated with plasma collected 24 h post-ozone exposure trended in the opposite direction to those observed in neurons treated with 0 h plasma (not statistically significant). *Ex vivo* ozone-induced effects on gene expression occurred in the absence of cytotoxicity, as there was no difference in LDH release between any plasma treatment groups (Figure 5.3c).

To determine whether the *ex vivo* ozone-induced effects were dependent on GCs, we examined *ex vivo* gene expression patterns in neurons treated with plasma from rats administered metyrapone, a GC synthesis inhibitor, prior to air or ozone exposure. The ozone-induced effects on neuronal expression of *SGK1*, *HIF3α*, *MT1* and *MT2 ex vivo* were prevented in the metyrapone treatment group, consistent with expression being GC-dependent (Figure 5.3b). Additionally, the temporal patterns in gene expression observed were consistent with the changes in plasma CORT levels, which were elevated in 0 h plasma and slightly decreased in 24 h plasma

(Supplementary Figure 5.S2). *Ex vivo* ozone effects also significantly correlated with responses in cells treated with plasma from rats administered CORT 1 h prior to exposure to clean air ($r = 0.94$, $p = 0.02$; Fig 5.3d), providing further support that effects were specific to CORT.

5.5.3 *Ex vivo* ozone-induced responses in neurons reproduce *in vivo* hippocampal effects

To assess whether responses observed in the *ex vivo* neuron model are representative of *in vivo* exposures, we compared *ex vivo* responses to those observed in whole hippocampal tissue from ozone-exposed rats (Figure 5.4). *Ex vivo* responses were significantly correlated with *in vivo* effects ($r = 0.83$, $p = 0.0002$).

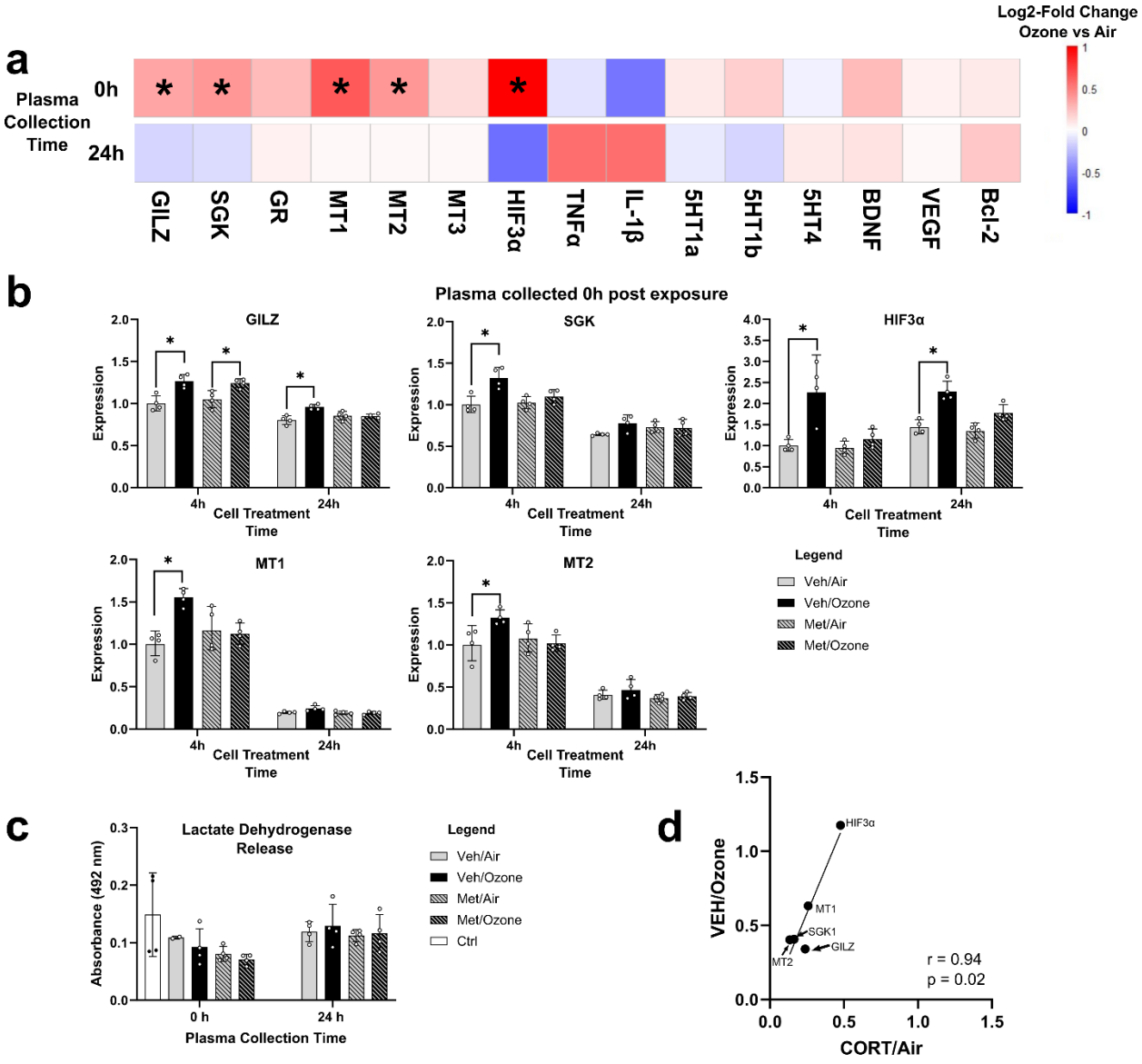


Figure 5.3 Neuronal gene expression responses to plasma from ozone-exposed rats. Primary rat hippocampal neurons were treated with pooled plasma [n = 5, 5%] from rats administered metyrapone (Veh = 0 mg/kg, Met = 50 mg/kg) prior to exposure to ozone (0.8 ppm) or clean air. a) Gene expression heat map representing Log2-Fold Changes of Veh/Ozone relative to Veh/Air for neurons treated for 4 h with plasma collected immediately (0 h) or 24 h post-exposure. b) Gene expression relative to Veh/Air (time matched) and normalized to repeat average. c) Lactate dehydrogenase release in neurons treated with plasma samples for 24 h. d) Pearson correlation between primary hippocampal neurons treated for 4 h with plasma (0 h) from ozone-exposed animals (Veh/Ozone) or animals administered corticosterone prior to exposure to clean air (CORT/Air), axes represent Log2-Fold Changes relative to Veh/Air. Statistical analysis (a,b,c) conducted with Three-Way ANOVA (factors: *Ozone, Metyrapone, Time*) with post-hoc Holm Sidak. * $p < 0.05$, n = 4. Bars represent geomean ± GSD.

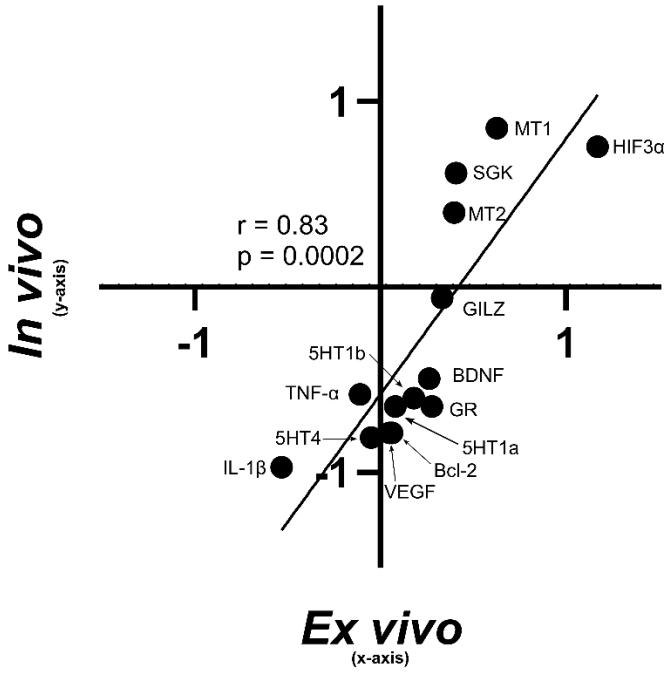


Figure 5.4 Correlation of ex vivo and in vivo ozone-induced effects. Log2-Fold Changes of Veh/Ozone relative to Veh/Air responses from the *ex vivo* model (primary rat hippocampal neurons treated with pooled plasma [5%] for 4 h) were compared to gene expression results in whole hippocampal tissue (*in* vivo) using Pearson correlation. Plasma samples used and whole hippocampal tissue collected immediately post-exposure. r = 0.83, p = 0.0002. n = 4 for *ex vivo,* n = 5 for *in vivo*.

5.5.4 Plasma from ozone-exposed rats alters microglial inflammatory responses

Given that we established that ozone-induced circulating factors, including GCs, modify responses in neurons, we examined whether such circulating factors may also modify functional responses in other brain cell types relevant to disease. Microglia are the resident innate immune cells in the brain, and dysregulation of inflammatory responses of these cells are linked to development of neurodegenerative conditions (Bachiller et al. 2018).

GILZ expression was increased in primary microglia treated with plasma collected from ozone-exposed rats immediately post exposure relative to plasma from air-exposed rats (4 h: *Ozone* main effect $p = 0.0004$, 24 h: *Ozone* main effect $p < 0.0001$; Figure 5.5a). Conversely *ex vivo* ozone exposure with 24 h plasma decreased GILZ expression (4 h: *LPS* x *Ozone* interaction $p = 0.02$, 24 h: *Ozone* main effect $p < 0.0001$; Figure 5.5b). No *ex vivo* effects were observed for *MT1* or *MT2*.

Treatment of cells with a low dose of LPS (0.5 ng/mL) substantially elevated expression of inflammatory cytokines *IL-1β* and *TNFα* (*LPS* main effect $p < 0.05$ for each), as well as iNOS which could not be detected in the absence of LPS (Figure 5.6a). In general, plasma (both 0 h and 24 h) inhibited the LPS-induced increase of these genes relative to media-only controls.

Plasma (0 h) from ozone-exposed rats inhibited expression of *TNFα*, *Il-1β* and *iNOS* (Figure 5.6b). Ozone significantly altered *Il-1β* expression *ex vivo* at 24 h post plasma treatment (*Ozone* main effect, $p = 0.001$), and at 4 and 24 h post plasma treatment for *TNFα* (*Ozone* main effect $p = 0.01$, < 0.001 respectively) and *iNOS* (*Ozone* main effect $p < 0.001$, Drug x Ozone interaction < 0.007 respectively). While there were no significant *LPS* x *Ozone* interactions, effects tended to be more pronounced in the presence of LPS. Similar *ex vivo* ozone-induced effects were observed in cells treated with plasma (0 h) from rats administered metyrapone prior

to exposure, suggesting these effects may be GC-independent. In contrast to the ozone-dependent reduction in inflammatory gene expression observed following treatment with 0 h plasma, treatment of cells with plasma collected from ozone-exposed rats after a 24 h recovery period increased expression of *TNFα* after 24 h treatment (Figure 5.6c), particularly in the presence of LPS (*LPS* x *Ozone* x *Drug* interaction, p = 0.04)

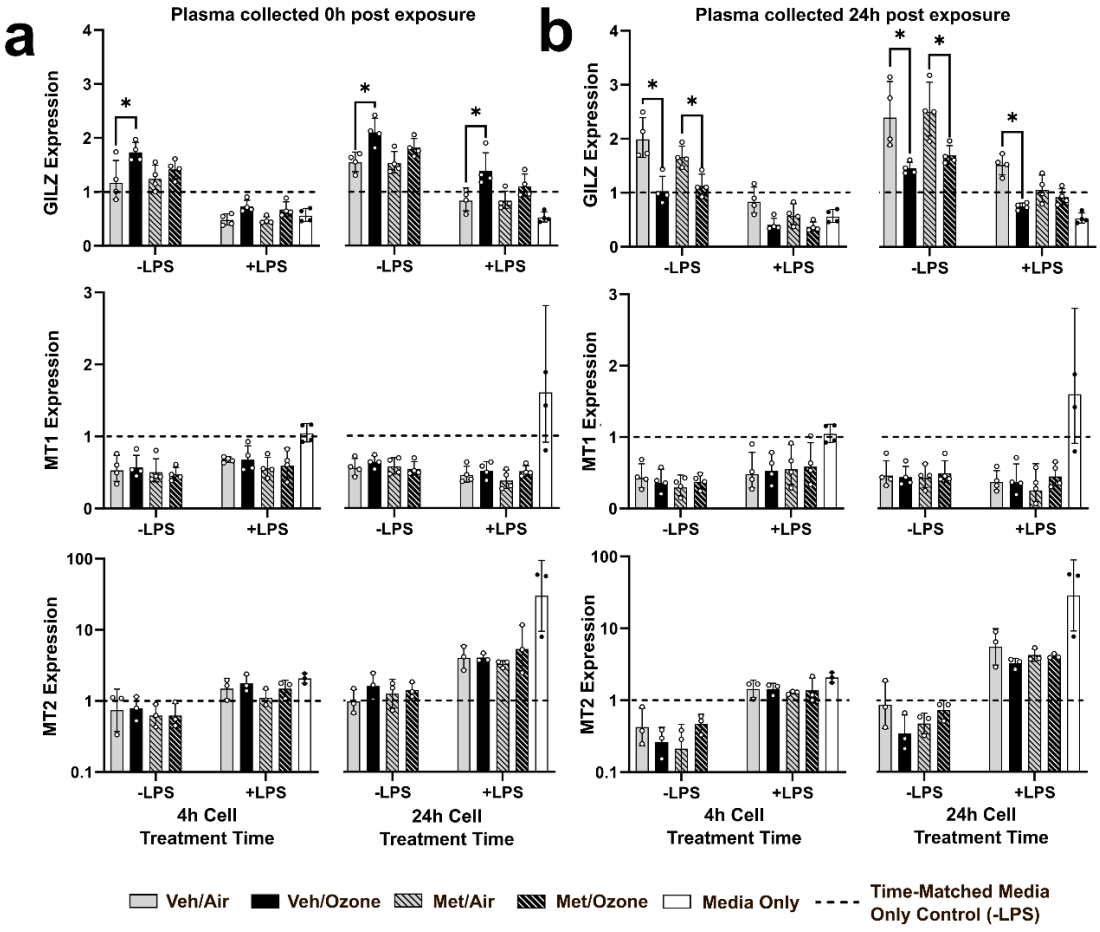


Figure 5.5 Ex vivo ozone-induced effects on mRNA levels of stress-sensitive genes in microglia. Primary rat microglial cells were treated with pooled plasma samples [n = 5; 5%] collected immediately (a) or 24 h (b) post exposure from rats administered metyrapone (Veh = 0 mg/kg, Met = 50 mg/kg) prior to exposure to ozone (0.8 ppm) or clean air. Cells were treated with plasma alongside LPS (0 or 0.5 ng/mL). Gene expression results normalized to time-matched media-only control. Statistical analysis conducted via Three-Way ANOVA (factors *Ozone, Metyrapone, LPS*) with post hoc Holm-Sidak. *p < 0.05, n = 4. Bars represent geomean ± GSD.

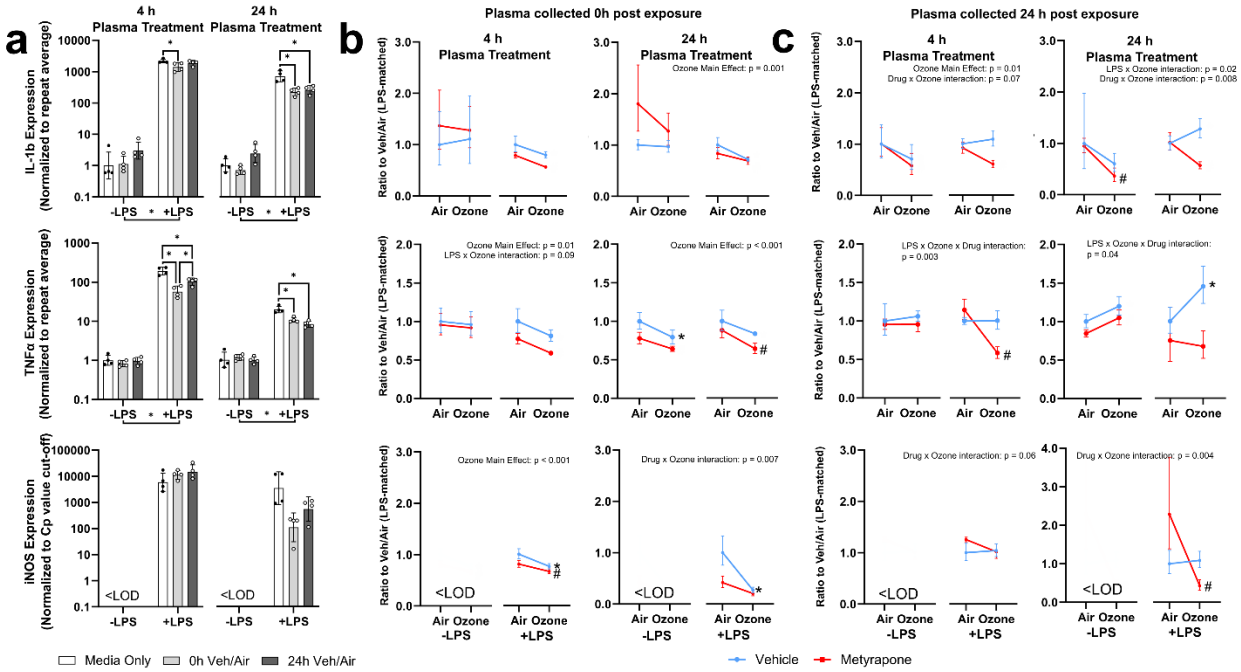


Figure 5.6 Ex vivo ozone-induced effects on inflammatory responses in microglia. Primary rat microglial cells were treated with pooled plasma samples [n = 5; 5%] collected immediately (b) or 24 h (c) post exposure from rats administered metyrapone (Veh = 0 mg/kg, Met = 50 mg/kg) prior to exposure to ozone (0.8 ppm) or clean air. Cells were treated with plasma alongside LPS [0 or 0.5 ng/mL]. a) Fold change in expression of inflammatory genes with LPS treatment and control (Veh/Air) plasma samples. Expression normalized to repeat average for IL-1β or TNF-α, and Cp value cut-off for iNOS. b,c) Ratio of gene expression relative to Veh/Air (LPS and time-matched) and normalized to repeat average. Statistical analysis performed via a) Two-way ANOVA (factors *LPS, Plasma*) with post hoc Holm Sidak or b,c) Three-way ANOVA (factors *LPS, Ozone, Metyrapone*) with post hoc Holm-Sidak. * $p < 0.05$, n = 4. LOD = limit of detection. Bars (a) and points (b,c) represent geomean ± GSD.

