Full Length Article

Polychlorinated biphenyls induce oxidative stress and metabolic responses in astrocytes

Mondona S. McCann [a,b], Harvey R. Fernandez [a], Sarah A. Flowers [a], Kathleen A. Maguire-Zeiss [a,b,*]

[a] Department of Neuroscience, Georgetown University Medical Center, Washington D.C., United States
[b] Interdisciplinary Program in Neuroscience, Georgetown University Medical Center, Washington D.C., United States

A B S T R A C T

Exposure to environmental toxicants is prevalent, hazardous and linked to varied detrimental health outcomes and disease. Polychlorinated biphenyls (PCBs), a class of hazardous organic chlorines once widely used for industrial purposes, are associated with neurodegenerative disease and oxidative stress in both *in vitro* and *in vivo* models. Here, we investigated the impact of Aroclor 1254, a commercially available PCB mixture, on primary murine astrocytes to determine the response to this once ubiquitously used toxicant on the most numerous cells of the central nervous system (CNS). Astrocytes are a critical component of homeostasis throughout the CNS, including at the blood-brain barrier, where they serve as the primary defense against xenobiotics entering the CNS, and at the synapse, where they are closely coupled to neurons through several metabolic pathways. We hypothesized that PCBs cause astrocytic oxidative stress and related dysfunction including altered metabolism. We exposed primary murine cortical astrocytes to PCBs and report an increased expression of antioxidant genes (*Prdx1, Gsta2, Gfap, Amigo2*) in response to oxidative stress. Our data show increased ATP production and spare respiratory capacity in astrocytes exposed to 10 μM (∼ 3 ppm) PCBs. This dose also causes an increase in glucose uptake that is not seen at a higher dose (50 μM) suggesting that, at a lower dose, astrocytes are able to engage compensatory mechanisms to promote survival. Together, these data suggest that exposure to PCBs impact astrocytic metabolism, which is important to consider both in the context of human health and disease and in *in vitro* and *in vivo* disease models.

1. Introduction

In recent years, our understanding of the central nervous system (CNS) has expanded to recognize the important role of glial cells. This significant shift away from a predominantly neuron-centric view of brain physiology, pathology, and implicated processes therein, has led to important discoveries in areas ranging from basic science to therapeutics (Blackburn et al., 2009; Liddelow and Barres, 2017). Astrocytes, a type of glial (non-neuronal) cell, are the most numerous cell type in the CNS (Han et al., 2013) and are unique in their homeostatic functions (Brown and Ransom, 2007; Souza et al., 2019). Many astrocytic functions underlie proper neuronal transmission and maintenance (Bélanger et al., 2011) and, while this aspect of astrocyte biology is the subject of increasingly more research, it is clear that these cells are important for

brain function in ways that exceed direct astrocyte-neuron interactions (Allaman et al., 2011). The CNS is a notably complex organ system that requires its constitutive structural, cellular and molecular machinery to work both independently and as part of complex, multi-system networks (Maffei and Fontanini, 2009; Kulkarni et al., 2018). In other words, proper communication between neurons and glia as well as glia-glia communication are critical for neuronal function, health, and well-being. This is particularly apparent when the brain is provoked, for example during exposure to endogenous and/or exogenous stressors.

Neuronal degeneration can result from the perturbation of endogenous processes (proteostasis, for instance) (Dasuri et al., 2013), exposure to exogenous compounds, or a combination of internal and external factors (Bossy-Wetzel et al., 2004; Fleming, 2017). For example, oxidative stress is causatively linked to neurodegeneration as a result of

both endogenous and exogenous stressors (Barnham et al., 2004; Migliore and Coppedè, 2009). Environmental toxicants represent a large category of exogenous human stressors, ranging from acute to chronic, life-long exposure. Organochlorines are a class of environmental toxicants known to cause oxidative stress. These chemicals are ubiquitous and, due to their high lipophilicity, chemical stability and long half-lives, particularly potent environmental toxicants (Hawker and Connell, 1988; Grandjean et al., 2008). Several members of this class, including polychlorinated biphenyls (PCBs), are linked to neurological dysfunction wherein neuronal oxidative stress is implicated, such as in Parkinson's disease (PD) (Hatcher-Martin et al., 2012; Bouchard et al., 2014). PCBs are also associated with neurodevelopmental deficits (reviewed in (Pessah et al., 2019)), neuroendocrine dysfunction (Gore, 2001) as well as oxidative stress and cellular dysfunction in multiple animal models (Seegal et al., 1994; Hennig et al., 1999; Lyng et al., 2007).

PCBs cause direct neuronal oxidative damage (Lee and Opanashuk, 2004) but the effects on glial cells remain understudied and are the focus of the work presented herein. In order to have a more complete understanding of the impact of compounds, including PCBs as a representative oxidative stress-inducing toxicant, on the brain in general and on neurons, it is important to understand the direct effect of those same compounds on astrocytes.

The physiological processes of astrocytes include, among others, various aspects of metabolism and antioxidant support. The metabolism of astrocytes plays a uniquely important role in meeting the high energy demands of the brain (Bélanger et al., 2011). Astrocytic end-feet abutting the cerebral vasculature enable astrocytes to quickly take up glucose, which is then metabolized through glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. Astrocytes have a higher glycolytic rate than other cells, suggesting that increased glucose uptake is a mechanism by which astrocytes can maintain this metabolic profile (Itoh et al., 2003; Herrero-Mendez et al., 2009; Bittner, 2010). The importance of the metabolic coupling between astrocytes and neurons is manifest in the antioxidant support astrocytes provide neurons. This relationship and the ways in which it can be disrupted is critical to our understanding of neuronal damage and neurodegeneration.