5.6 Discussion

Our study shows that an *ex vivo* approach involving exposure of brain cells to plasma samples collected from pollutant-exposed individuals can be used to study effects of air pollution on the brain. Models were sensitive to pollutant exposure, and to temporal differences in response post-exposure, providing the capacity to assess the progression of responses to exposure. Furthermore, models were sensitive to specific systemic mediators of effects, demonstrating the utility of the approach for investigation of underlying mechanisms. Importantly, the *ex vivo* responses reproduced effects observed *in vivo*, providing a basis for use of this approach to model human exposure scenarios.

In vitro models used to study effects of pollutants on human brain cell populations have typically focused on direct effects of PM (Wei et al. 2017; Solaimani et al. 2017; Kang et al. 2021; Lin et al. 2022; Li et al. 2022). While such models enable direct actions of pollutants to be examined, they do not incorporate the systemic response that pollutants induce and that contribute importantly to extrapulmonary effects (Block and Calderón-Garcidueñas 2010; Wang et al. 2017; Thomson 2019; Kodavanti et al. 2023). For example, it was the commonality in responses to both PM and ozone in a multi-tissue study that included the brain that led to identification of GCs as systemic mediators of effects (Thomson et al. 2013). The strong correlation of *ex vivo* ozone-induced transcriptional responses to hippocampal responses shown in the present study demonstrates that an *ex vivo* approach can be used to model systemic effects of pollutant exposure. Two prior studies studied effects of sera from ozone-exposed rats on neural cells. One found that sera from ozone-exposed rats modulated levels of PI3K-signaling proteins, as well as synaptic-plasticity related proteins PSD95 and synaptophysin in the neuronal-like cell line PC12 (Cao et al. 2022), while the other found altered microglial responses to inflammatory stimuli (Mumaw et al. 2016). However, our study is the first to our knowledge

to demonstrate a direct relationship between *ex vivo* ozone-induced transcriptional responses and effects observed in actual brain tissue. Establishing that this *ex vivo* approach resembles *in vivo* exposure effects is critical to not only isolate the role of circulating factors in mediating pollutant-induced effects observed previously, but provides a basis to extend this approach to model human responses to pollutant exposures.

In addition to recapitulation by cultured neurons of gene expression profiles observed *in vivo*, the model also enabled assessment of how plasma-derived factors contribute to functional changes. Dysregulation of microglia, including modification of their inflammatory responses, plays a role in disease outcomes (Perry and Holmes 2014). The observation that plasma from ozone-exposed rats modified inflammatory responses in primary microglial cells suggests that circulating factors can alter microglia cell immune function. This aligns with prior work by Mumaw et al. in which they propose that circulating factors following ozone exposure prime microglia to be more susceptible to inflammatory stimuli (Mumaw et al. 2016). While the present study demonstrated an inhibitory effect on inflammatory responses with plasma collected immediately post exposure, cells treated with plasma collected 24 h post exposure show elevation of *TNFα* particularly in cells challenged with LPS, similar to what was observed by Mumaw et al. Modification of immune cell responses to inflammatory stimuli has also been shown outside the brain, for instance LPS-induced cytokine production in bronchoalveolar lavage fluid and lung tissue was exacerbated in mice pre-exposed to diesel exhaust (Kim et al. 2021-1-06). Several toxicological studies conducted *in vivo* have found modulation of microglial responses by pollutants. For instance, chronic exposure to pollutants increases microglial activation (Levesque et al. 2011; Greve et al. 2020; Valdez et al. 2020; Ehsanifar et al. 2023) as well as expression of inflammatory markers in multiple brain regions (Levesque et al. 2011;

Greve et al. 2022; Ehsanifar et al. 2023). The *ex vivo* observations in this paper provide further perspective to these findings by confirming a role for circulating factors in mediating pollutant-induced responses on microglial cells, and provide a means to study the temporal nature of responses. Moreover, given that LPS is an important bioactive constituent of airborne exposures that may be implicated in CNS impacts of air pollutants (Calderón-Garcidueñas et al. 2008) our results suggest that the model may be useful to study how systemic mediators of pollutant effects may in turn modify the response to other pollutants and/or to their secondary messengers (e.g. inflammatory cytokines).

GCs are widely considered to be of importance to brain health (Lucassen et al. 2014). Elevated GC levels have been found in plasma samples of patients with diseases such as dementia and depression, and have been associated with accelerated cognitive decline (Ouanes and Popp 2019). Importantly, GCs may be an important mediator of pollutant-induced effects on brain health, as several studies have demonstrated increases in circulating GC levels following exposure to pollutants in both rodents (Thomson et al. 2013, 2016) and humans (Miller et al. 2016a; Thomson et al. 2021). GCs can readily cross the blood-brain barrier to interact directly with brain cells, and several studies have shown GC-induced pathology in neuronal cells (Shi et al. 2019; Donoso et al. 2019; Liu et al. 2020; Zhang et al. 2021; Hyun et al. 2022) and modulation of inflammatory responses in microglial cells (Jacobsson et al. 2006; Nakatani et al. 2012; Liu et al. 2018). While these studies often observe effects at doses higher than what may be physiologically relevant (often 200 μM and greater for neuronal effects), we demonstrated that even low doses of CORT are capable of modifying gene expression responses in primary rat hippocampal neurons and microglia, confirming that these cell types are sensitive to physiologically-relevant pollutant-induced increases in circulating GCs. Indeed, *ex vivo* effects

on GC and cellular stress responsive genes in neurons were GC-dependent and, notably, temporal patterns of gene expression aligned with circulating CORT levels. This supports *in vivo* observations that GCs mediate ozone-induced transcriptional effects in the brain (Henriquez et al. 2019; Thomson et al. 2019; Rose et al. 2020).

Interestingly, in contrast to the GC-dependent effects observed in neurons, the *ex vivo* ozone-induced effects in microglia observed in this study appear to be GC-independent. There are several possible circulating factors that may mediate these microglia effects. For instance, lipoproteins present in plasma samples can reduce potency of LPS-induced responses due to binding of the LPS (Flegel et al. 1989) and indeed we observed that plasma samples from control groups exposed to clean air reduced LPS-induced expression of inflammatory genes. While low density lipoprotein and high density lipoprotein levels are not elevated in plasma from ozone-exposed rats (Supplementary Figure 5.S3), other unmeasured proteins capable of binding LPS may be elevated. Other inflammatory or oxidative-stress related signals may also be responsible although both ourselves (data not shown) and Mumaw et al. 2016 have found a lack of changes in circulating cytokines in plasma/sera samples used. *Ex vivo* ozone effects may be partially mediated by the MAC-1 receptor, as Mumaw et al. found that blockade of this receptor prevented *ex vivo* ozone-induced effects on *TNFα* production in microglia. Future work utilizing an *ex vivo* model could further elucidate the specific mechanisms. Given this capacity to demonstrate GC -dependent and independent actions, the *ex vivo* model is sensitive to specific mediators of *in vivo* effects. This is critical as it demonstrates that the approach can be used to isolate the role of such mechanisms, which could be investigated in a human-relevant system by applying pharmacological intervention to cells, such as through use of either GC-receptor antagonists, or by applying antioxidants to investigate the role of oxidative stress pathways.

Given the potential applications of this *ex vivo* approach in studying the effects of air pollution on the brain, understanding of the strengths and limitations of such a model is crucial. This *ex vivo* approach provides an opportunity to examine the role of systemic effects of air pollution exposure on the brain using an *in vitro* system. Not only does this allow extension to a human-relevant model of tissues that are not easily accessible, but it also allows examination of cell-type specific effects that would be difficult to disentangle *in vivo*, as well as opportunities to manipulate experimental conditions to gain further understanding of the mechanisms involved. Additionally, use of this system eliminates the uncertainty in *in vivo* systems regarding mechanisms involved in causing certain effects (i.e. circulating factors, direct interactions with particles, or nervous system activation) as well as uncertainty regarding the temporal nature of effects over the course of the exposure. While the 2D monoculture systems used in this study are rather simple models of the brain, they nevertheless reproduced effects observed *in vivo*. The approach should be amenable to application in more complex 3D co-culture and "organ-on-a-chip" microfluidic systems as warranted. Finally, this model is only suitable to assess mediators of pollutant-induced effects that are carried by plasma, and will not capture other potential mechanisms such as direct effects of particles translocated via the olfactory nerve or nervous system activation that could be important for adverse health effects of pollutants. While this proof-of-concept study specifically aimed to model an acute ozone exposure scenario, the approach should be broadly applicable to exposures that elicit a systemic response given that the treatment is with the plasma rather than the substance itself.

In conclusion, we show that an *ex vivo* pollution exposure model - in which *in vitro* culture systems are treated with plasma from ozone-exposed animals - is capable of reproducing *in vivo* effects in the brain. This provides an exciting opportunity to explore the effects of air

pollution using human-relevant models of the brain that incorporate effects of systemic mediators. As well, we demonstrate the utility of this system to examine functional responses in brain cells such as microglia to pollutant-induced circulating factors. By employing plasma as the exposure, this approach allows investigation into how pollutant-responses may differ based on inter-individual variability in the population, providing a means to assess characteristics that may contribute to sensitivity to pollutant exposure. Use of human models – including models of disease – coupled with pharmacological intervention to test involvement of specific mediators and biological pathways, provides a means to evaluate involvement of specific systemic mediators of the adverse effects on brain health of pollutant exposure. Air pollution may be a critical modifiable factor relevant to neurodegenerative conditions such as AD and PD, amongst others. The capacity to evaluate effects and underlying mechanisms in *ex vivo* models should provide further insight into the link between pollutant exposure and disease processes, in support of risk assessment initiatives.

5.7 Acknowledgements

Funding for this study was provided by Health Canada through the Clean Air Regulatory Agenda and the Addressing Air Pollution Horizontal Initiative.

5.8 Supplemental Figures

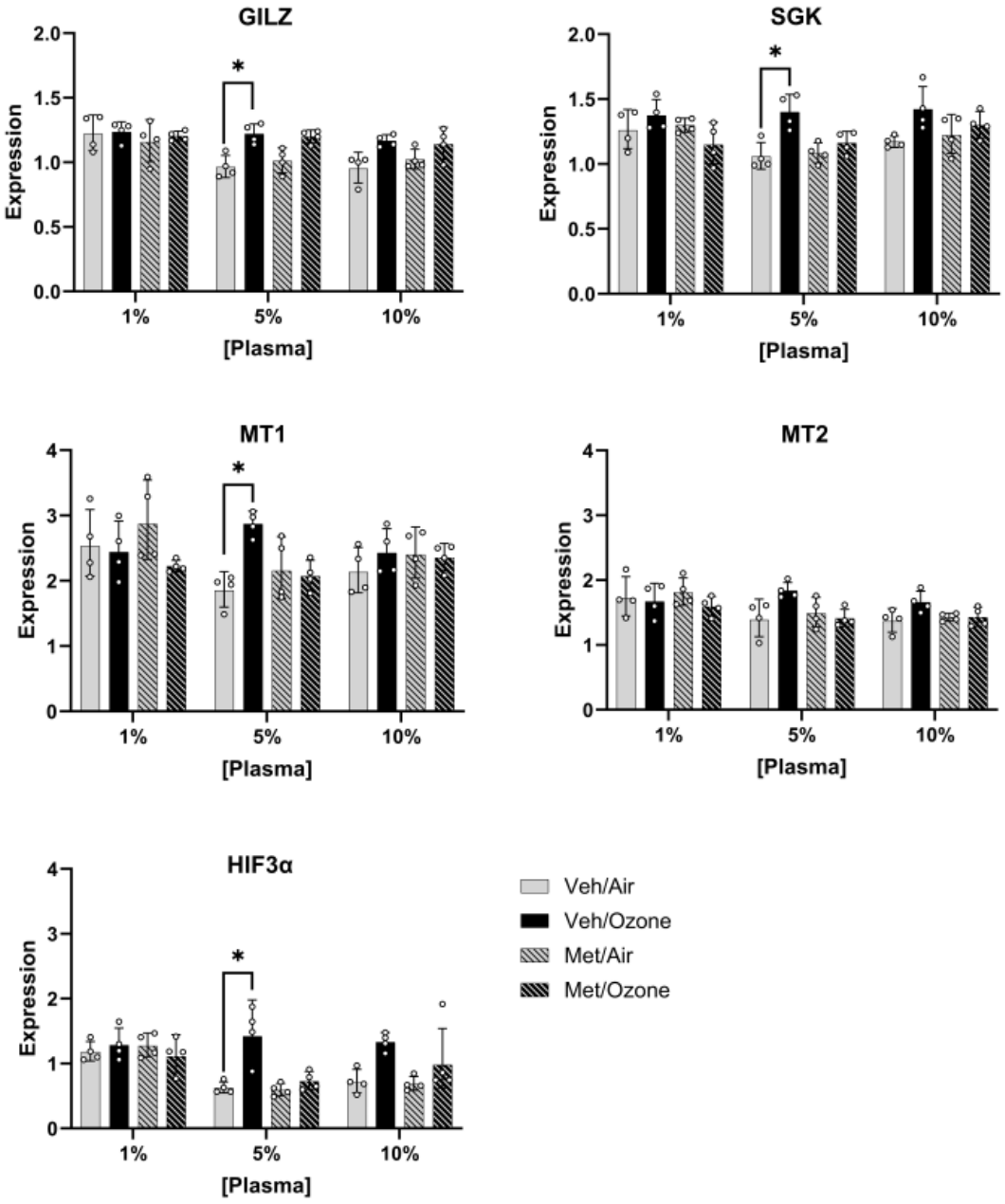


Figure 5.S1. *Ex vivo* ozone-induced effects on neuronal gene expression at various plasma concentrations. Primary hippocampal neurons were treated for 4h with pooled plasma [n = 5, 1, 5, 10%] from rats administered metyrapone (Veh = 0 mg/kg, Met = 50 mg/kg) prior to exposure to ozone (0.8 ppm) or clean air. Plasma samples collected immediately post exposure. Gene expression normalized to daily average and PP1A as a reference gene. Statistical analysis conducted via three-way ANOVA (factors: *Ozone, Metyrapone, Plasma %*) with post-hoc Holm Sidak. *$p < 0.05$, n = 4.

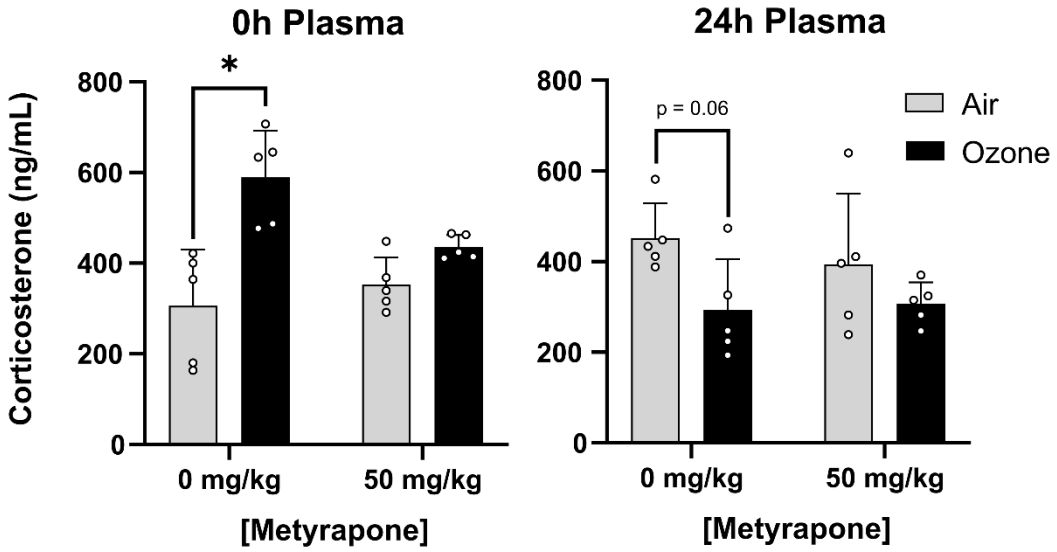


Figure 5.S2. Effects of ozone exposure on plasma corticosterone levels. Plasma concentrations of corticosterone were measured in fisher-344 rats exposed to ozone (0.8 ppm) or clean air for 4 hours following administration of metyrapone [0, 50 mg/kg] 1 h prior to exposure. Plasma samples collected immediately (0h) or 24h post exposure. Statistical analysis conducted via two-way ANOVA (factors: *Ozone*, *Metyrapone*) with post-hoc Holm-Sidak. *$p < 0.05$, $n = 5$ / group.

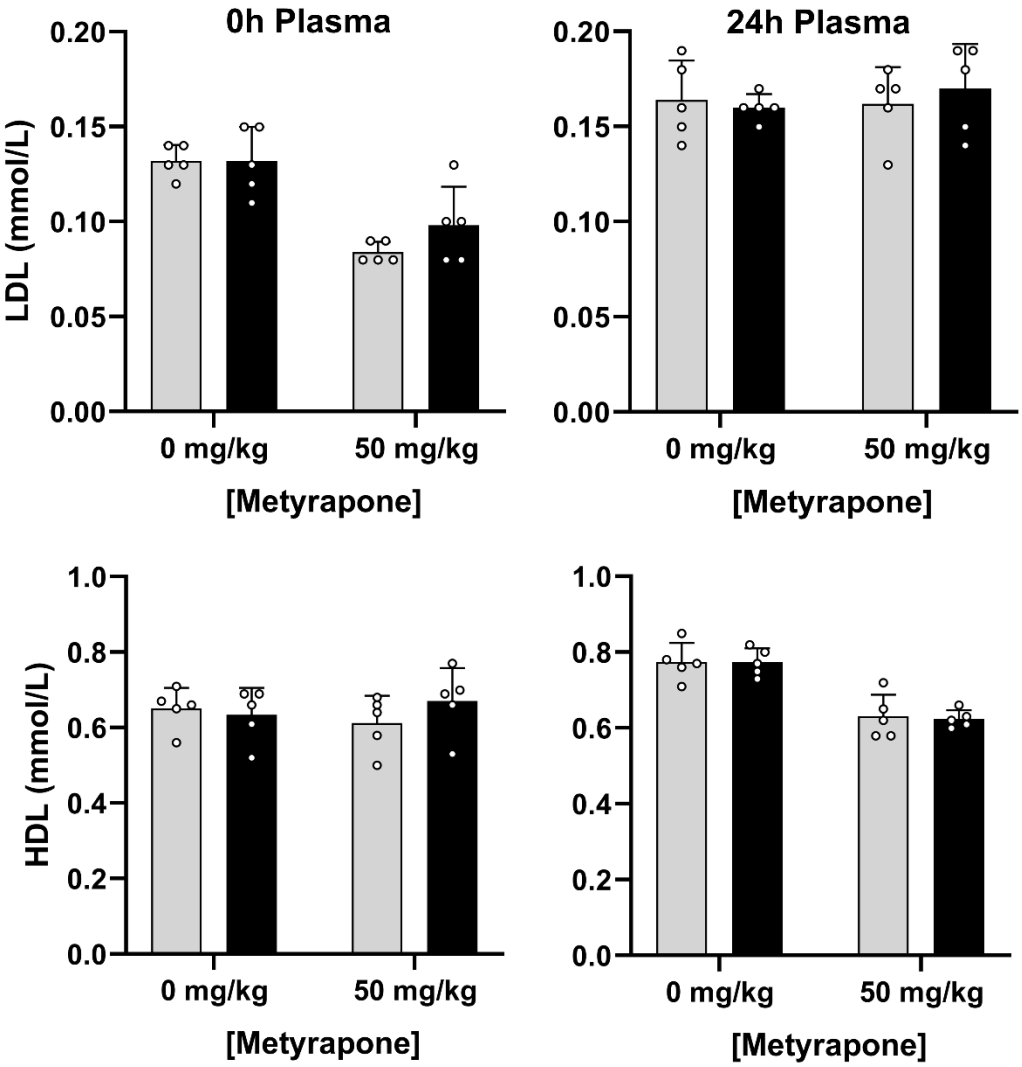


Figure 5.S3. Plasma levels of high-density and low-density lipoprotein following ozone exposure. Plasma concentrations of high-density lipoprotein (HDL) and low density lipoprotein (LDL) were measured in fisher-344 rats exposed to ozone (0.8 ppm) or clean air for 4 hours following administration of metyrapone [0, 50 mg/kg] 1 h prior to exposure. Plasma samples collected immediately (0h) or 24h post exposure. Statistical analysis conducted via two-way ANOVA (factors: *Ozone*, *Metyrapone*) with post-hoc Holm-Sidak. *$p < 0.05$, $n = 5$ / group.

Chapter 6: Diesel exhaust-induced systemic mediators alter transcriptomic profiles in human iPSC-derived neurons in a sex-specific manner

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Manuscript submitted for peer review

6.1 Authorship Contributions

The study described in the present manuscript was designed by MR, SALB, and EMT. The controlled diesel exhaust exposure study was conducted by CR and CC. Cell culture work and *ex vivo* exposures were performed by MR. Immunocytochemistry was prepared by MR and imaging performed by SB. Preparation of RNA sequencing libraries was performed by MR, and sequencing was performed by AN. TS, LB, and MM were responsible for processing and quality control of RNA sequencing data. AW performed statistical analysis. Figures were prepared by MR. The manuscript was written by MR, with review and editing by EMT and SALB.

6.2 Abstract

Exposure to air pollution – including traffic-related air pollution – is associated with increased risk of neurodegenerative and psychiatric diseases. Elucidating mechanisms and susceptibility factors remains challenging, in part due to a lack of human models that capture contributions of systemic processes to pollutant-induced effects. We have shown that an *ex vivo* approach – in which hippocampal neurons are treated with plasma from pollutant-exposed rats – can reproduce *in vivo* effects. Here we translate this approach to the human context to assess acute effects of diesel exhaust (DE) exposure. Induced pluripotent stem cell (iPSC)-derived neurons were treated with plasma from a randomised double-blind crossover study of participants [n = 16; 11 with asthma (6 females, 5 males), 5 without (3 females, 2 males)] exposed to filtered air or DE [300 $\mu g/m^3$ $PM_{2.5}$] for 2 h; participants took N-acetylcysteine [NAC; 600 mg] or placebo for 6 days prior to DE exposure. Transcriptional responses were assessed via RNA-sequencing. Plasma collected following DE exposure altered neuronal gene expression relative to participant-matched filtered-air exposures, notably oxidation-dependent pathways (MTORC1 signaling, oxidative phosphorylation). Pathway analysis revealed sex-dependent effects, including alteration of protein secretion and cellular stress-related pathways in males and cellular metabolism-related pathways in females; these play important roles in neuronal functioning, communication, and plasticity. NAC prevented a subset of DE-induced effects, and modulated pathways generally aligned with expected effects of the medication. These findings support the notion that systemic processes can contribute to pollutant-induced effects in the brain, and provide insight into underlying mechanisms and inter-individual differences.

6.3 Introduction

Exposure to air pollution is the second largest risk factor to human health globally, and the largest environmental risk factor. In 2023, an estimated 7.9 million deaths and 232 million healthy years lost were attributed to air pollution (Health Effects Institute 2025; Hay et al. 2025). While the effects of air pollution on respiratory and cardiovascular disease are well recognized, there is a growing understanding that air pollution also impacts the brain. Several epidemiological studies have found an association between air pollution exposure and increased risk of neurodegenerative (Xu et al. 2016; Power et al. 2016) and neuropsychiatric diseases (Zundel et al. 2022), such as Alzheimer's disease (AD), Parkinson's disease (PD), and depression. Thus, in 2025, the latest Global Burden of Disease (GBD) study included, for the first time, dementia as a health issue in the estimate of air pollution exposure outcomes. Specifically, dementia attributable to air pollution exposure was estimated to contribute to 626,000 excess deaths and 11.6 million healthy years of life lost in 2023 (Health Effects Institute 2025). With an aging population, and increasing prevalence of depression (Shorey et al. 2022; Daly 2022) and dementia (Nichols et al. 2022), the public health burden is likely to increase. Gaining a better understanding of how air pollution affects the brain, especially the underlying mechanisms involved, would provide insight into vulnerable populations and may inform risk estimates.

The lack of human-relevant laboratory models represents a primary challenge to elucidating how air pollution alters brain function. Exposure studies conducted in rodents have made significant progress in demonstrating that air pollution can induce cognitive impairment and behavioural deficits (Fonken et al. 2011; Mokoena et al. 2015; Woodward et al. 2018; Ehsanifar et al. 2023; Li et al. 2024), neuroinflammation (Fonken et al. 2011; Mumaw et al. 2016; Woodward et al. 2018; Hajipour et al. 2020; Ehsanifar et al. 2023), decreased neurogenesis

(Woodward et al. 2018) and altered neurotransmitter systems (Mokoena et al. 2015; Rose et al. 2020). However, rodents (often inbred strains) lack the heterogeneity observed within the human population that may modulate susceptibility to pollutants and thus disease risk. Interspecies differences may also complicate interpretation of findings to the human context given marked differences in rodent and human metabolic rates (Sharma and McNeill 2009; Kummitha et al. 2014). For these reasons, models that more directly represent human exposure scenarios and biology may be needed to better characterise underlying mechanisms and effects.

Use of *in vitro* cell culture systems is a potential avenue for modelling pollutant effects on human brain cells in controlled exposure environments. Such studies have previously focused on examining direct effects of particulate matter (PM) on human brain cell populations (Wei et al. 2017; Lin et al. 2022; Li et al. 2022), demonstrating that direct exposure to $PM_{2.5}$ reduces cell viability, increases inflammatory responses and oxidative stress, and impacts energy metabolism and mitochondria bioenergetics in the SH-SY5Y neuroblastoma cell line.

However, these models do not account for the role that pollutant-induced systemic responses may play in causing effects in the brain. While PM may translocate to the brain through trigeminal or vagal nerve pathways (Lewis et al. 2005; Elder et al. 2006; Wang et al. 2008), or the bloodstream (Oberdörster et al. 2008; Kreyling 2016; Qi et al. 2022), both PM and gaseous pollutants can also react with cells in the lungs, which may induce downstream systemic responses in peripheral organs leading to the release of bioactive factors into circulation (Thomson et al. 2013, 2019; Miller et al. 2016b; Pope et al. 2016; Rose et al. 2020). These circulating factors, such as inflammatory signals, hormones such as glucocorticoids (GCs), or oxidative stress products, may crucially drive several pollutant-induced effects on the brain. For instance, through pharmacological intervention studies in rodents we have previously shown that

elevation of GCs due to ozone exposure impacts expression of several neurotrophic factors and serotonin receptors in the hippocampus (Rose et al. 2020). Curcumin, a substance with anti-oxidant and anti-inflammatory properties, has been shown to protect against PM- and ozone-induced effects, including effects on the brain, behavioural impairments, and elevation of serum cytokine levels (Nery-Flores et al. 2018; Vastegani et al. 2023). Elevated GC levels, chronic systemic inflammation, and oxidative stress are hallmarks of neurodegenerative diseases and psychiatric conditions (Lucassen et al. 2014; Bayraktaroglu et al. 2025), and may importantly contribute to disease pathology. Therefore, accounting for contributions of systemic processes in evaluating the impacts of air pollution on the brain would be required to improve our understanding of disease risk.

Plasma is a relatively accessible biological sample, and contains circulating factors that may be transported to the brain. We previously demonstrated with a rat ozone-exposure model that an *ex vivo* approach in which neurons were exposed to plasma reproduced gene expression patterns observed in brain tissue (Rose and Thomson 2025). In the present study, our objectives were to: a) translate this approach to the human context by studying the effects of plasma from participants who were exposed (in a controlled exposure study) to diesel exhaust (DE) on human induced pluripotent stem cell (iPSC)-derived forebrain neurons, b) examine whether *ex vivo* DE responses differ based on sex and asthma status, and c) evaluate how treatment with an antioxidant N-acetylcysteine (NAC) modifies any DE-induced responses.

6.4 Methods

6.4.1 Diesel exhaust exposure

The study was approved by the University of British Columbia, Vancouver Coastal Health Research Institute, and Health Canada ethics review boards (H08-02288, 2023-051H). Written consent was obtained from each participant. Full study details are provided in Carlsten et al., 2014. Briefly, participants [n = 16, 11 with doctor-diagnosed asthma (6 females, 5 males) and 5 without (3 females, 2 males); all free from current use of inhaled corticosteroids or long-acting β_2 agonists, from regular use of bronchodilators, and from vitamin A, C, or E supplementation] took *N*-acetylcysteine (NAC; 600 mg) or placebo capsules thrice daily for 6 days. On day 6, participants were exposed to either filtered air or DE (300 $\mu g/m^3$ $PM_{2.5}$). A randomised, double blind, crossover design was implemented that included a minimum 2-week washout period between exposures, and that utilised three experimental conditions: 1) filtered air with placebo (FAP), 2) diesel exhaust with placebo (DEP), and 3) diesel exhaust with NAC (DEN). Archived plasma samples collected immediately pre- (0 h) and post- (2 h) exposure were used in the present study.

6.4.2 Generation of iPSC-derived forebrain neurons

Human iPSC-derived neural progenitor cells (NPCs; STEMCell Technologies, Vancouver, British Columbia, Canada, Catalogue #200-0620) were used to generate forebrain neuron-like cell cultures. NPCs (passage 3) were thawed on hESC-qualified Matrigel-coated (Corning, Tewksbury, Massachusetts, Catalogue #354277) 12-well cell culture plates at a density of 2 x 10^5 cells/cm^2 in STEMDiff Complete Neural Progenitor Media (STEMCell Technologies, Catalogue #05833). Cells from independent repeats were derived from the same lot but thawed from separate vials post initial expansion. Cells were incubated at 37 ˚C + 5% CO_2, and media was exchanged in full daily for 6 days. At 7 days after seeding, NPCs were replated on poly-L-

ornithine (15 µg/mL; Sigma-Aldrich, Oakville, Ontario, Canada, Catalogue #P4957-50ML) and laminin (10 µg/mL; Sigma-Aldrich, Catalogue #L2020)-coated 12-well plates at a density of 2 x 10^5 cells/cm^2 in STEMDiff Complete Neural Progenitor Media. Accutase (STEMCell Technologies, Catalogue #07920) was used to dissociate NPCs from the plate surface. The following day, 100% of the media was removed and replaced with STEMDiff Forebrain Neuron Differentiation Media (STEMCell Technologies, Catalogue #08600) to initiate the differentiation process. Media was exchanged in full daily for the next 5 days. At 7 days after re-seeding, cells were replated on poly-L-ornithine and laminin coated 96-well plates at a density of 37,500 cells/cm^2 in STEMDiff Forebrain Neuron Maturation Media (STEMCell Technologies, Catalogue #08605) and media was exchanged in full every 2-3 days until day *in vitro* (DIV) 21.

6.4.3 Immunocytochemistry

To confirm cell culture composition, NPCs were seeded on Matrigel-coated 96-well PhenoPlates (Revvity, Groningen, The Netherlands) at a density of 1 x 10^5 cells/cm^2, or poly-L-ornithine and laminin coated 96-well PhenoPlates at a density of 37,500 cells/cm^2 for neuronal differentiation until DIV21. Cells were fixed with 4% paraformaldehyde (Thermo Scientific, Burlington, Ontario, Canada) for 15-20 min at room temperature, followed by three washes with Dulbecco's phosphate buffered saline containing calcium and magnesium (DPBS; Gibco, Burlington, Ontario, Canada). Cells were then permeabilized with Triton X-100 (0.3% v/v in DPBS; Fluka Chemicals) for 5 min, followed by three washes in DPBS (5 min incubation per wash). The final DPBS wash was left on the cells, and cell culture plates were sealed with parafilm and stored at 4 ˚C for up to 1 month. Goat serum solution (10% v/v in DPBS; Abcam, Toronto, Ontario, Canada) was added for 1 h at room temperature to block. Goat serum solution was replaced with primary antibodies (see Table 1) diluted in 10% goat serum and left to incubate for 1 h at room temperature. Primary antibody solutions were removed and cells were

washed three times with DPBS (5 min incubation per wash). Secondary antibody solutions in 10% goat serum (see Table 1) were added and left to incubate for 1 h at room temperature in the dark. Secondary antibody solution was removed, and cells were washed three times with DPBS (5 min incubation per wash, in the dark). Cells were then stained with Hoescht 33342 (2 µg/mL in H_2O; Thermo Scientific) for 5 min in the dark. Cells were washed once with DPBS (5 min incubation in the dark), and stored in DPBS in parafilm-sealed plates at 4 ˚C until imaged.

Immunofluorescence images were acquired using an Opera Phenix High Content Screening System (Revvity). For each well, 16-20 non-overlapping fields were captured using identical exposure settings within each experimental replicate using a 20x water objective. Each channel was a separate exposure to avoid bleed through with the following laser/emission filter combinations: Hoechst, 375 nm laser/435-515nm filter, Alexa Fluor 488, 488nm laser/500-550 nm filter, Alexa Fluor 594, 561 laser/570-630 filter. Images were uploaded to the Children's Hospital of Eastern Ontario Research Institute Columbus Image Analysis server for remote visualization.

Fluorescence intensity thresholds were set to be greater than secondary antibody-only controls. The anti-OCT4 threshold was set above wells stained with anti-PAX6 or anti-nestin, without OCT4, as some nonspecific binding of the secondary antibody was observed. Anti-TUJ1 and anti-GFAP thresholds were set above average intensities observed in NPC cultures. Images were analyzed using QuPath v.0.5.1 (Bankhead et al. 2017).

Table 6.1. Antibodies.

Antibody	Dilution	Manufacturer	Catalogue #
Mouse anti-Octamer binding transcription factor 3/4 (OCT3/4)	200	STEMCELL Technologies	1550
Rabbit anti-nestin	100	Abcam	ab105389
Rabbit anti-Paired Box 6 (PAX6)	500	STEMCELL Technologies	100-1340
Rabbit anti-Glial fibrillary acidic protein (GFAP)	500	Sigma-Aldrich	G9269
Mouse anti- βeta Tubulin III, tuj1 clone (TUJ1)	2000	RnD Systems	MAB1195
Rabbit anti-Vesicular glutamate transport 2 (VGLUT2)	500	Synaptic Systems	135 402
Rabbit anti-Synapsin	500	Abcam	ab254349
Rabbit anti-post synaptic density protein 95 (PSD95)	500	Invitrogen	51-6900
Goat anti-Mouse Alexafluor 488	1000	Invitrogen	A-11001
Goat anti-Rabbit Alexafluor 594	1000	Invitrogen	A32740

6.4.4 *Ex vivo* exposure

At DIV21, neuronal cultures were treated with plasma samples (5% in STEMDiff Forebrain Neuron Maturation Media) collected from participants from the DE study (FAP, DEP, and DEN conditions, at 0 h and 2 h time points, n = 16 participants). Plasma samples were filtered using Millipore Ultrafree-MC Centrifugal filters (sterile) with a 0.22 µm Durapore membrane (Millipore Sigma, Oakville, Ontario, Canada), and centrifuged at 1000 x g for 2 minutes at room temperature. Cells were incubated for 4 h at 37 ˚C + 5% CO_2, after which media was removed and frozen at -80 ˚C and cells were lysed with Qiagen RLT Buffer (Qiagen,

Toronto, Ontario, Canada) + 1% β-mercaptoethanol (Sigma-Aldrich). Three replicate wells per condition were combined, and lysates were stored at -80 ˚C. Two independent experimental repeats (T1 and T2) were conducted.