The reactivity state of astrocytes in response to injury and in neurodegenerative disease can exacerbate inflammation and neuronal dysfunction. Therefore, it is not surprising that astrocytes play an important role in PD pathogenesis (reviewed in (Booth et al., 2017)). Recent work indicates that astrocytes may have multiple functions in PD including acting as antigen presenting cells (Rostami et al., 2020) and as non-cell autonomous mediators of neurodegeneration (di Domenico et al., 2019). Furthermore, growing evidence from PD patient brains show α-synuclein accumulates in astrocytes (Wakabayashi et al., 2000; Braak et al., 2007). In PD, where the dopaminergic neurons of the substantia nigra degenerate, many of the genes associated with monogenic cases of PD are expressed by astrocytes. For example, *PARK7* (DJ-1) is linked to oxidative stress and mitochondrial function in astrocytes (Zhang et al., 2016). Interestingly, in addition to region-specific transcriptional profiles, the substantia nigra is known to contain fewer astrocytes than the ventral tegmental area, where dopaminergic cell bodies involved primarily in mesocorticolimbic systems reside (Damier et al., 1993; Nair-Roberts et al., 2008; Kostuk et al., 2019). The reactive astrocyte phenotype described by Liddelow et al. (2017) and discussed above is also relevant to PD. In a recent study, blocking the ability of microglia to activate the A1 phenotype in astrocytes reduced behavioral deficits and the loss of dopamine neurons in a mouse model of sporadic PD (Yun et al., 2018). Characterization of the specific phenotype of astrocytes during PD pathogenesis is still a nascent field of study but the role of these cells can perhaps be best understood by careful examination of their response(s) to specific perturbations.

Here, we utilize gene expression, oxidative stress, glucose uptake, and metabolism assays to interrogate the effects of PCBs on primary murine astrocytes to determine how this cell type, with specific roles in homeostasis, responds to this environmental toxicant as a representative oxidative stressor. We demonstrate that PCBs directly: increase astrocyte oxidative stress, promote the mRNA expression of a subset of antioxidant response element regulated genes, alter mitochondrial metabolism, and affect glucose uptake. Our work highlights the importance of understanding astrocytic responses to stressors in the maintenance of brain health.

2. Results

2.1. Polychlorinated biphenyls induce oxidative stress in astrocytes

PCBs cause oxidative stress in several disease models and immortalized cell-lines (Hennig et al., 2002; Lyng and Seegal, 2008; Zhu et al., 2009) including a dopaminergic neuronal-like cell line (MN9Ds; Lee and Opanashuk, 2004; Lee et al., 2006) and cerebellar granule cells (Mariussen et al., 2002). The doses of PCBs (administered in the form of mixtures [Aroclors, for example] and individual congeners) used in both *in vitro* and *in vivo* studies have varied greatly (Ulbrich and Stahlmann, 2004; Klocke and Lein, 2020) making a thorough assessment of the toxicity and implicated pathways challenging. For example, stress and depletion of glutathione reserves was observed after 24 hour exposure to 10 ppm Aroclor 1254 in MN9Ds (Lee and Opanashuk, 2004) while cellular viability was significantly decreased in neuroblastoma cells (SH-SY5Y) after exposure to 5 ppm for 6 hours (Cocco et al., 2015).

Taking into account the body of existing *in vitro* data, here we exposed primary murine astrocytes to Aroclor 1254 (1.25–50 μM) for 24 h, which is equivalent to 0.4–16 ppm. Following Aroclor 1254-exposure, total reactive oxygen species were quantified using 2′-7′-dichlorodihydrofluoresceine diacetate (H$_2$DCFDA), a nonfluorescent compound that is converted to a highly fluorescent compound, dichlorofluorescein (DCF), in the presence of reactive oxygen species (Fig. 1a). The PCB-exposed cells had a significantly (p < 0.05) higher level of fluorescence (10 μM: 1.75×10^6 +/− 2.48×10^4 [SEM]; 20 μM: 1.78×10^6 +/− 3.059×10^4 [SEM]; 50 μM: 1.85×10^6 +/− 4.5×10^4 [SEM]) compared to the vehicle astrocytes (1.5×10^6 +/− 1.68×10^4 [SEM]) demonstrating that exposure to a biologically relevant dose of Aroclor 1254 (10 μM or 3 ppm) results in significant oxidative stress in astrocytes.

Given the observed increased oxidative stress in astrocytes with exposure to PCBs, glutathione, a tripeptide antioxidant abundant in astrocytes (Raps et al., 1989; Dringen and Hirrlinger, 2003) was analyzed. In the presence of reactive oxygen species, reserves of reduced glutathione (GSH) are oxidized to form oxidized glutathione (GSSG). As such, GSSG was quantified to understand the engagement of this important cellular defense pathway. Primary murine astrocytes were exposed to PCBs (10 μM, 24 h) as described above and a GSSG detection assay was used to quantify the concentration of oxidized glutathione in PCB and vehicle-exposed astrocytes. Astrocytes exposed to PCBs had a statistically significant (p = 0.04) increase in the concentration of GSSG compared to vehicle-exposed cells (Fig. 1b). These results suggest that a depletion of GSH reserves may be an important avenue of response in PCB-induced toxicity and confirms that PCBs cause an increase in oxidative stress in astrocytes.

In order to determine whether cell death is increased with PCB exposure, a cell viability, MTT, assay was performed on a range of doses (1.25–20 μM). Hydrogen peroxide (H$_2$O$_2$) was included as a positive control for oxidative stress-induced decreased viability (Whittemore et al., 1995). Primary murine astrocytes were exposed to vehicle, 1.25, 2.5, 5, 10 or 20 μM of PCBs for 24 h before the cell viability assay was performed. At doses ≤20 μM, there was no significant change in cellular viability (Fig. 1c). This finding was confirmed using a complementary cytotoxicity assay which quantifies lactate dehydrogenase (LDH) release following 24 h with 10, 25 and 50 μM PCB exposure (Fig. 1d). LDH is a cytoplasmic enzyme found in all cells and is released into the cellular
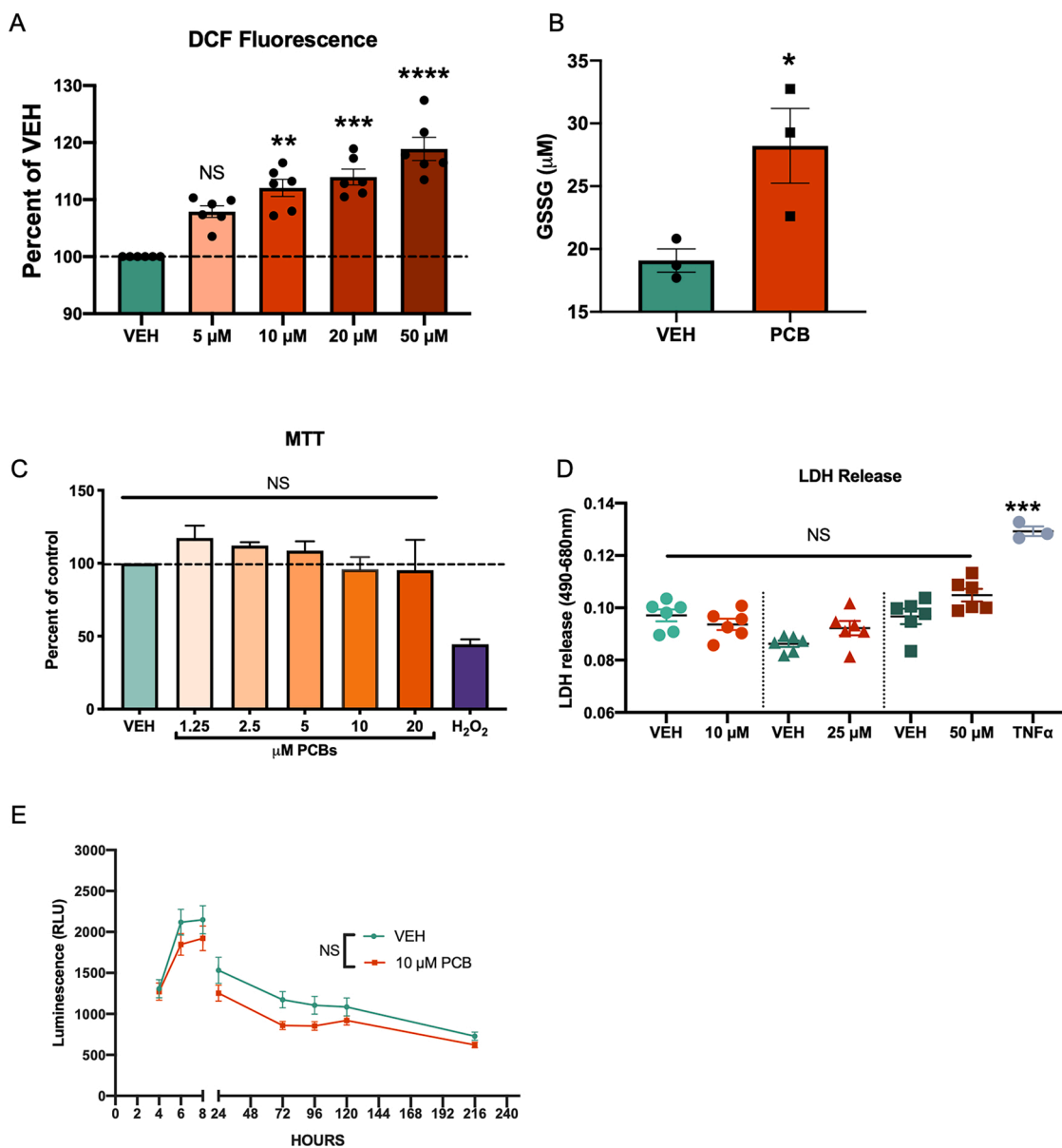
Fig. 1. Polychlorinated biphenyls induce oxidative stress in astrocytes without impacting cellular viability.
A. DCF quantification of vehicle and PCB-exposed astrocytes (24 h), n = 6, Data analyzed *via* one-way ANOVA**p = 0.0015, ***p = 0.0002, ****p < 0.0001. **B.** GSSG quantification of vehicle and PCB-exposed astrocyte lysates (24 h), n = 3, *p = 0.04. **C.** Formazan quantification with an MTT assay of PCB or methanol-exposed astrocytes (24 h) and hydrogen peroxide (100 μM) positive control, n = 6. Methanol concentration is matched v/v to highest 50 μM PCB dose. **D.** LDH release (24 h) including TNF-α positive control, data analyzed *via* one-way ANOVA ***p = 0.0015. **E.** RealTime-MT glo assay quantifying cellular viability over a 216 h period. Data analyzed by two-way ANOVA (time x treatment) and Šidák's multiple comparisons test; 4 -h (p > 0.99), 6 h (p = 0.86), 8 h (p = 0.9), 24 h (p = 0.78), 72 h (p = 0.169), 96 h (p = 0.45), 120 h (p = 0.85) and 216 h (p = 0.6). All data represented as mean ± SEM.