Samples were processed in batches (cell culture plate, RNA extraction, sequencing library preparation) within each replicate experiment. All plasma samples from the same participant were used to treat cells plated on the same 96-well plate. Cells treated with plasma samples from the same participant were processed together for both RNA extraction and sequencing library preparation. During T1, samples from two separate participants were never processed in the same batch at multiple steps (e.g. two participants whose samples were processed together during RNA extraction, had sequencing libraries prepared at different times). During T2, samples were processed as the same batch through all steps. T1 therefore had 3 technical batch variables (cell culture plate, RNA extraction, sequencing library preparation) whereas as T2 had one batch variable.

6.4.5 RNA-sequencing

RNA was extracted from cell lysates using Qiagen RNeasy Micro Kits (Qiagen), according to the manufacturer's protocol. RNA was quantified using Ribogreen RNA Quantitation Reagent and Kit (Molecular Probes, Eugene, Oregon, USA), and quality was assessed using the Tapestation High-Sensitivity RNA Screentape (Agilent, Mississauga, Ontario, Canada). RNA Integrity Numbers were all > 8. RNA sequencing libraries were prepared using the Illumina Stranded mRNA Prep, Ligation Kit (Illumina, San Diego, California, USA) according to manufacturer protocol. Approximately 50 – 90 ng of RNA per sample was used as input. Final library quantities were determined using the Tapestation D1000 Screentape (Agilent) and Qubit High Sensitivity dsDNA quantification kit (Invitrogen, Burlington, Ontario, Canada).

Libraries were diluted to 2 nM and combined. The pooled library was sequenced (single-end) with the NextSeq2000 (Illumina) using a P2 and P4 flow cell. An average sequencing depth of 22-25 million reads per sample was achieved. Library preparation and sequencing was performed separately per independent repeat.

Reads were demultiplexed from the BCL files and processed into FASTQ files using DRAGEN analysis v1.3.0 on the Illumina BaseSpace Sequence Hub (Illumina). Processing and quality control of the FASTQ files, and generation of gene-level count data, were performed using the R-ODAF analysis pipeline (https://github.com/R-ODAF/R-ODAF_Health_Canada), which manages workflows using Snakemake v8.0 (Mölder et al. 2021) and relies on scripts implemented in R v4.3.2 (R Core Team 2025). Reads were trimmed using fastp v0.23.2 (Chen et al. 2018) and aligned to the human GRCh38 reference genome using STAR v2.7.8a (Dobin et al. 2013). A samples-by-genes count matrix was produced using RSEM v1.3.3 (Li and Dewey 2011). Study-wide quality control was performed on the count matrix following the general approach described by Harrill et al., 2021. Samples were excluded if they had fewer than 2,000,000 mapped reads (10% of target reads) or less than 50% uniquely mapped reads. Samples with a Gini coefficient greater than 0.99 were also excluded. Sample consistency was assessed using pairwise Spearman correlations, and samples where Spearman's $\rho < 0.9$ were excluded. No samples were excluded based on these criteria. In addition, samples falling outside Tukey's outer fences, defined as three times the interquartile range, for the number of detected genes or the number of genes accounting for the top 80% of the signal were flagged for exclusion. One sample did not meet this specific criteria, but was retained on the basis of passing all other QC filters.

6.4.6 Statistical analyses and data visualisation

Read counts were size-factor normalized using DESeq2 (Love et al. 2014). Batch adjustment was applied using a linear model on the $\log_2$(normalized counts + 0.5); in T1 two batch variables were included in the model (cell culture batch, RNA extraction batch), in T2 there was just one batch variable. A within-participant $\log_2$ fold change (delta score) was calculated for each transcript by subtracting the $\log_2$-normalized count at 0 h from the $\log_2$-normalized count at 2 h, for each exposure condition. Two-Way ANOVAs were performed (factors *Exposure, Sex* or *Exposure, Asthma*) via R v.4.4.3 (R Core Team, 2025); post-hoc pairwise comparisons of interest (t-tests) were evaluated using the esticon function from the doBy package (Halekoh and Højsgaard 2026). Calculated $\log_2$ fold changes and their respective standard errors were back-transformed to linear fold changes, with standard errors approximated using the delta method (Oehlert 1992). Differentially expressed genes (DEGs) were classified as significant if they had a significant ($p < 0.05$) main effect or interaction effect (depending on comparison), and post-hoc comparison was unadjusted $p < 0.05$, and $|\text{fold change}| > 1.2$. The following R packages were used for visualisation and data processing and analysis: pheatmap (Kolde 2019), PCAtools (Blighe et al. 2024), VennDiagram (Chen and Boutros 2022), tidyverse (Wickham et al. 2019), enrichplot (Yu 2025). Gene set enrichment analysis (GSEA) was performed via clusterProfiler (Yu 2024) with the Molecular Signatures Database hallmark gene set (Liberzon et al. 2015). A false discovery rate correction of 5% using a Benjamin-Hochberg procedure was applied. Genes were pre-ranked according to the following equation: sign(fold change) × (-ln(pvalue)).

6.5 Results

6.5.1 iPSC-derived forebrain neurons express markers of neuronal maturation and glutamatergic origin

The human IPSC-derived NPCs ($PAX6^+$ / $Nestin^+$ / $OCT4^-$) utilised for this study were differentiated into forebrain-like neuron cultures that were comprised of mature glutamatergic neurons ($VGLUT2^+$ / $Tuj1^+$), with some astrocytes present ($GFAP^+$) (Figure 6.1). Neuron cultures also expressed pre-synaptic (Synapsin) and post-synaptic (PSD-95) markers consistent with synapse formation by DIV21 (Figure 6.1). As an additional confirmation of synaptic function and glutamatergic origin, iPSC-derived neuron cultures respond to kainic acid, a glutamate analog, measured through increased expression of the target gene *cFOS* (Supplementary Figure 6.S1).

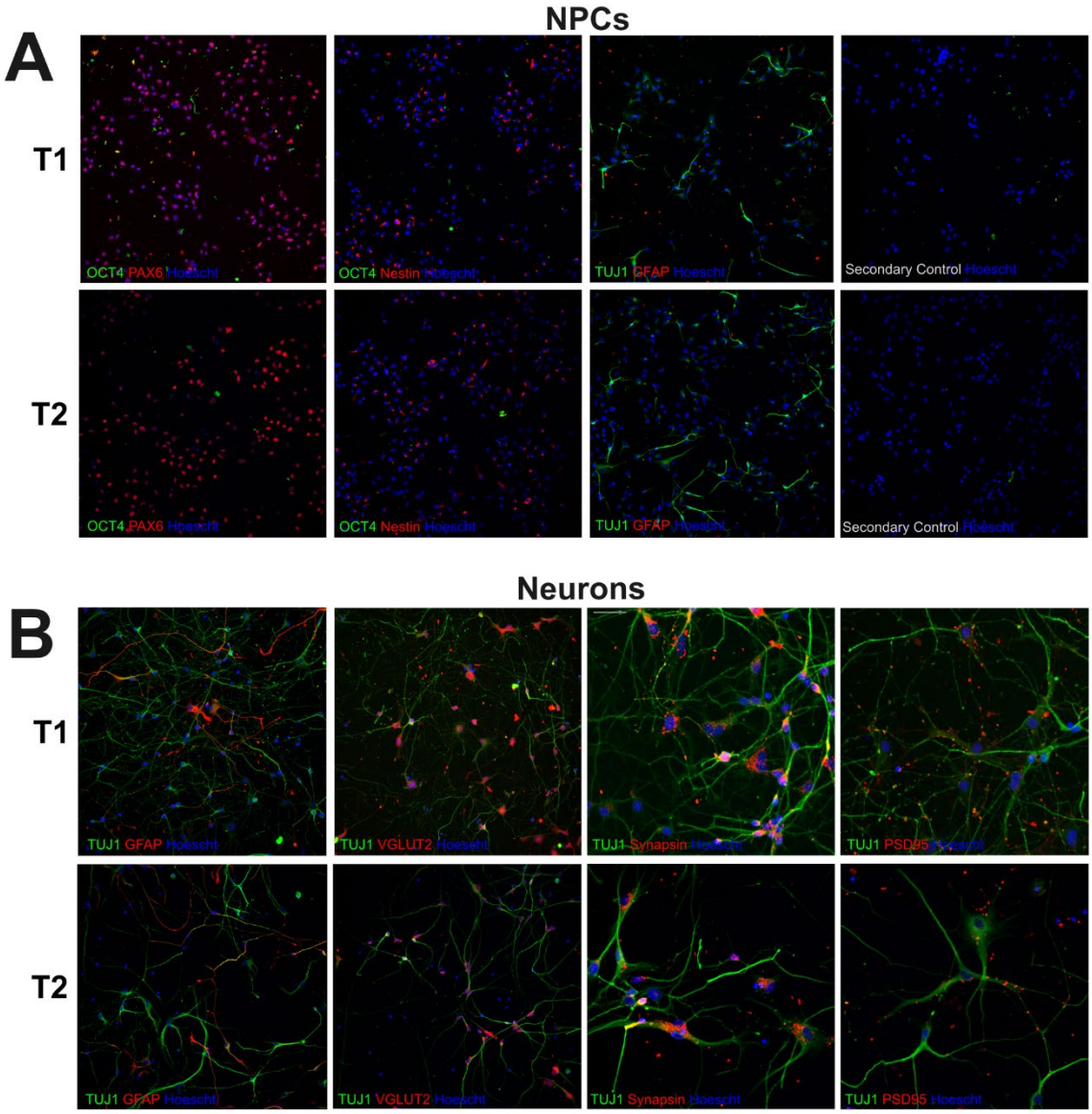


Fig 6.1. Differentiation and characterization of iPSC-derived neuron cultures. iPSC-derived NPCs were differentiated into forebrain-like neuron cultures. A) NPCs stained positively for Pax6 (red) and Nestin (red), and expressed negligible Oct4 (green). B) Differentiated neuron cultures at DIV21 primarily consisted of Tuj1+ (green) and vGLUT2+ (red) cells, with a small percentage of GFAP+ (red) cells. Tuj1+ cells also expressed Synapsin (red) and PSD-95 (red). Cells were counterstained with Hoescht (blue). NPC = neural progenitor cell; T1= independent repeat 1; T2 = independent repeat 2.

6.5.2 Effects of *ex vivo* diesel exhaust exposure on neuronal gene expression

To assess how circulating factors following DE exposure (with or without NAC treatment) modified gene expression in iPSC-derived neurons, we ran a two-way ANOVA (factors *Exposure, Sex*) on delta scores for the DEP and DEN conditions relative to FAP, with independent repeats assessed separately. A total of 578 genes were found to have an *Exposure* main effect ($p < 0.05$) for T1, and 1038 for T2. A PCA restricted to genes with an *Exposure* main effect showed separation between the three exposure conditions, indicating that the DEP and DEN conditions displayed distinct effects relative to FAP (Figure 6.2A)

For the DEP exposure condition, 90 DEGs (35 up/55 down) were significant in T1, and 221 (68 up/153 down) were significant in T2. For the DEN exposure condition, there were 76 DEGs (31 up/45 down) for T1 and 93 DEGs (40 up/53 down) for T2. There was very little overlap of DEGs within each exposure condition between T1 and T2 (Figure 6.2B).

Despite the differences between replicate experiments in terms of specific gene alterations, DE elicited similar pathway level changes (GSEA) between the independent repeats for MTORC1 signaling, oxidative phosphorylation, androgen response and protein secretion pathways (Figure 6.2C), implicating these pathways as potential targets for DE-induced effects on neurons. The only significant pathway found for DEN was protein secretion in T1.

While the DEP and DEN conditions elicited largely distinct effects, a small subset of DEGs were common between conditions (27 for T1, 30 for T2). The fold changes for this subset of genes were strongly correlated between the exposure conditions ($r = 0.95$, $p < 0.001$ for T1, $r = 0.93$, $p < 0.001$ for T2, Figure 6.3).

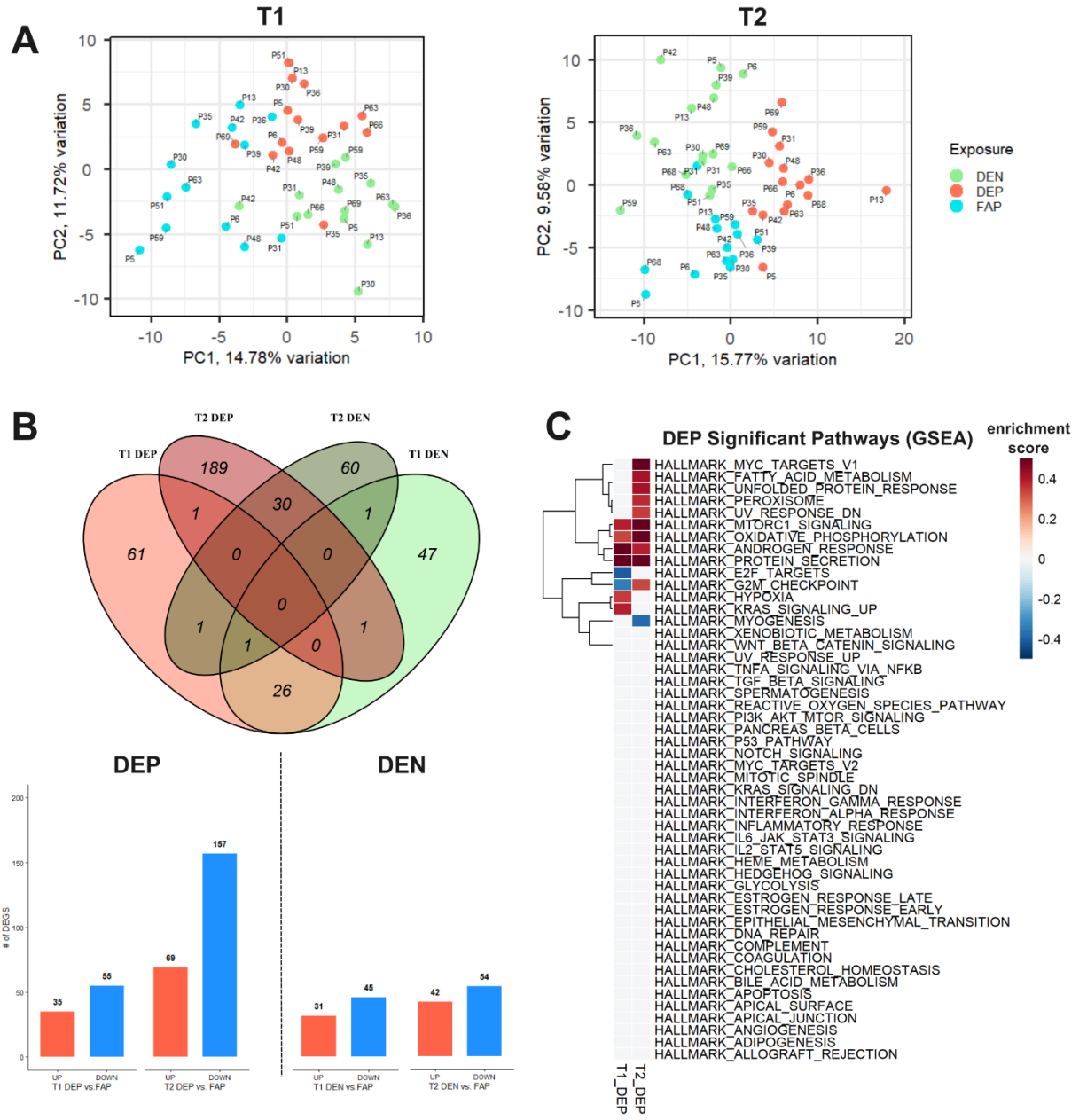


Figure 6.2. Diesel exhaust and N-acetylcysteine elicit transcriptional responses in iPSC-derived neurons. iPSC-derived forebrain neurons were treated with plasma from participants exposed to diesel exhaust +/- N-acetylcysteine. Two-way ANOVA (factors: *Exposure, Sex*), followed by pairwise analysis per comparison was conducted. A) PCA on delta scores of genes with a significant *Exposure* main effect ($p < 0.05$). Labels indicate participant ID. B) Venn diagram of DEGs (*Exposure* main effect $p < 0.05$, pairwise comparison unadjusted $p < 0.05$, $|FC| > 1.2$) from each exposure condition and independent repeat (T1, T2). Bar graph represents number of upregulated and downregulated DEGs. C) Heatmap of enrichment scores from a gene set enrichment analysis (GSEA) of the DEP exposure condition. Only enrichment scores from pathways with a $p < 0.05$ following FDR correction are shown. FAP: filtered air + placebo; DEP: diesel exhaust + placebo; DEN: diesel exhaust + N-acetylcysteine. T1: first independent repeat; T2: second independent repeat.