media when the plasma membrane is damaged, as is the case in necrotic and apoptotic cell death (Kumar et al., 2018). Tumor necrosis factor alpha (TNFα) was included as a positive control verifying both that the primary astrocytes were able to release LDH and that the released LDH was detectable (Park and Bowers, 2010; Chan et al., 2013; Olmos and Lladó, 2014). Together these data suggest that astrocytes exposed to 10 μM PCBs undergo increased oxidative stress in the absence of reduced cellular viability.

Given the chronic and persistent nature of exposure to many environmental toxicants, including PCBs, cell viability was analyzed over a longer exposure time to PCBs at a range of doses. A non-lytic continuous read cell viability assay (RealTime-Glo™MT Cell Viability assay, Promega #G9711) was used which allowed for longer PCB exposure times (up to 216 h) at various doses (2.5, 5,10, 20 or 50 μM PCBs). There

were no significant differences between vehicle and PCB concentration at any time point (data not shown for 2.5, 5, 20 and 50 μM exposure). Comparison of the 10 μM PCB-exposed astrocytes and vehicle revealed no significant difference in cell viability at any time point throughout the assay (Fig. 1e). This data indicates that PCB-exposed astrocytes have an increased burden of ROS at 24 h but remain viable, suggesting that they are engaging cellular pathways to mitigate damage.

2.2. Upregulation of antioxidant genes in response to PCB exposure

In order to better understand which cellular pathways might be engaged in response to PCBs, and because PCBs and other organochlorines are associated with PD (supplemental Fig. S2), we performed an *in silico* screen using the Comparative Toxicogenomics Database (CTD,

data queried on Feb 17ᵗʰ, 2019) (Davis et al., 2021) (Fig. 2a). Three parameters were used to determine genes of interest. In order to determine functional relevance for the central nervous system, we defined one of the three parameters as genes linked to PD (Hatcher-Martin et al., 2012). The second parameter was genes associated with PCB exposure. The third parameter, genes associated with antioxidant activity, was based on our findings outlined in Fig. 1 since PCB exposure caused oxidative stress in astrocytes. These *in silico* analyses identified 54 overlapping genes (supplementary Fig. S3). Genes of interest were identified by taking tissue and cellular expression as well as isoform redundancy into account.

Three genes were selected (*Gsta2, Prdx1* and *Sod1*) from this screen and we added an additional six genes that are associated with astrocyte reactivity (*Aqp4, Nox1, Slc10a6, C3, Amigo2, Gfap*) (Liddelow et al., 2017). All nine genes were examined using quantitative real-time reverse transcription PCR (qRT-PCR). PCB exposure of astrocytes resulted in a significant increase in antioxidant genes including glutathione-s-transferase 2 (*Gsta2,* 1.6 fold increase), and peroxiredoxin 1 (*Prdx1,* 8.6 fold increase). Of the genes relating to astrocyte reactivity, *Gfap* (p = 0.003, 1.8 fold increase) and *Amigo2* (p = 0.02, 1.5 fold increase) showed small but significant increases following exposure to PCBs; C3 (p = 0.1, 2.7 fold increase) and *Sod1* (p = 0.06, 2.9 fold increase) were trending towards increased but not statistically significant. There was no significant change in the antioxidant gene NADPH oxidase 1 (*Nox1*), or the astrocyte reactivity related genes, aquaporin 4 (*Aqp4*) and solute carrier family 10 member 6 (*Slc10a6*) (Fig. 2b).

2.3. Metabolic changes with PCB exposure

Given that the oxidative state of astrocytes can impact their metabolic function and homeostatic processes (reviewed in (McCann and Maguire-Zeiss, 2021)), we next investigated the metabolic profile of PCB-exposed primary murine astrocytes using the Seahorse Extracellular Flux analyzer. We examined mitochondrial oxygen consumption rate (OCR) in 10 µM PCB-exposed astrocytes and found no significant differences in basal respiration, proton leak, or maximal respiration (Fig. 3a–c). Interestingly, there were significant increases inATP production in the 10 µM PCB-exposed astrocytes (Fig. 3d). We tested a higher dose of PCBs (50 µM) and found no significant differences in OCR, basal and maximal respiration or ATP production (Fig. 3e–h). There were no significant differences in proton leak with either treatment (Fig. 3i), however there was a significant increase in the spare respiratory capacity of the 10 µM PCB-exposed astrocytes (Fig. 3j). This data suggests that at the lower (10 µM) PCB dose, the astrocytes are engaging mechanisms to overcome metabolic dysfunction whereas this mechanism fails at the higher dose (50 µM).

2.4. Increased glucose uptake with low dose exposure to PCBs

Throughout the CNS, astrocytes play important roles at synapses, where they partake in an intensive metabolic exchange with neurons that is essential for neuronal survival, and at the blood-brain barrier, where astrocytic endfeet ensheathe the vasculature and express GLUT1, a glucose transporter[23]. The metabolic processes that regulate glucose

A

B
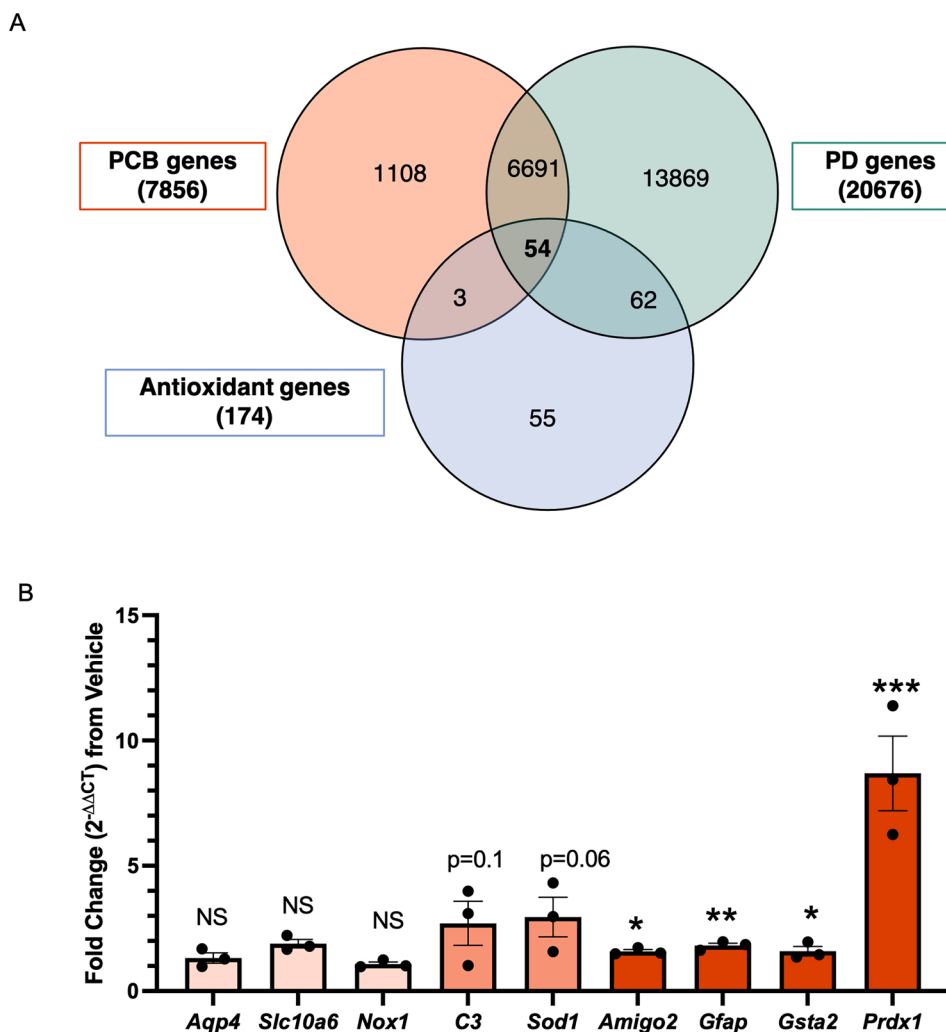
Fig. 2. Upregulation of antioxidant genes in response to PCB exposure.
A. Comparative toxicogenomics database screen of antioxidant genes with published associations to both PCBs and Parkinson's disease. **B.** qRT-PCR (mRNA), fold change of 10 µM PCB exposed astrocytes compared to vehicle (methanol) exposure. Data analyzed *via* Student's *t*-test, represented as mean ± SEM with 3 biological replicates and was replicated in 2–3 independent experiments, *p = 0.02, **p = 0.003, ***p = 0.0003.
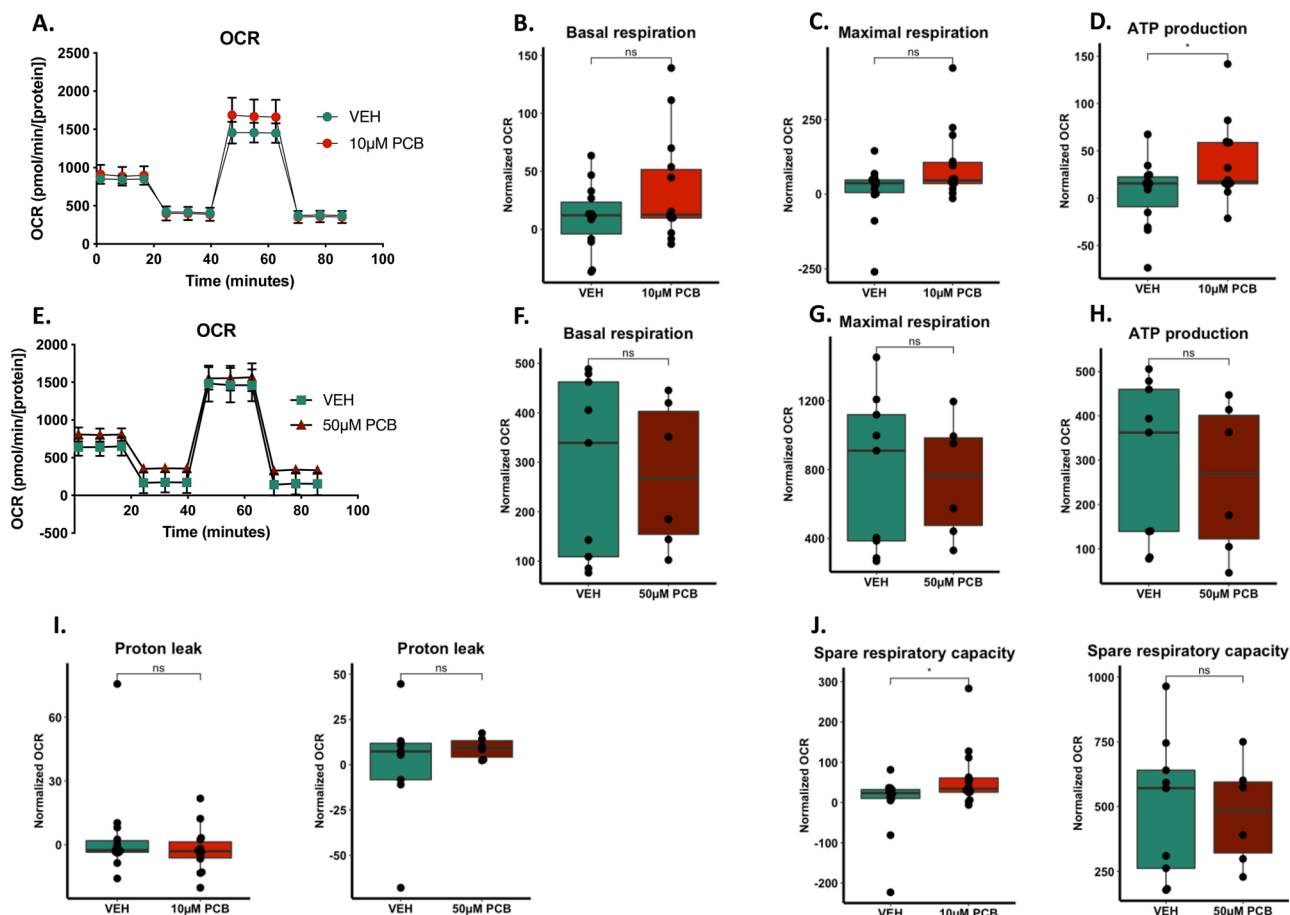
Fig. 3. Metabolic changes with PCB exposure.