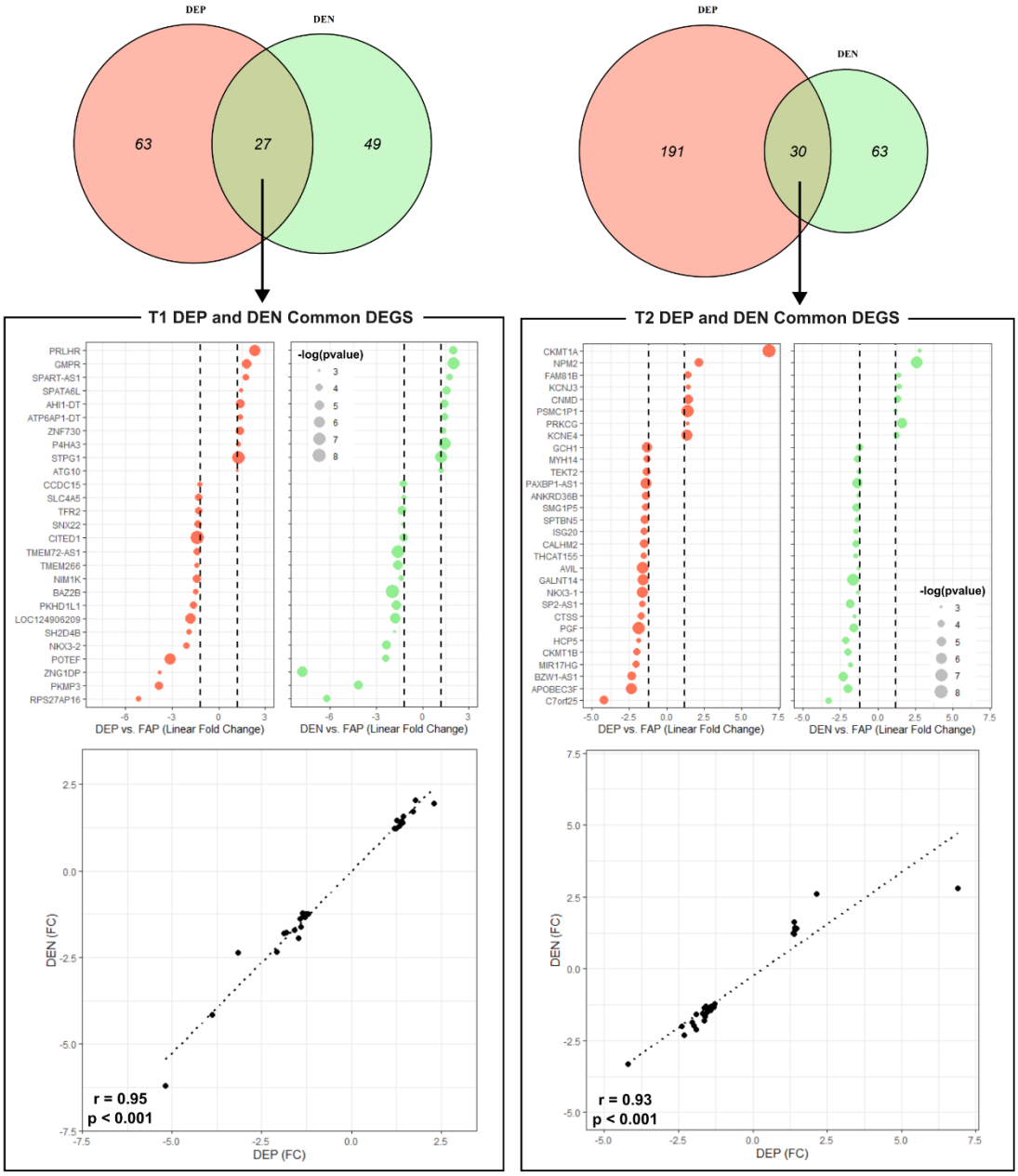


Figure 6.3. DEGs common to diesel exhaust with and without N-acetylcysteine are highly correlated. Plasma from participants exposed to diesel exhaust (+/- N-acetylcysteine) was used to treat iPSC-derived forebrain neurons. Two-way ANOVA (factors: *Exposure, Sex*) followed by pairwise analysis per comparison was conducted. Venn diagram displays DEGs (*Exposure* main effect < 0.05, pairwise comparison unadjusted $p < 0.05$, $|FC| > 1.2$) for each exposure condition, per independent repeat (T1, T2). Dot plots and scatter plots represent fold changes for DEGs significant in both the DEP and DEN exposure condition. Pearson correlation was conducted to determine correlation coefficients between DEP and DEN. DEP: diesel exhaust + placebo; DEN: diesel exhaust + N-acetylcysteine. T1: first independent repeat; T2: second independent repeat.

6.5.3 Sex modifies responses to diesel exhaust and N-acetylcysteine exposure

Our analyses revealed a number of sex-specific DEGs (*Exposure* x *Sex* interaction, $p < 0.05$) within each exposure condition, suggesting that plasma composition is different in females and males in response to DE and NAC exposure. The sex dependent effects in the DEP and DEN conditions appear to be largely independent, as the separation of sexes within DEP compared to DEN occurred across different principal components in a PCA restricted to significant *Exposure* x *Sex* DEGs (Figure 6.4A).

For cells treated with plasma from male participants, the DEP condition had 259 and 433 DEGs for T1 and T2, respectively. The DEN condition had 127 and 313 DEGs for T1 and T2. For cells treated with plasma from female participants, the DEP condition had 104 DEGs for T1 and 96 for T2. For the DEN condition, there were 86 DEGs in T1 and 275 DEGs in T2 (Figure 6.4B).

Both sexes had a subset of genes commonly altered in both exposure conditions, although in males the proportion of overlapping genes was larger (males: 21% / 33% for DEP and 44% / 46% for DEN; females: 16% / 19% for DEP and 19% / 7 % for DEN; T1/T2 of total DEGs per exposure condition), suggesting that NAC may prevent more DE-induced effects in females compared to males.

Pathways altered were sex-specific (Figure 6.4C). For males, DE elicited positive enrichment of protein secretion and UV response (down) pathways, and negative enrichment of the UV response (up) pathway in both repeats. For females, DE elicited positive enrichment of oxidative phosphorylation, adipogenesis, xenobiotic metabolism, cholesterol metabolism, fatty acid metabolism, MTORC signaling, and MYC target pathways in both repeats. For the DEN exposure condition, common between both repeats was negative enrichment of TNF-α signaling

via NF-κB, MYC targets, oxidative phosphorylation, hypoxia, and UV response (up) in males, along with positive enrichment of protein secretion. In females we observed positive enrichment of the TNF-α signaling via the NF-κB and MTORC1 signaling pathways.

6.5.4 Asthma modifies responses to diesel exhaust and N-acetylcysteine exposure

In addition to the impact of sex on modifying responses to DE, we also examined whether responses differed between participants diagnosed with asthma or those with no asthma. It should be noted that males and females were combined for this analysis as there was an insufficient sample size to stratify by both sex and asthma.

Several asthma-dependent effects were observed. There were 499 DEGs with a significant *Exposure* x *Asthma* interaction in T1, and 668 in T2. Plasma from participants with asthma resulted in 34 and 46 DEGs in T1 and T2 respectively for the DEP exposure condition, and 33 and 25 DEGs in T1 and T2 respectively for the DEN exposure condition. Plasma from participants without asthma resulted in 82 and 129 DEGs in T1 and T2 respectively for the DEP exposure condition and 81 and 131 DEGs in T1 and T2 respectively for the DEN exposure condition (Figure 6.5B).

Protein secretion was positively enriched in the DEP condition for both independent repeats, regardless of asthma status. No other pathways were detected in both repeats for the DEP exposure conditions. For the DEN exposure, TNF-α via NF-κB inhibition, epithelial mesenchymal transition, and apical junction pathways were negatively enriched in cells treated with plasma from individuals without asthma (Figure 6.5C).

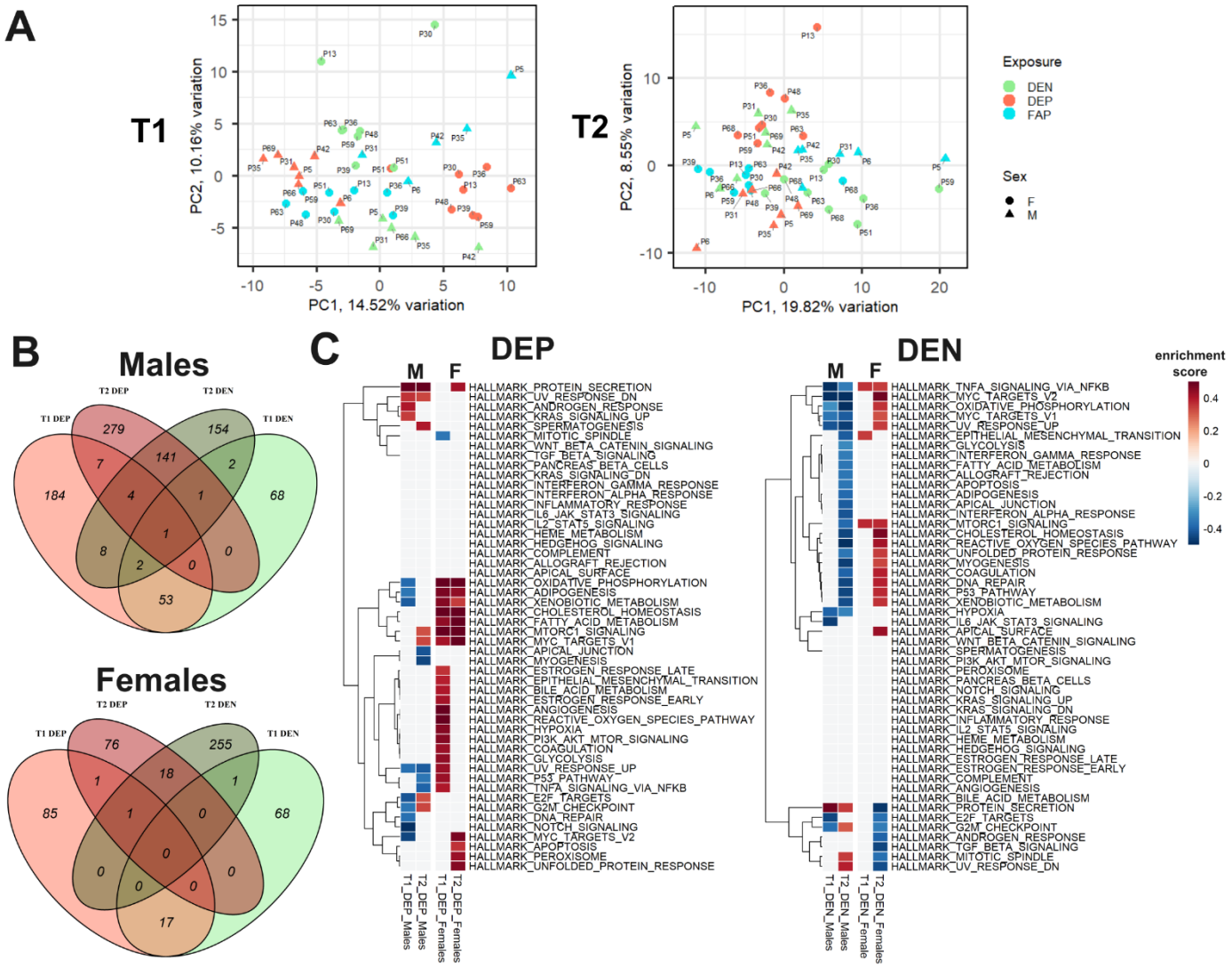


Figure 6.4. Sex modifies *ex vivo* responses to diesel exhaust (± N-acetylcysteine). Plasma from participants exposed to diesel exhaust (± N-acetylcysteine) was used to treated iPSC-derived forebrain neurons. Two-way ANOVA (factors: *Exposure, Sex*) followed by pairwise analysis per comparison was conducted. A) PCA on delta scores of genes with a significant *Exposure* x *Sex* interaction ($p < 0.05$). Labels represent participant IDs. B) Venn diagrams of DEGs (*Exposure* x *Sex* interaction $p < 0.05$, pairwise comparison unadjusted $p < 0.05$, $|FC| > 1.2$) for males and females by exposure condition, per independent repeat. C) Enrichment scores from a gene set enrichment analysis (GSEA). Enrichment scores only displayed for pathways with $p < 0.05$ following FDR correction. FAP: filtered air + placebo; DEP: diesel exhaust + placebo; DEN: diesel exhaust + N-acetylcysteine. T1: first independent repeat; T2: second independent repeat. F: female; M: male.

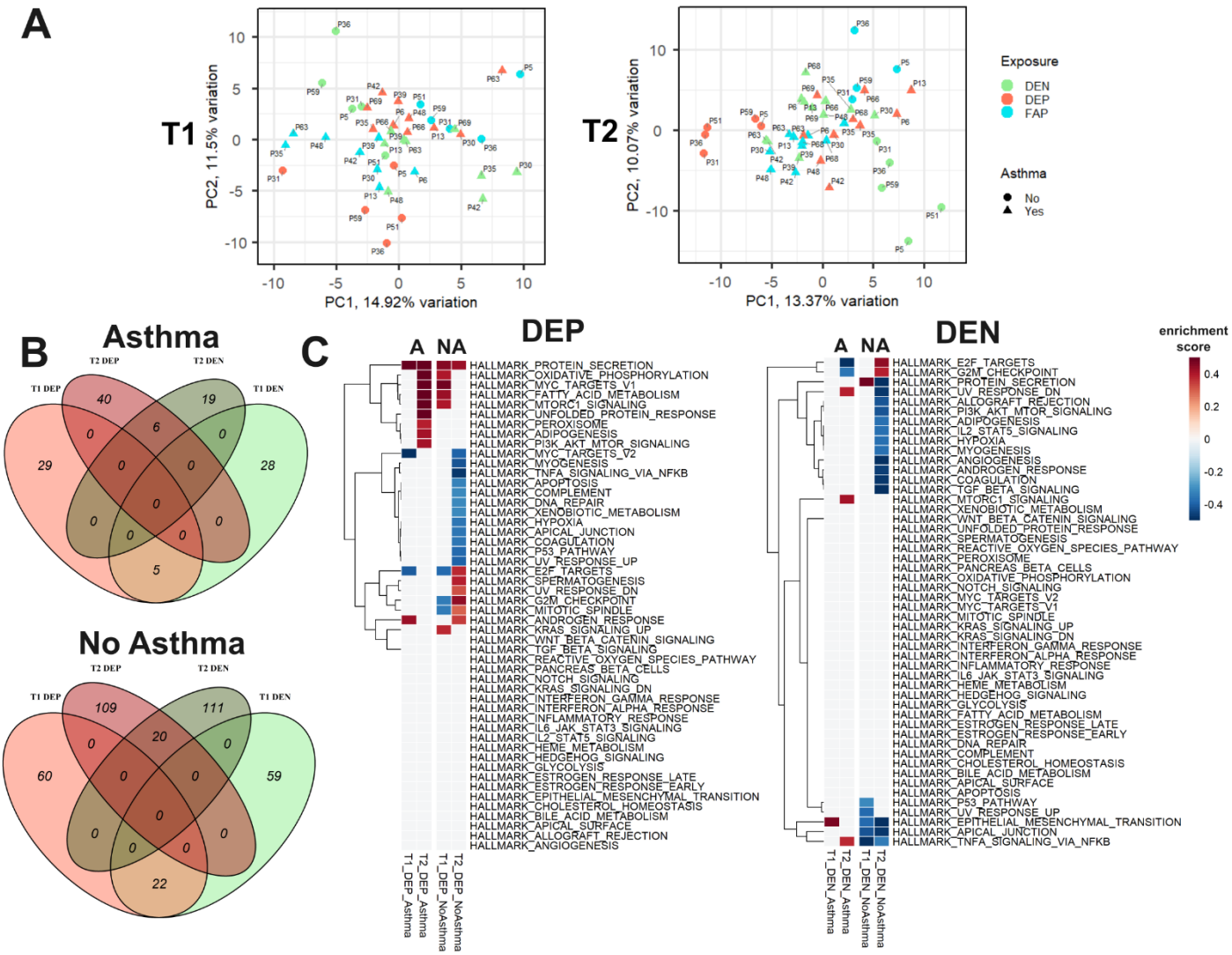


Figure 6.5. Asthma modifies *ex vivo* responses to diesel exhaust (± N-acetylcysteine). Plasma from participants exposed to diesel exhaust (± N-acetylcysteine) was used to treated iPSC-derived forebrain neurons. Two-way ANOVA (factors: *Exposure, Sex*) followed by pairwise analysis per comparison was conducted. A) PCA on delta scores of genes with a significant *Exposure* x *Asthma* interaction ($p < 0.05$). Labels represent participant IDs. B) Venn diagrams of DEGs (*Exposure* x *Asthma* interaction $p < 0.05$, pairwise comparison unadjusted $p < 0.05$, $|FC| > 1.2$) for participants with asthma and with no asthma by exposure condition, per independent repeat. C) Enrichment scores from a gene set enrichment analysis (GSEA). Enrichment scores only displayed for pathways with $p < 0.05$ following FDR correction. FAP: filtered air + placebo; DEP: diesel exhaust + placebo; DEN: diesel exhaust + N-acetylcysteine. T1: first independent repeat; T2: second independent repeat. A: asthma; NA: no asthma.

6.6 Discussion

In the present study, we applied an *ex vivo* approach to assess whether systemic mediators may contribute to pollutant-induced effects in neurons. This is the first study, to our knowledge, to use an *ex vivo* approach to examine the impacts of air pollutants on human neurons. It builds on previous work that established that differential gene expression in a rodent *ex vivo* model is consistent with gene expression responses in brain tissue following pollutant exposure (Rose and Thomson 2025). Human iPSC-derived neuronal cells were responsive, at the transcriptional level, to DE and NAC- induced changes in bioactive factors present in plasma. Effects varied according to sex and asthma status, confirming the usefulness of this approach in identifying differences in responses across population groups. Additionally, many DE-induced effects were absent in cells treated with plasma from participants administered NAC prior to DE exposure, further implicating oxidative stress and inflammatory processes as underlying mechanisms.

Circulating factors present following DE exposure altered several transcriptional pathways that importantly contribute to neuronal development, function, synaptic plasticity and axonal elongation (Harris et al. 2012; Rumpf et al. 2023). The protein secretion pathway, especially in males, was upregulated in response to DE, both with and without NAC treatment. Several of the core enrichment genes within this pathway are related to protein trafficking and transport of vesicles, a critical function necessary for proper communication of neurons (Horton and Ehlers 2004; Kennedy and Hanus 2019). In females only, DE exposure resulted in enrichment of genes associated with metabolic pathways, which tended to relate to energy generation and biomolecule synthesis.