A-D. 10 μM PCB-exposed astrocytes have increased ATP production after 24 h compared to vehicle but no significant differences in basal or maximal respiration (data is normalized to cell density). **E–H.** 50 μM PCB exposure for 24 h does not result in significant differences in basal respiration, maximal respiration or ATP production. **I.** Proton leak is not impaired by either 10 or 50 μM PCB exposure. **J.** Spare respiratory capacity is increased in the 10 μM PCB exposed astrocytes but not the 50 μM condition. Data analyzed *via* generalized linear model, data represented as mean ± SEM with 3–5 biological replicates from three independent experiments.

levels are critical for brain function, including synaptic transmission and global homeostasis (Allaman et al., 2011; Han et al., 2013; Chai et al., 2017; Verkhratsky et al., 2019). The ability of astrocytes to serve as sensors of metabolic changes in the microenvironment places these cells in the unique position of regulating glucose dynamics within the CNS and from the periphery into the brain (García-Cáceres et al., 2016). In order to determine whether astrocyte function is affected by PCB exposure, we examined glucose uptake in astrocytes. Interestingly, 10 μM PCB-exposed astrocytes demonstrate increased glucose uptake compared with vehicle-exposed cells (Fig. 4a) whereas 50 μM PCB-exposed astrocytes do not (Fig. 4b). This finding suggests that in response to low-dose (10 μM) PCB exposure, astrocytes are responding to metabolic demand.

3. Discussion

Together, the work presented here shows that astrocytes exposed to PCBs are subjected to increased oxidative stress, display an antioxidant response, and are able to increase glucose uptake and ATP production. Glucose, the main substrate for energy in the brain, is metabolized by three successive processes: glycolysis, the tricarboxylic acid (TCA) cycle, and oxidative phosphorylation. This final process, oxidative phosphorylation, is the largest driver of cellular ATP production. Our data suggests that at a 10 μM exposure to PCBs, astrocytes are able to increase glucose uptake and drive ATP production to compensate for increases in reactive oxygen species. This mechanism is not engaged in the higher
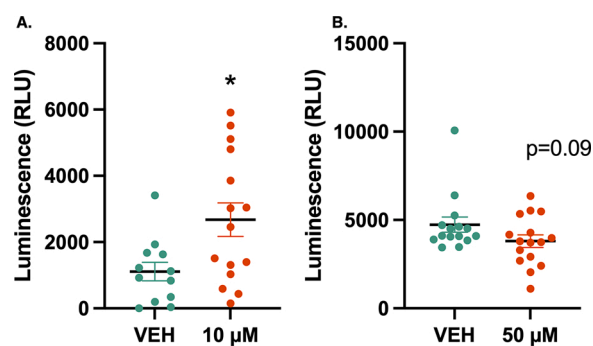

Fig. 4. Increased glucose uptake with low dose exposure to PCBs.

A. 10 μM PCB exposure results in increased astrocyte glucose uptake compared to vehicle. **B.** 50 μM exposure of astrocytes does not significantly alter glucose uptake. Data represented as mean ± SEM with 3–6 biological replicates from 3 independent experiments, unpaired two-tailed t-test, *p = 0.01.

dose (50 μM) exposure, where the astrocytes do not increase glucose uptake and are unable to utilize increased energy expenditure. This suggests that the mechanisms engaged at the lower dose are less effective at a higher dose. As indicated by increased spare respiratory capacity (Fig. 3j), it is possible that the 10 μM-PCB exposed astrocytes are adapting to utilize increased oxidative phosphorylation in an attempt to meet energy and antioxidant demands. Extending these studies to

determine the impact of both low and high PCB exposure on glycolysis as well as a more detailed examination of GLUT expression and function is an important future direction. Furthermore, a comparison of the expression and function of both high- and low-affinity glucose transporters in response to PCB exposure represents a next step to address the mechanism by which this shift occurs (Jouroukhin et al., 2018; Koepsell, 2020). Importantly, glutathione detoxification is an ATP-consuming process and, as such, it is possible that an increase in ATP is required to drive production of glutathione, the synthesis of which is increased in response to oxidative stress (Dringen et al., 2015). Addressing these mechanisms will deepen our understanding of astrocyte responses to toxicants in order to determine more completely how exposure to this toxicant alters the metabolic profile of astrocytes.