The brain tends to equip itself to respond to potential harm by undergoing structural and functional reorganization, an energy intensive process (Gazerani 2025). Brains of individuals

with mild cognitive impairment tend to be hypermetabolic (Ashraf et al. 2015), and exhibit upregulation of genes involved in energy generation, anabolic and catabolic pathways, and protein trafficking, which is hypothesized to be a compensatory action to overcome early pathology. This contrasts with a pattern of downregulation of these pathways and a hypometabolic phenotype in brains from AD patients (Berchtold et al. 2014). A study examining chronic stress responses found that stressed mice had elevated expression of genes involved in oxidative phosphorylation in the prefrontal cortex, which may have been to compensate for the reduced mitochondrial respiration and brain glucose levels, which occur in the context of behavioural impairments (Weger et al. 2020). Similarly, the brain may respond to certain plasma mediators by activating these pathways to respond to stressors. Several upstream regulators - such as insulin or brain derived neurotrophic factor are present in circulation that can activate these metabolic processes in neurons (Marosi and Mattson 2014; Takei and Nawa 2014). Chronic dysregulation of these pathways may also lead to pathological outcomes. For instance, overactivation of mTOR signaling, which promotes protein synthesis and cell growth and inhibits autophagy, has also been observed in post-mortem brains of individuals with AD (Li et al. 2005; Tramutola et al. 2015), and treatment with mTOR inhibitors has been shown to reduce amyloid beta loads in transgenic AD mouse models and rescue early cognitive deficits (Caccamo et al. 2010; Jiang et al. 2014; Cassano et al. 2019; Hussong et al. 2021). While the present study reflects acute responses to a single short-term exposure, which in and of itself may not be expected to lead to a pathology, it is notable that results from acute crossover exposure studies (including under the same exposure conditions as the present study) demonstrate that even acute exposures can lead to temporary impairments in cognitive performance (Mallach et al. 2023; Curran et al. 2025) and functional connectivity of the default mode network (Gawryluk et al.

2023). Our results suggest that such effects may be accompanied by alterations in expression of genes involved in pathways relevant to neuronal communication, function, development, and plasticity.

The largely sex-dependent *ex vivo* effects of DE align in a general sense with epidemiological literature reporting sex-based differences in susceptibility to pollutant exposure for neurocognitive health outcomes (Chen et al. 2025), although patterns of effects vary between population cohorts and pollutants assessed, and may be confounded by several societal factors. Additionally, rodent studies show elevated susceptibility to neuropathology in males compared to females following exposure to ozone (Akhter et al. 2015) or DE (Cole et al. 2016; Coburn et al. 2018; Ehsanifar et al. 2021). However, which risk factors govern sex-based differences in response remains especially unclear. As *ex vivo* DE effects separated based on whether plasma was collected from males or females, plasma composition must differ by sex following DE-exposure.

Examining the effects of NAC, a drug known primarily for its antioxidant and anti-inflammatory properties, in modulating DE-induced effects provided insight regarding potential mechanisms driving these sex-dependent differences. Administration of NAC prior to DE-exposure resulted in several distinct *ex vivo* effects compared to preceding DE exposure with placebo, suggesting oxidative stress and inflammatory pathways may contribute to DE-induced effects. This notion is further supported by the established role of oxidative stress in regulating pathways modified by DE such as MTORC1 signaling and oxidative phosphorylation (Heberle et al. 2015). While NAC prevented a subset of DE-induced effects in both males and females, a greater proportion of DE-induced effects were prevented by NAC in females compared to males. This may indicate that males have a systemic response to DE that is less modified by NAC, or

may be due to sex-dependent effects of NAC on these pathways. Interestingly, responses in the DEN condition, particularly for males, largely aligned with expected biological responses to NAC. Negative enrichment of inflammatory pathways and oxidative stress pathways were observed, in line with the antioxidant and anti-inflammatory capacity of the drug, as well as c-Myc signaling, with which NAC has been shown to interact (Alizadeh et al. 2022; Yao et al. 2023). These NAC-induced effects however, were not observed in cells treated with plasma collected from females, implying sex-dependent differences in response to NAC. While some studies have reported sex-dependent effects of NAC (Nie et al. 2016; Goenaga et al. 2020), a general lack of literature examining how responses to NAC may differ by sex – especially in the brain, or with respect to plasma mediators – prevents further interpretation of these results. Of note, oxidative stress responses are sex-dependent, with females being generally more resistant to oxidative stress than males (Kander et al. 2017), which may contribute to the sex-specific effects of DE and NAC observed.

In addition to sex-dependent effects, our data provide some evidence that asthma may modify susceptibility to effects of air pollution on the brain. This is consistent with work by Cho *et al.* who found that individuals with asthma were at elevated risk of emergency department visits for a depressive episode following pollution exposure, compared to non-asthmatics (Cho et al. 2014). Participants from the present study with asthma exhibited greater elevation of circulating cortisol following DE exposure compared to those without asthma (Thomson et al. 2021), again suggesting differential sensitivity according to disease status. While certain neuronal *ex vivo* responses did differ based on asthma status, there were very few effects observed in the asthma group. The only pathway commonly enriched by DEP in both experimental repeats was protein secretion, for both asthma and non-asthma groups alike.

However, it should be noted that this relative lack of effects may be a result of combining the sexes together – necessary due to sample size considerations – potentially obscuring any effects of asthma that differ by sex.

iPSC-derived neurons share functional similarities with neurons found in the brain, making them a valuable, yet accessible model to study neuronal responses. Unlike traditional cell lines which are more homogeneous, iPSC-derived neurons are highly sensitive to the differentiation process. This sensitivity is reflected in mixed cultures of neurons with other cell types (usually glial cells), and large variability between transcriptomic profiles – even between cells cultured under the same protocol – when compared across laboratories and between technical replicates induced on separate days within laboratory (Volpato et al. 2018). Previous work has shown that such variability led to very little overlap of detectable DEGs between laboratories when comparing iPSC-derived cells originating from a healthy control individual to a familial AD patient (Volpato et al. 2018). Given this expected variability, the strong correlation of commonly altered genes between DEP and DEN exposure conditions in our study supports the notion that cells are responsive to plasma factors altered by DE exposure in a highly reproducible manner within a given experimental repeat; however, independent experimental repeat remained the largest source of variation in our study (Supplementary Figure 6.S2), despite transcriptomic profiles being highly correlated for all samples. iPSC-derived neurons are generally cultured under serum-free conditions to allow for proper differentiation; addition of plasma to cell cultures significantly modifies the baseline transcriptomic profile (Supplementary Figure 6.S3), which may also contribute to variability observed. Despite the gene-level variation, it is noteworthy that there was similarity in biological response at the pathway level. This is consistent with the notion that biological processes may be more readily reproduced than

changes in individual genes (Weis and Members of the Toxicogenomics Research Consortium 2005).

In conclusion, we have shown that human iPSC-derived neurons are responsive to bioactive factors present in plasma following pollution exposure, and that, while expression of specific genes vary, pathway-level changes in gene expression are reproducible between independent experimental repeats, providing insight into biological effects. The *ex vivo* model described here may be a useful tool to study systemic contributions to air pollution-induced effects on the brain, using accessible human samples (plasma). As sex and asthma modified *ex vivo* responses, this approach also allowed for the examination of how individual characteristics may influence susceptibility to air pollution-induced health outcomes. While outside the scope of the present study, coupling this approach with pharmacological intervention would provide a means to elucidate contributions of specific underlying mechanisms and improve mechanistic understanding of effects. Given the prevalence of neurodegenerative and psychiatric diseases within the population, air pollution, a ubiquitous exposure, may be a critical modifiable factor contributing to these diseases. Application of *ex vivo* models provides a tool to support the identification of mechanisms and susceptibility factors governing the effects of air pollution in contributing to these diseases.

6.7 Acknowledgements

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6.8 Data availability

The RNA-sequencing data underlying in this publication have been deposited in NCBI's Gene Expression Omnibus and are openly available under GEO accession GSE351067 [https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE351067].

6.9 Supplemental figures

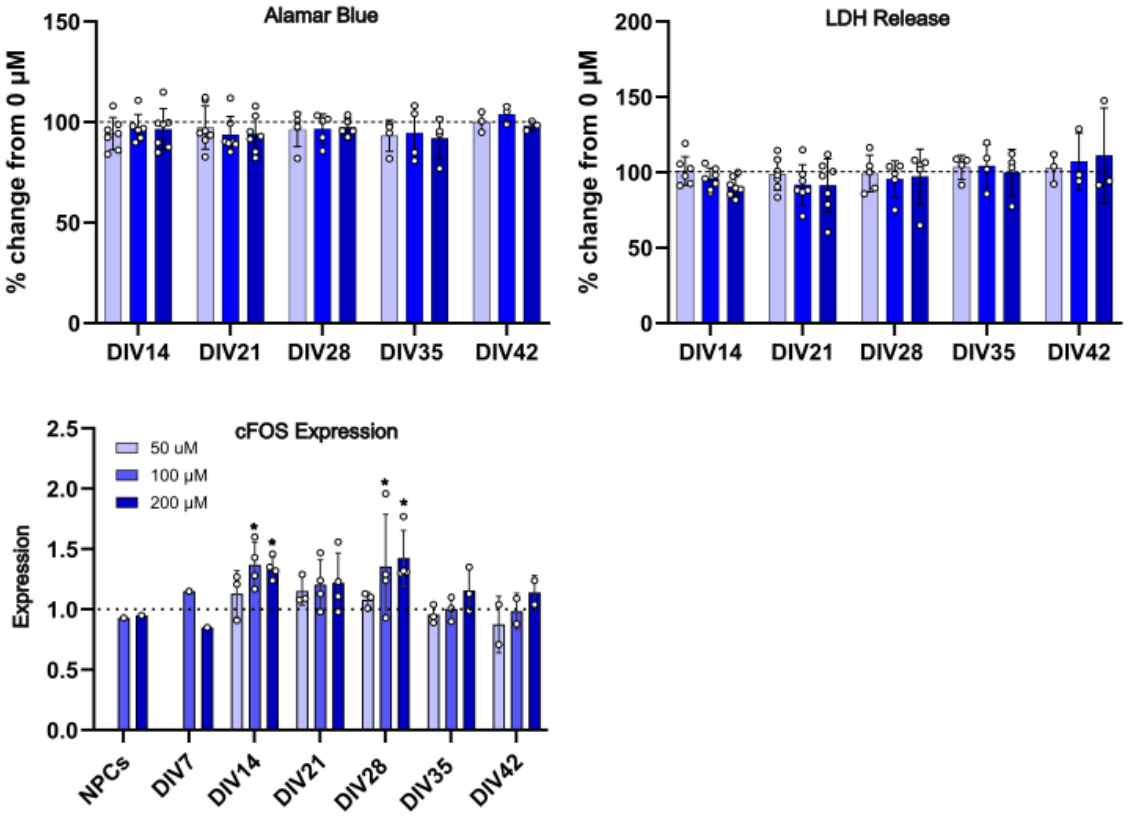


Figure 6.S1. Response of iPSC-derived neurons to kainic acid. NPCs or iPSC-derived neurons (DIV 7, 14, 21, 28, 35, 42) were treated with kainic acid [0, 50, 100, 200 µM] for 1 h. LDH release, resazurin reduction (alamar blue assay) were measured following exposure, and cFOS expression was measured in cell lysate via RT-qPCR. Statistical analysis performed via Two-Way ANOVA (factors: *Kainic Acid, DIV*) followed by post-hoc Holm Sidak for multiple comparisons, for DIV14-42 cells. NPCs and DIV7 cells excluded from statistical analysis due to *N* of 1. Bars represent geomean ± SD. Points represent independent repeats. * $p < 0.05$ relative to DIV-matched 0 µM dose (dotted line). NPC: neural progenitor cell; LDH: lactate dehydrogenase.

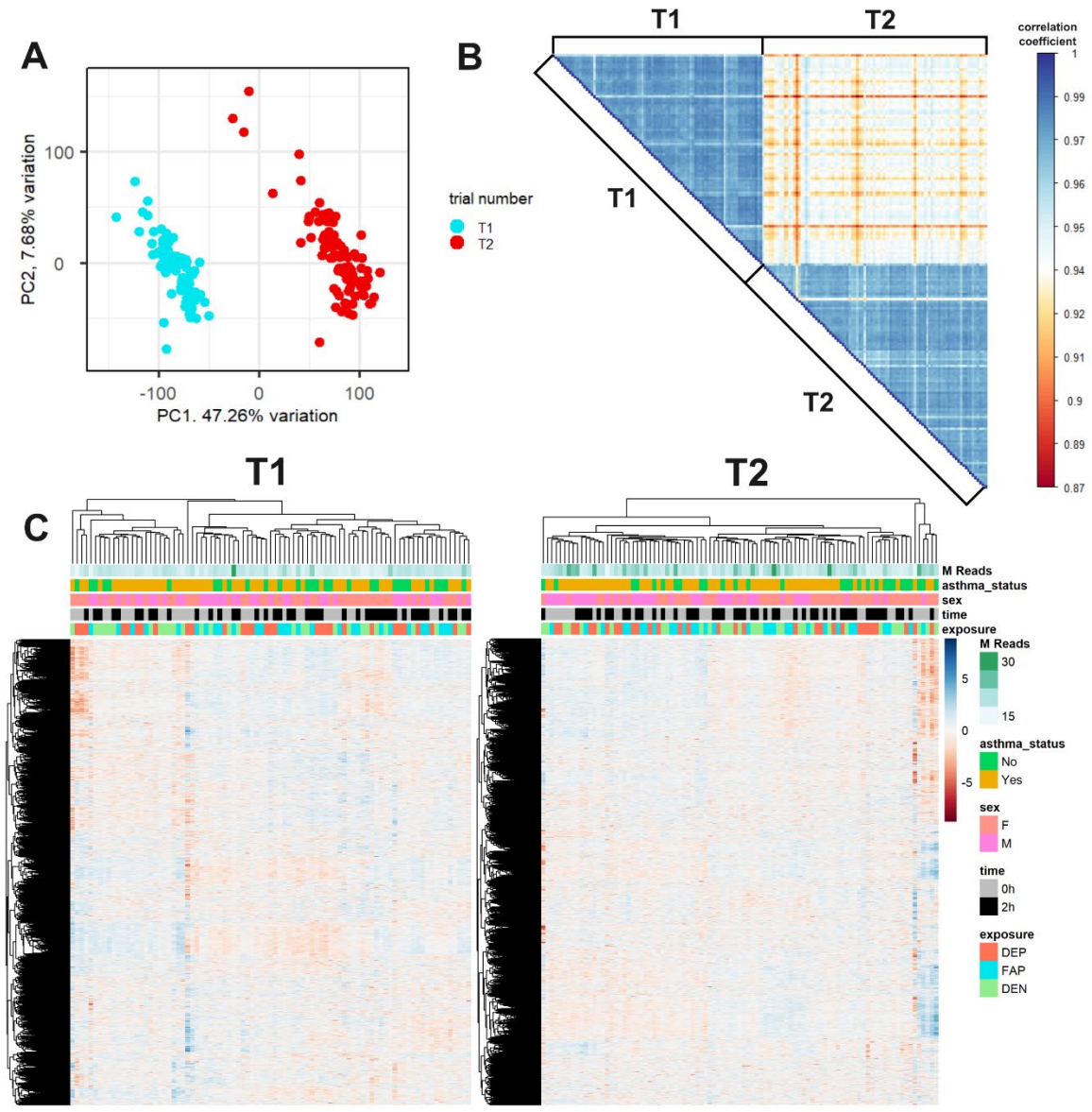


Figure 6.S2. Comparison of transcriptomic profiles across and within independent experimental repeats. Normalized counts of transcriptomic data from iPSC-derived neurons, treated with plasma from individuals the diesel exhaust study, in two independent experimental repeats (T1 and T2). A) PCA of normalized counts. B) Correlation plot displaying Pearson correlation coefficients of normalized counts for all samples in both repeats. C) Heatmap of normalized counts (z-score), annotated by intra-repeat variables. T1: first independent repeat; T2: second independent repeat.