Our findings reported here are concordant with the hormesis principle of toxicology wherein stimulation is observed at low doses of exposure to chemical agents that cause toxicity at high doses, resulting in a biphasic response (Tapia, 2006; Calabrese et al., 2007; Mattson, 2008). Hormesis, which is related to terms such as "adaptive response" or "preconditioning" that also describe nonlinear responses, is increasingly shown to be an important consideration in the evaluation of dose-response models in biological systems (Calabrese et al., 1999, 2007; Calabrese and Baldwin, 2003). While this concept as it pertains to public health decisions is controversial, and has great potential for adverse health outcomes if applied in a regulatory context, it is clear that "nonmonotonic" dose responses are important to consider in the context of basic science and interpretation of data, with an emphasis on understanding the biological machinery underpinning these responses (Thayer et al., 2005).

With regards to PCBs, hormeticeffects have been shown in several models including human lung fibroblasts (Hashmi et al., 2015) and in iron sequestration in the striatum of PCB-exposed mice (Lee et al., 2012). When considering the impact of PCBs on primary neuronal cultures, where decreased cellular viability is shown to occur after a 4 h exposure to PCBs at concentrations between 30 and 100 μM in rat cortical cultures (Sánchez-Alonso et al., 2004) and there is evidence of differential outcomes between hippocampal and cortical neurons (Howard et al., 2003), it is critical to consider the impact of the attendant astrocytes in these culture systems. At higher doses (>10 μM, as we show here), it is possible that astrocytes fail to engage compensatory mechanisms to promote their own survival in addition to that of neurons. As it becomes increasingly evident that astrocytic phenotypes differ with anatomical regions, this heterogeneity may contribute to an explanation of differing neuronal vulnerabilities (Matyash and Kettenmann, 2010; Xin and Bonci, 2018; Matias et al., 2019).

Although much of the work regarding toxicants in the brain has focused on neurons, it is clear that astrocytes are an important component of the brain's response to this xenobiotic compound. Since neurons are often co-cultured with astrocytes *in vitro*, our work supports the need to understand individual cell-type responses (neurons and glia separately) before interpreting the neuronal-glia combined responses to environmental toxicants. This is important because astrocytic metabolism and redox status are intricately tied to that of neurons andthe brain in general. As such, this research suggests that the ability of PCBs to alterastrocyte function will have long-term, detrimental consequences for the brain as a whole.

PCBs are persistent environmental pollutants and, despite their production being banned in most countries since 1985, are continually linked to novel instances of disease and environmental disasters. These compounds are part of a larger class of organochlorines, including compounds like trichloroethylene, which is still used in dry cleaning, therefore understanding the impact that PCBs have on astrocytes can inform our understanding of other compounds. Furthermore, the predicted lipid-normalized concentration of PCB-153 (2,2′,4,4′,5,5′-hexachloro-biphenyl) is approximately 50 ng/g lipid (Quinn et al., 2011), and up to approximately 10 ng/g lipid in the brain (Mitchell et al., 2012) and although there are many factors influencing an individuals' life time

exposure to PCBs (including geography, occupation, diet), the impact of these compounds on human health is significant. For neurodegenerative diseases, like PD, where sporadic cases far outnumber familial cases, it is possible that chronic exposure to hazardous compounds over the course of an individual's lifetime coincide with age-related vulnerabilities and cellular dysfunction resultingin disease. Importantly, the prevalence of neurodegenerative diseases is increasing (Brookmeyer et al., 2011; Kowal et al., 2013; Hebert et al., 2013) and, as individuals who were exposed to PCBs prior to regulation continue aging, this represents an impactful avenue of research.

From both experimental and epidemiological models of disease, there is compelling evidence that exposure to organochlorine environmental toxicants is associated with oxidative stress and the development of PD (Seegal et al., 1994; Lee and Opanashuk, 2004; Richardson and Miller, 2004; Hatcher-Martin et al., 2012). There is also evidence of increased susceptibility of dopaminergic neurons to oxidative stress as a result of metabolites produced during dopamine synthesis, suggesting that oxidative stress and related cellular sequelae are important for both the development and progression of PD (Graham, 1978; Jenner, 2003). Importantly, as the antioxidant functions of astrocytes are critical for mounting a productive antioxidant response, so too is astrocytic oxidative stress important for the propagation of oxidative injury and PD pathogenesis (Rizor et al., 2019). In addition, *in vitro* studies using the C6 glioma cell line, demonstrate that PCBs disrupt the protein kinase C pathway and increase expression of neurotrophic factors, suggesting a role for astrocytes in multiple mechanisms associated with PCB exposure (Gurley et al., 2007; Adornetto et al., 2013). As astrocytic functions and processes are increasingly associated with various aspects of PD, including neuroinflammation and metabolic dysfunction, uncovering the role of these cells in disease pathogenesis will aid in our comprehensive understanding of this disorder. Furthermore, as environmental toxicants like PCBs impact astrocytic processes, considering the nexus of PCB exposure and astrocytic processes implicated in PD can inform future research directions.