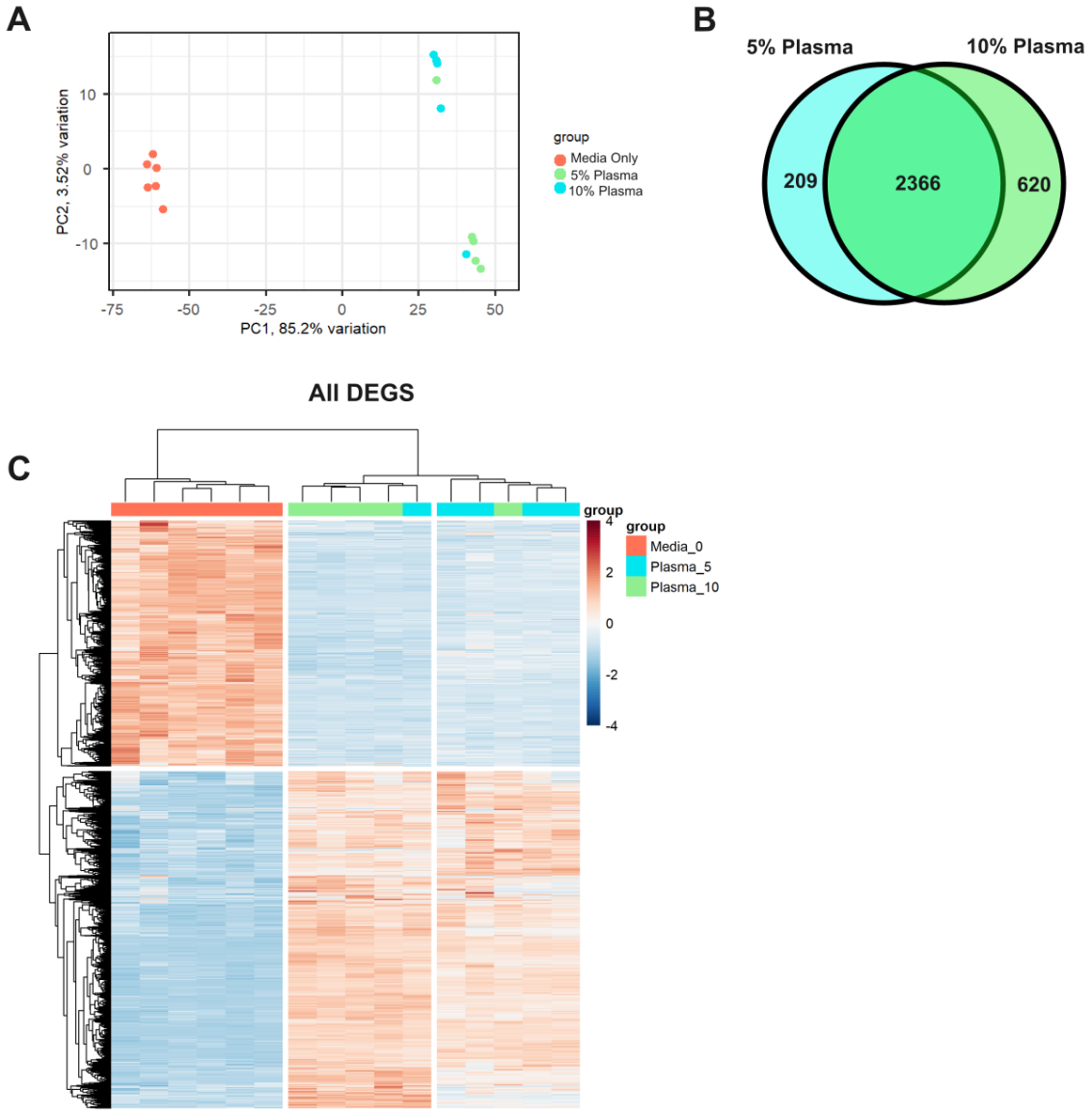


Figure 6.S3. Effect of plasma treatment on iPSC-derived neurons. iPSC-derived neurons (DIV21) were treated for 4 h with a plasma sample [0, 5, 10% in cell culture media] pooled from samples collected from study participants 18 h prior to first exposure. DEGS were defined as |FC| > 1.5 and FDR-adjusted p-value > 0.05. A) PCA restricted to DEGS, B) Venn Diagram showing # of DEGS of each plasma dose relative to media only controls, C) Heatmap of all DEGS, representing z-score of counts per million.

Chapter 7: General Discussion

7.1 Overview

The work described in this thesis demonstrates that a) air pollution alters transcriptional processes in the brain, b) HPA axis activation is an important mediator of air pollution induced effects on the brain, c) tissue-specific modification of metabolic outcomes are also a result of glucocorticoid-dependent and independent actions following pollution exposure, and d) an *ex vivo* approach is a suitable tool to investigate air pollution health effects in a human-relevant model that reflects systemic contributions. Overall, this thesis illustrates the importance of systemic processes as drivers of health impacts in the CNS due to air pollution exposure, and establishes a means to collect human-relevant data regarding the biological impacts of pollution on the brain.

These findings are placed in context with the fact that air pollution exposure is associated with increased risk of neurodegenerative and neuropsychiatric diseases (Power et al. 2016; Allen et al. 2017), which present a significant population health burden. While several rodent toxicological studies have provided evidence that air pollution exposure leads to neuropathological outcomes and disease phenotypes, there remains a need to better understand the mechanisms that drive these effects, especially in human-relevant models. Systemic mechanisms activated by pollution exposure, such as HPA axis activation, may be important mediators, impacting the brain through direct actions or through changes in associated metabolic outcomes. Studying the role of these mechanisms in a human context is especially challenging, due to a current lack of methods that can incorporate systemic mediators into the study of brain effects in humans. In this thesis, I sought to address these challenges by 1) investigating the contribution of GCs in mediating air pollution-induced effects on the brain and peripheral

metabolic outcomes, and 2) establishing an *ex vivo* approach to assess the systemic effects of air pollution exposure on the brain using human-relevant cell culture models.

Thus, using samples collected from an *in vivo* ozone exposure model with pharmacological intervention, I established that GCs drive both air pollutant-induced effects in the brain and peripheral metabolic outcomes that include changes to insulin signaling and lipid metabolism. I then demonstrated the utility of an *ex vivo* approach in studying pollutant-induced effects in human-relevant brain cell cultures. I established that *ex vivo* responses reproduce effects in the brain with a rodent model of acute air pollution exposure, then extended this approach to the human context by distinguishing systemic responses following DE exposure in a human iPSC-derived neuronal cell culture model. This chapter will contextualize these findings in a broader scope, synthesizing knowledge from the prior chapters and scientific literature.

7.2 Air pollution exposure alters transcriptional processes in the brain via systemic mediators

To examine how acute exposure to air pollutants might alter pathways of interest within the brain, I assessed transcriptional responses using *in vivo* and *ex vivo* approaches. Gene expression is a sensitive and responsive measure that can be used to assess initial perturbations following acute pollutant exposure as gene expression changes often precede other toxicity endpoints (Thomson et al. 2013; Joseph 2017). While changes to gene expression do not always correspond to phenotypic changes, several critical enzymes and proteins are regulated at the gene expression level, and toxicological data supports correspondence between transcriptomic responses and other toxicity measures in target organs (Waring et al. 2001; Sellamuthu et al. 2011, 2012).

Acute ozone exposure led to alterations of several genes of interest within the hippocampus, a stress-sensitive region of the brain responsible for learning and memory formation. In line with observations in other tissues (Thomson et al. 2016), GC and cellular stress responsive genes *SGK1, MT-1, MT-2* and *HIF3α* were elevated, whereas proinflammatory factors *TNFα* and *IL-1β* were inhibited. Expression profiles of *SGK1* and *MT-1* were also broadly consistent across different brain regions, as measured in a prior publication using samples from the same exposure study (Thomson et al. 2019). Several serotonin receptors, neurotrophic factors (*BDNF, TRKB, VEGF, BCL-2*) and members of the hedgehog signaling pathway (*GLI, SMO*; Supplementary Figure 2.S1) were altered as well, aligning with the hypothesis that air pollution may impact neurogenesis and cell death (Rivas-Arancibia et al. 2015; Woodward et al. 2018; Hedges et al. 2019). These findings substantiate and expand on prior evidence (Henriquez et al. 2019; Thomson et al. 2019) that the brain is responsive to systemic processes such as HPA axis activation following pollutant exposure, which is discussed further in section 7.3.

The human *ex vivo* model used in this thesis also confirmed that systemic factors following pollution exposure can modify transcriptional responses in brain cells. Exposure to DE resulted in *ex vivo* modification of pathways associated with energy generation and biomolecule synthesis, which are critical for synaptic plasticity and neuronal functioning. Activation of these pathways may compensate for insult, as individuals with mild cognitive impairment demonstrate an upregulation of these pathways and a hypermetabolic phenotype (Berchtold et al. 2014), and mice exposed to chronic stress demonstrate upregulation of genes involved in oxidative phosphorylation, despite reduced mitochondrial respiration, brain glucose levels, and behavioural impairments (Weger et al. 2020). Chronic dysregulation of these pathways may also lead to

pathological outcomes; overaction of mTOR signaling for instance associates with AD-pathology (Li et al. 2005; Tramutola et al. 2015; Cassano et al. 2019; Hussong et al. 2021).

While the genes that were altered in the *in vivo* model by ozone were not changed *ex vivo* by DE, this difference in responses between the two models could be due to several factors including species, timing of exposures, or pollutant used (ozone vs. DE). Regardless of the differences between these models, both provide clear evidence that systemic processes can contribute to air pollution induced effects on the brain and provide insight into potential pathways which may contribute to in a chronic exposure setting to disease-related processes.

7.3 Activation of the HPA axis is a key contributor to air pollution-induced effects

In this thesis, a pharmacological inhibition model was employed to investigate whether air pollution-induced effects on the brain and peripheral metabolic outcomes are dependent on increases in circulating GCs following exposure. Metyrapone, an 11β-hydroxylase inhibitor, abrogates the ozone-induced increase in circulating GCs (Thomson et al. 2016). Several ozone induced effects on the hippocampus were prevented by metyrapone treatment, including regulation of serotonin receptors (*5-HT2A, 5-HT2C, 5-HT7*), neurotrophic factors (*IGF1, BCL2, TRKB*), and genes involved in kynurenine metabolism (*MAO-A, KMO*). Additionally, a subset of metabolic outcomes (lipid metabolites and insulin signaling pathway genes) following acute ozone exposure were also prevented by metyrapone. Furthermore, treatment of an additional groups of rats with exogenous CORT prior to exposure to clean air reproduced ozone-induced effects on both hippocampal gene expression and lipidomic changes (after 24 h recovery) providing additional confirmation of the role of GCs in mediating these effects. This aligns with several previous publications from our laboratory and others that GCs mediate air pollution-induced effects throughout the body in rodent exposure studies (Thomson et al. 2016; Thomson

2019; Henriquez et al. 2019). These GC-mediated effects are further substantiated by scientific literature supporting a role for GCs in regulation of neurogenesis (Gould et al. 1997; Tanapat et al. 2001; Coe et al. 2003) and serotonin receptor expression (Holmes et al. 1997; Le Corre et al. 1997; Dwivedi et al. 2005; Lee et al. 2009).

Beyond the *in vivo* findings, the *ex vivo* model was responsive to pollutant-induced increases in GCs as well. Treatment of both primary hippocampal neurons and primary microglia with plasma from ozone exposed rats elevated expression of GC-responsive genes (*GILZ, SGK1, MT1, MT2, HIF3α*), which was not observed in cells treated with the plasma from the rats administered metyrapone. Similarly to what we detected *in vivo*, gene expression changes in neurons treated with plasma from ozone exposed rats correlated with those observed in neurons treated with plasma from rats exogenously administered CORT prior to air exposure. The current data does not allow elucidation of whether these *ex vivo* effects are directly due to GCs, or whether they are a result of other factors released into the plasma as a secondary response to HPA axis activation. However, given that treatment of cells with physiologically relevant GC concentrations induced similar expression patterns in cells, that GCs can cross the BBB (McEwen et al. 1968), and that effects were observed in plasma collected immediately post exposure in which GC levels are elevated, but which may precede other metabolic effects such as lipidomic changes, it seems likely that GCs may be directly responsible. Addition of GC receptor antagonists, such as the GR antagonist RU486, to cell culture in addition to plasma could shed further light on the exact mechanisms responsible.

Together, the data from this thesis indicate that GCs may be an important early initiator of disease processes in the brain following exposure to air pollution. Notably however, a subset of effects – both within the brain and periphery – were not prevented by metyrapone, implying that

GCs are not solely responsible, but rather act in tandem with other systemic processes to lead to effects in the brain and potentially increase risk of disease.

An important consideration is that this study utilises an acute exposure model to investigate early initiating events without complication by potential disease-induced effects following a chronic exposure paradigm. Several of the findings presented within this study, as well as previous data collected from this model (Thomson et al. 2016), appear to indicate a general anti-inflammatory phenotype which is contradictory to the general consensus that air pollution exposure leads to chronic proinflammatory responses. GCs are well-known anti-inflammatory agents. It is therefore unsurprising that under acute exposure conditions, a rise in GCs corresponds with a general anti-inflammatory phenotype. This is substantiated by findings that preventing the ozone-induced increase in GCs with metyrapone exaggerated inflammatory responses compared to vehicle controls, both in the lungs and plasma (Thomson et al. 2016). However, under chronic stress conditions in which GCs are persistently elevated, the body can develop GC resistance (Stark et al. 2001; Cohen et al. 2012), a condition in which GCs no longer effectively downregulate immune responses. This can exacerbate symptoms and development of inflammatory-diseases such as asthma (Barnes and Adcock 2009), as well increase susceptibility to infection, and may explain the coexistence of both elevated cytokines and cortisol levels in individuals with Major Depressive Disorder (Perrin et al. 2019). While this thesis utilizes acute exposure models, in the "real-world" we are all chronically exposed to air pollution throughout our lifetime representing a form a chronic stress. Therefore, air pollution exposure may induce GC resistance, contributing to dysregulation of inflammatory responses and development of chronic low-grade inflammation. Some human exposure studies indicate that air pollution may reduce effectiveness of inhaled corticosteroids on asthma symptoms; however these are only

associations, and may have other explanations such as worsening asthma severity (reviewed in Rider & Carlsten, 2019). Cigarette smoke is also shown to induce GC resistance in individuals with asthma (Chaudhuri et al. 2003) or chronic obstructive pulmonary disease (Wang et al. 2025). Air pollution exposure may also modify use of inhaled corticosteroids, with one study showing that exposure to PM correlated with increased use in a German cohort (Klot et al. 2002), representing another mechanism through which pollutant may confer GC resistance. Currently, there are no experimental animal studies that characterize whether chronic exposure to air pollution induces GC resistance.

7.4 Changes in peripheral metabolic features following pollution exposure may provide insights into potential brain health effects

Beyond the direct impacts that GCs (and other systemic processes) may have on the brain, they can also provoke further peripheral changes which could disrupt brain functioning. Metabolic diseases such as T2D, insulin resistance and obesity often coincide with neurodegenerative and neuropsychiatric conditions (Barbagallo and Dominguez 2014; Yue et al. 2025), and these diseases feature several commonly affected pathways such as insulin signaling and lipid metabolism (Kulas et al. 2020). Notably, disruptions of HPA axis activation are common amongst metabolic, neurodegenerative and psychiatric diseases alike (Di Dalmazi et al. 2012; Ouanes and Popp 2019). It is therefore possible that air pollution, through actions of systemic mediators such as GCs, may induce widespread metabolic changes that contribute to both peripheral and CNS-based disease development. To gain a better of understanding of how air pollution may modify risk of these diseases, I examined systemic changes in these commonly dysregulated metabolic features following air pollution exposure, leveraging samples collected from a rat acute ozone exposure model, and studied the role of GCs in mediating these effects.

Rats acutely exposed to ozone had modifications to expression of several genes in the insulin signaling pathway across the liver, brown and white adipose, and skeletal muscle. Results generally point to inhibition of the insulin signaling cascade but activation of glycolytic pathways, particularly in the liver. Transcriptomic alterations of these key genes may be an important method through which air pollution impairs glucose tolerance and confers insulin resistance, as these occur in the absence of any changes in circulating insulin (Thomson et al. 2016) or glucose within this acute exposure model. Interestingly, only very few effects on insulin signaling pathway genes were observed in the hippocampus (Supplementary Figure 3.S3). This is despite the hippocampus being a target for changes in expression of serotonergic receptors and neurotrophic factors following ozone exposure (Chapter 2). This may reflect temporal differences, regional differences in the brain or more cellular-specific action which could not be picked up in whole tissue homogenate. The brain is an insulin-responsive organ, and impairment of insulin signaling is associated with worse cognitive outcomes, atrophy, attenuation of synaptic plasticity, and elevated Aβ plaque load (reviewed in Lee et al., 2016). It would therefore be of interest for future studies to further investigate whether air pollution exposure directly impacts this pathway within the brain as this could be a potential mechanism of disease development. Alternatively, it is possible that air pollution does not specifically impact these pathways in the brain, but rather metabolic impairments in the periphery could secondarily lead to insulin resistance in the brain or other pathological effects over time.

In addition to gene expression changes, ozone exposed rats also demonstrated perturbations of circulating metabolic features, such as sphingolipid metabolism, kynurenine and tryptophan, in addition to the previously reported decrease in levels of glucagon and ghrelin, and increase in the antifibrinolytic factor PAI-1 (Thomson et al. 2016). Altered peripheral

concentrations of several of these metabolites are associated with neurocognitive outcomes and may play roles in inducing or exacerbating pathology. For instance, kynurenine pathway metabolites regulate neuroinflammation and neuronal excitability – kynurenic acid and quinolinic acid are N-methyl-D-aspartate receptor antagonists and agonists, respectively (Comai et al. 2025). Elevated kynurenine/tryptophan ratios in circulation are associated with AD (Widner et al. 2000) and suicide (Bradley et al. 2015). Circulating PAI-1 is also found to be elevated in AD patients (Angelucci et al. 2023), and may contribute to AD pathology by inhibiting plasmin, an enzyme which degrades Aβ (Barker et al. 2010) and cleaves proBDNF to mature BDNF (Pang et al. 2004).

While lipidomic changes following acute ozone exposure did not correlate with those found in a transgenic AD rat model, sphingolipid levels following ozone exposure did negatively correlate to those associated with aging. This may be a result of the anti-inflammatory actions of GCs, which regulated many of the lipidomic effects observed. Following chronic exposure however, GC resistance could result in disruption of these pathways more in line with pro-inflammatory, aging-like phenotype, which has been observed in other chronic exposure and population-based studies (Miller et al. 2015, 2016a; Liu et al. 2022; Xu et al. 2022b; Zhu et al. 2023; Zhang et al. 2024a).

Notably, plasma alterations by ozone which did not appear to be regulated by GCs – kynurenine, tryptophan, and PAI-1 – align with those reported for several disease states, including both metabolic and neurodegenerative conditions (Widner et al. 2000; Bradley et al. 2015; Messaoud et al. 2018; Khoddam et al. 2026), suggesting that GC-independent mechanisms following pollution exposure may lead to metabolic disruptions that could contribute to elevated risk of brain pathology.

These findings, in addition to work by previous studies, paint a picture of widespread metabolic dysregulation following exposure to air pollution, which may explain the increased risk of metabolic diseases observed in populations exposed to higher levels of air pollutants. Although this thesis does not establish whether changes to lipid, kynurenine or insulin signaling pathways directly contribute to pollution-induced effects on the brain, this work provides evidence that air pollution acts on metabolic pathways common to disease, and provides a basis for future studies to further investigate the connection between metabolic changes and brain health outcomes following air pollution exposure.

7.5 A human relevant *ex vivo* model allows for study of systemically-mediated effects of air pollution on the brain

As this thesis illustrates throughout, systemic mechanisms are key drivers of pollution-induced brain effects. Chapters 5 and 6 of this thesis focused on the development of a model to investigate how air pollution affects human brain cells, that could incorporate systemic mediators. Previously, rodent exposure models have been used to investigate the toxicological effects of air pollution exposure. However for several reasons, including ethical concerns, inter-species differences and inability to reflect the heterogeneity of the human population (Pound and Ritskes-Hoitinga 2018), there is a need for a human-relevant approach that can be used to study the effects of air pollution on the brain.

The development of *in vitro* human cell culture models has allowed for the toxicological study of air pollutants on human cells, including brain cells using either neuroblastoma cell lines (Wei et al. 2017; Lin et al. 2022; Li et al. 2022) or iPSC-derived neural cells (Zhang et al. 2022; Park and Choi 2023; Jäntti et al. 2024). However, current models only allow for the study of the direct effects of PM on cells and thus do not incorporate systemic processes. In chapter 5 of this thesis, I demonstrated that an *ex vivo* model in which primary hippocampal neurons were treated

with plasma from ozone-exposed rats reproduces gene expression changes in the hippocampal tissue. This confirms that circulating factors drive the ozone-induced effects observed in the brain (Chapter 2), and establishes the utility of the *ex vivo* model in investigating air pollution-induced effects on the brain. Additionally, this model was able to shed light on effects in other cell types, as plasma from ozone-exposed rats also modified immune-responses of microglial cells. While another study has applied a similar approach in the past to understand how treatment with plasma of ozone-exposed animals affects microglial cells (Mumaw et al. 2016), this is the first time that *ex vivo* findings have been directly related to *in vivo* observations in the brain.

Having established that the *ex vivo* approach can reproduce *in vivo* effects, I extended this approach to the human context. In chapter 6, I show that factors present in plasma following exposure to DE could modify transcriptional responses in human iPSC-derived neuronal cells, with pathway-level changes being reproducible between independent repeats of the experiment, despite gene-level variability. This presents a new opportunity to investigate the impacts of air pollution on the human brain that incorporates systemic processes. Importantly, this can also allow study of how individual characteristics may modify response to air pollution, as we found responses differed by sex and asthma status, aligning with epidemiological literature that generally supports that both sex (Chen et al. 2025) and asthma status (Cho et al. 2014) may influence susceptibility to neurocognitive outcomes following pollution exposure.

This model is not without limitations however, particularly due to the challenges involved in culturing iPSC-derived neurons. Being highly sensitive to the differentiation process, iPSC-derived neuron cultures can vary significantly when induced on separate occasions, even from the same cell source (Volpato et al. 2018). This is a critical factor to consider when interpreting results from this model and understanding its potential uses. While we were able to identify

pathways reproducibly altered by DE and NAC *ex vivo* between independent experimental repeats, gene-level effects varied substantially between repeats. Therefore, it may be difficult to confidently apply this model to a study of select genes of interest. Dynamics of the BBB were also not incorporated into this model, representing a limitation in inferring systemic impact of air pollutants from periphery to the CNS. The BBB is highly selective, and while some pollutant-induced circulating factors, including GCs, are permeable, others may not be able to access the brain. Further, pollutants can impact BBB function, increasing permeability by reducing expression of tight junction proteins in animal models (Woodward et al. 2018; Wang et al. 2026). However studies investigating BBB function in humans following air pollution exposure are extremely limited. One study employing a human 3D BBB model found that nanoparticle exposure disrupted the antioxidant system (Li et al. 2020b). In future studies, the *ex vivo* approach described here could be useful method for further investigation of how pollutant exposure affects the BBB as it involves exposure to plasma following *in vivo* exposure, which better represents the biological system as compared to direct application of particles. Finally, while iPSC-derived neurons do have several physiological properties characteristic of neurons such as synapse formation and electrical activity, it is important to note that *in vitro* cell culture models are not representative of the complex development and architecture of the brain. Thus, this *ex vivo* model can provide useful insight into the role of systemic mediators in governing responses, as well as potential alterations which may confer risk of disease, or differences in response between population groups; however, it nor any other *in vitro* approach can definitively establish whether air pollution exposure results in physiological outcomes or contributes to disease development in individuals.

Beyond the use for examining air pollution toxicological effects in the brain, this *ex vivo* approach could be used to investigate effects in other tissue types and may have broader applications for the field of toxicological research in general. Human iPSC-derived neurons were not only sensitive to changes due to diesel exhaust, but also to the pharmacological agent NAC, which affected pathways aligned with expected biological effects of the drug. While a challenge of this approach is that it does require plasma from individuals exposed to a toxicant, plasma is a relatively accessible biological fluid and can be obtained multiple times from the same individual. Using this approach to characterize health impacts of other environmental pollutants such as pesticides, recreational substances such as cannabis, or pharmaceutical products could be of benefit, especially as it provides an alternative to traditional *in vitro* toxicological screening methods which only consider direct interactions of a chemical with the cell.

7.6 Responses to air pollution exposure are of a temporal and tissue specific nature

A particular strength of the approaches taken in this thesis is that they allowed for exploration of the temporal and tissue specific nature of responses to air pollution exposure. Rather than draw conclusions based on a single endpoint, time frame or biological matrix/tissue, I took a multifaceted approach to investigate how air pollution impacts the body.

Interestingly, ozone exposure altered insulin signaling pathway genes across all four tissue types examined, yet responses displayed tissue-specific patterns of regulation. While the liver had the most numerous genes altered, the brown and white adipose, muscle, and hippocampus displayed far fewer. One explanation for regional transcriptomic differences may be cell type differences in response to systemic mediators such as GCs. The liver, brown adipose and white adipose had evidence of GC-dependent effects, whereas the muscle had none. This is in line with findings by other groups that GCs display cell-type specific actions on transcription

(Franco et al. 2019). Similarly, our *ex vivo* model revealed some cell-type specificity to pollution-induced circulating factors in the brain. The metallothionein genes *MT1* and *MT2* were found to be upregulated in hippocampal neurons treated with plasma from ozone exposed rats, through GC-dependent actions. This was not the case in microglial cells, despite these genes being similarly responsive to corticosterone in both cell types. Cell and tissue specific regulation of gene expression by mechanisms such as the actions of GCs is therefore an important consideration in interpreting pathophysiological responses to air pollution exposure.

Timing of responses to air pollution exposure also varied across endpoints. Modification of gene expression (both *in vivo* and *ex vivo*) and alterations to tryptophan metabolism were early responses to ozone, as effects were observed immediately following exposure but largely returned to control values following a 24 h recovery period. This aligns with circulating corticosterone levels, which were increased by two-fold immediately post exposure, but then were slightly attenuated 24 h post exposure compared to controls. Activation of the HPA axis and subsequent gene expression changes represent a rapid response, with GC concentrations peaking 1-2 h following a stressor, and often resolving quickly even if the stressor is still ongoing (Herman et al. 2016-3-15). In contrast, in this research, the majority of ozone-induced changes on circulating lipid species were observed after the 24 h recovery period, with far fewer effects being captured immediately post exposure. These responses, while occurring when corticosterone levels were no longer elevated, were still largely GC dependent, and the timing of these response patterns correlated with those observed in rats administered exogenous corticosterone. These kinetics may reflect a delayed response to HPA axis activation as downstream metabolic changes are occurring, requiring coordination of several biological events to modify enzyme levels / activity, and secretion of substrates from tissues into circulation.

Taken together, these observations illustrate the need for holistic assessment of air pollution health outcomes, evaluating endpoints across several tissues, at various times during or post exposure, to properly contextualise findings in experimental models. Evaluating insulin signaling responses across tissues collected from the same animals in this thesis allowed us to conclude that the differences we observed are part of a coordinated whole body response, rather than a result of differences in experimental paradigm as would be the conclusion if assessed in separate studies. These considerations are especially important in the case of the brain, where several studies have noted regional differences in transcriptomic and lipidomic profiles in response to air pollution exposure in isolation (Lee et al. 2020, 2022; Qin et al. 2023). Drawing conclusions based on a singular measure, tissue type or time frame may miss key effects. Additionally, comparison between air pollution toxicological studies is already complicated due to the use of different pollutants, exposure scenarios, and animal strains. Making broad assumptions based on a single data point complicates interpretation of findings, making health outcomes less clear and impacting reproducibility.

7.7 Interindividual differences may govern susceptibility to health outcomes of air pollution exposure

A benefit to the *ex vivo* model described in this thesis is that it allows the study of how interindividual differences may govern susceptibility to health outcomes on the brain following pollution exposure. Certain population groups may be more or less susceptible to air pollution-induced health impacts based on preexisting acquired (lifestyle, social determinants, other environmental exposures, disease status) and intrinsic (genetic predisposition, sex) factors. As these effect modifiers may also coincide with (or be a result of) existing comprised health status or disease risk, air pollution exposure may subsequently further elevate risk of disease in these groups. For instance, O'Neill et al. argued that, in the case of socioeconomic position, air

pollution exposure may contribute to poorer health outcomes in lower socioeconomic groups due to either greater exposure, pre-existing compromised health status, or a combination of both these factors (O'Neill et al. 2003). Similarly, several epidemiological studies suggest that associations of respiratory outcomes and exposure are greater in women during adulthood, which may be due to inherent sex-based biological differences, gendered societal reasons influencing behaviours, exposure conditions, or stress, or the interplay of all of these factors combined (Clougherty 2010-2). While animal studies can provide insight into how biological variables may impact responses, these do not reflect the full scale of heterogeneity in the human population, nor can they represent the social determinants that may predispose certain populations to greater health effects.

Within the population cohort exposed to DE described in chapter 6, *ex vivo* responses of neurons to DE-exposure varied between individuals. Responses were largely sex-dependent, with some differences based on asthma status being observed as well. Plasma from males exposed to DE modified pathways associated with protein secretion, such as vesicle transport, as well as DNA repair and cellular stress response (UV response pathways). Conversely, plasma from females exposed to DE tended to modify pathways associated with energy metabolism, and biomolecule synthesis. While only significant in one of the two independent repeats, plasma from DE-exposed males activated the androgen response pathway, whereas plasma from DE-exposed females activated the estrogen response pathways, suggesting DE-exposure may also influence sex hormone related pathways. Females also had a greater proportion of *ex vivo* effects prevented by NAC treatment than males. This data indicates a difference in systemic responses between males and females to DE exposure, which may lead to differential responses in the brain. Beyond effects of sex or asthma, responses to DE varied between individuals within this

study, both in terms of *ex vivo* responses, as well as levels of circulating cortisol (Thomson et al. 2021). For instance, plasma from three male participants tended to cause greater *ex vivo* responses compared to the other males, which was consistent between both repeats of the study. While it is unclear why plasma from these individuals elicited greater responses *ex vivo*, subsequent analyses of both demographic information (e.g., gene polymorphisms, age, BMI) and plasma composition could provide insight on what may be driving these responses. These findings illustrate the variability in human responses to air pollution exposure and highlight the need for studies to examine and identify potential risk factors.

7.8 Future directions

The work presented in this thesis provides a basis for several future avenues of research to advance knowledge of how exposure to air pollution elevates risk of neurodegenerative and psychiatric diseases. This section will introduce some potential future directions which have not yet been presented within this chapter.

7.8.1 Relating human *ex vivo* findings to cognitive measures following controlled exposures

While establishment of the *ex vivo* approach with a rodent model provides confidence that the *ex vivo* approach represents *in vivo* effects following an exposure scenario, it is not known whether *ex vivo* findings in the human model directly cause or contribute to cognitive effects. However, human *ex vivo* responses can be related to outputs such as cognitive tests to examine whether the *ex vivo* responses correspond with cognitive changes. A controlled exposure study evaluated cognitive performance following 2 h controlled exposure to DE, using a similar experimental design to that described in Chapter 6. Participants completed neuropsychological tests of the Cambridge Neuropsychological Test Automated Battery before and after exposure, and DE exposure was found to be associated with prolonged reaction times in the Reaction Time Index task (Curran et al. 2025). Plasma samples were also collected during this study, which

could be used to conduct an *ex vivo* exposure. *Ex vivo* responses could then be compared to performance of participants during the cognitive tests to establish whether there is an association between the two measures following DE exposure, and to establish whether circulating factors following short-term pollution exposure may lead to temporary cognitive deficits. A project to assess this relationship has already been funded and will represent future directions for our laboratory building on the body of work presented in this thesis.

7.8.2 Determining how multi-pollutant exposures modify brain health outcomes

Here, we conducted exposures to an individual pollutant (i.e. ozone) or to a pollutant from a single source (i.e. DE); however, there is a significant need for multi-pollutant exposure studies. A mixture of different gaseous and particulate pollutants is present in the air at all times, but little is understood about how mixtures influence health effects and susceptibility. Few studies have examined the effects of pollutant mixtures on the brain, but those that have indicate that mixtures may exaggerate pathological effects. Co-exposure of mice to ozone and either $PM_{2.5}$ (Yang et al. 2025a), or polystyrene nanoplastics (Dai et al. 2025) has been reported to exacerbate cognitive deficits, markers of neuroinflammation, BBB damage, and other pathological outcomes compared to single pollutant exposures. Understanding how mixtures may affect brain health outcomes relative to single pollutant exposures is imperative for better risk assessment and policy decision making. Both *in vivo* and *ex vivo* models could be utilised to study multi-pollutant effects on the brain.

7.9 Summary

The findings presented in this thesis provides evidence for the crucial mechanistic role that systemic mediators play driving air pollution-induced effects on the brain. Here, I demonstrate that activation of systemic mechanisms by air pollution exposure, particularly the

HPA axis, can alter transcriptional responses in the brain and modify peripheral metabolic features that, in context with the literature, associate with brain health outcomes.

This work also establishes an approach which can include the role of these peripheral systems in study of effects on *in vitro*, human-relevant cell culture models of the brain. Traditionally, animal models were considered necessary to incorporate systemic effects into studying air pollution health effects, as human *in vitro* models could only allow for study of direct interactions of particles. The *ex vivo* model presented here provides a novel approach to study the effects of air pollution on human brain cell culture models, which includes systemic mediators. This approach has applications beyond air pollution to the broader toxicology field and could provide a tool in combination with other NAMS to shift away from animal experiments towards human-relevant, *in vitro* models.

Air pollution exposure poses a significant risk to human health, yet we are likely underestimating the full scope of health impacts that result from polluted air. With an increasing understanding that there is no safe level of air pollution, improving health estimates to better inform policy decisions to reduce exposure are necessary. Understanding how air pollution may elevate risk of neurodegenerative and neuropsychiatric conditions, both of which pose enormous societal health impacts, is critical. This thesis addresses key challenges in this field by (a) shedding light on underlying mechanisms that link brain to peripheral responses to air pollutant and (b) developing a model that enables the study of how systemic mediators impact the brain in a human-relevant manner.

Appendix A: Primer Sequences

Table A.1 Primer Sequences.

Gene	Accession #	Sense Primer	Antisense Primer	Amplicon Size
TSC22D3 (GILZ)	NM_031345.1	gggatgtggtttccgttaaac	tgattcttcactagatccatggc	100
SGK1 (SGK)	NM_019232.3	cacgcctgagtatctcgct	ttgtcatacatctcggctgtg	147
NR3C1 (GR)	NM_012576.2	aactggaataggtgccaagg	ggtctcattccagggcttg	113
HIF3α	NM_022528.1	ggtatcccaggcaacagtct	gcaggtctgggctacaaaaa	121
MAOA	NM_033653.1	gccatcactctggatgacac	ctttccgggcaagtatgaag	73
MT1	NM_138826.4	atgtgcccagggctgtgt	gcagcactgttcgtcacttca	76
MT2a (MT2)	NM_001137564.1	tgcacctcctgcaagaaaa	cacttgtccgaagcctcttt	95
MT3	NM_053968.2	cctactggtggttcctgcacctgct	cttggccccctcttcgcctttg	150
TNFα	NM_012675.3	cgtagcccacgtcgtagc	ggttgtctttgagatccatgc	104
IL-1β	NM_031512.2	acagaccccaaaagattaagga	cgagatgctgctgtgagatt	93
Nos2 (iNOS)	NM_012611.3	tggcaggatgagaagctga	gaagcaaagaacaccgcttt	77
HTR1a (5HT1a)	NM_012585.1	ggcaccttcatcctctgct	gtggcagctgctttcacag	75

HTR1b (5HT1b)	NM_022225.1	tggaggagcagggtattcag	ggagaggttggcgagagg	77
HTR2A (5HT2A)	NM_017254.1	caacggtccatccacagag	tgatggactgcatcgtcct	61
HTR2C (5HT2C)	NM_012765.3	ccgagtccgtttctcgtcta	ctatgcttgcaggtaatagttgaca	94
HTR4 (5HT4)	NM_012853.1	ggatccactcatgtgctaagg	tgtgaggtgacaccgactct	69
HTR6 (5HT6)	NM_024365.2	tctttatgcgggacttcaagag	accgctgtgagaggtcca	112
HTR7 (5HT7)	ENSRNOG00000018827.9	tgcgtgatcagcatcgac	ggtaaggtgatggaggcaga	128
BDNF	NM_012513.3	ttgagcacgtgatcgaagag	tccgcgtccttatggtttt	76
KMO	NM_021593.1	tgaccatgcaatttcagacc	ggatggcattagtgcatgaag	121
VEGF	NM_031836.2	gagttaaacgaacgtacttgcaga	tctagttcccgaaaccctga	90
Bcl2	L14680.1	tgcagagatgtccagtcagc	caccccatccctgaagagt	91
TRKB	ENSRNOT00000042145	gggatgtatacagcaccgactac	catcggatgggcaacatt	61
Igf1	NM_138880.2	ccaaggggcttttacttcaa	acaatgcccgtctgtggt	74
PPIA	NM_017101.1	tgctggaccaaacacaaatg	cttcccaaagaccacatgct	88

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