In this study, we investigated a range of doses (1.25–50 μM) and used 10 μM Aroclor 1254 for the majority of our studies, as it is the lowest dose with significantly elevated reactive oxygen species. Additionally, we sought to use a paradigm that enabled us to study the cellular response in the absence of cell death. The data discussed here show that a significant increase in OCR at 10 μM is concomitant with increased glucose uptake, indicating an increase in metabolic activity that promotes cellular survival. The 10 μM PCB-exposed astrocytes also upregulated the expression of specific genes involved in cellular detoxification (*Gsta-2, Prdx-1*), suggesting that a subset of Nrf2-ARE gene pathways are involved in the cellular response to PCBs in astrocytes.

The glutathione system, which is particularly robust in astrocytes (Dringen and Hirrlinger, 2003), is rapidly engaged in xenobiotic conjugation and detoxification. Furthermore, oxidized glutathione (GSSG) can be utilized as a proxy for the degree of oxidative stress within a cell. Here, we showed that there was a significant increase in GSSG in PCB-exposed astrocytes, as well as an increase in *Gsta-2* by qRT-PCR. As astrocytes provide neurons with essential precursors for neuronal glutathione synthesis (CysGly) (Dringen and Hirrlinger, 2003), the PCB-induced depletion of reduced glutathione (GSH) suggests that astrocytic ability to properly detoxify this compound is impaired, yet the astrocytes are able to survive. It is important to point out that in this study, we did not examine whether long-term low dose exposure alters mitochondrial function and metabolism. We also have not yet examined the broader impact of PCBs on the intact brain. The field currently lacks consensus on the appropriate *in vivo* dose; these are important next steps.

As the world's population ages, we have yet to see the full effect of PCB-toxicity on humans. Approximately 43 % of the world's population was exposed to PCBs prior to the latest date of their ban (Council of the European Union, 1996). The impact of this ubiquitous toxicant is likely to be profound andtherefore, ongoing research focused on the

intersection of neurodegeneration and environmental toxicant exposure should take into account the important role of glial cells.

4. Methods and materials

4.1. Animals

Adult C57/Bl6J mice were bred and housed in accordance with the Georgetown University Institutional Animal Care and Use Committee and the ethical guidelines of the National Institutes of Health. Animals were housed with two females and one male per cage with food and water *ad libitum*.

4.2. Aroclor 1254

Aroclor 1254 (11097-69-1, AccuStandard, #C-254S-M) was diluted from a stock concentration of 1000 μg/mL in methanol. For exposure, Aroclor 1254 was diluted in serum-free astrocyte medium to the appropriate concentration as noted. Methanol was used as the vehicle (see figure legends for details).

4.3. Primary astrocyte culture

Primary astrocytes derived from C57/Bl6J mice were prepared as previously described (Daniele et al., 2014). In brief, brains were isolated from p0-p2 mice, meninges removed and cortices microdissected. Cortices were then minced in media (HBSS supplemented with 100 μg/mL P/S [penicillin/streptomycin]), allowed to settle, 2 mL of media removed, and resuspended in complete media (Minimum Essential Medium Earle's [MEM] supplemented with 1 mM L-glutamine, 1 mM sodium pyruvate, 0.6 % v/v D(+)-Glucose, 100 μg/mL P/S, 4% v/v fetal bovine serum [FBS], and 6% v/v horse serum), centrifuged, and resuspended again. The mixed glia were dissociated by gentle trituration and cultured in T-75 flasks in a humidified incubator (37 °C, 5% CO₂). At 14–21 days *in vitro* (DIV), astrocytes were isolated following a shaking procedure: 4 h shake on a rotary shaker (200 RPM, 37 °C), media and non-adherent cells removed, adherent astrocytes washed twice with warmed PBS and isolated following incubation in 1X trypsin/EDTA at 37 °C. Astrocytes were subsequently passaged into a T-175 flask and cultured in astrocyte medium (MEM supplemented with 100 μg/mL P/S, 1 mM L-glutamine, and 10 % FBS). Before plating for experiments, T-175 flasks were shaken overnight (200 RPM, 37 °C) to remove contaminating non-astrocytic cells, the media removed, cells washed twice with warmed PBS, astrocytes isolated by trypsin digestion and plated for experiments in astrocyte medium as described below. Astrocyte cultures were periodically verified as 90–95 % pure using AQP4, GFAP and DAPI immunofluorescence.

4.4. Exposure to PCBs or vehicle

Astrocytes were exposed to PCBs in the form of Aroclor 1254 (supplier and stock information above) or vehicle (methanol). The concentrations and time of exposure is noted in the figure legends. The cells were plated for the format of the experiment (cell number and well-format noted in the respective sections) and, four days later, were washed with serum-free media (MEM supplemented with 100 μg/mL P/S, 1 mM L-glutamine) and then incubated with serum-free media for 1 h. Next, the serum-free media was removed, and the appropriate treatment solution was added to the well in the following volume: 3 mL for 6-well plate (qRT-PCR), 2 mL for 12-well plate (GSH/GSSG), 300 μL for 96-well plate (H₂DCFDA, MTT, LDH and glucose uptake), and 100 μL for Seahorse 96-well plate.

4.5. Reactive oxygen species quantification

Total reactive oxygen species were quantified using 2′,7′-dichlorodihydrofluorescein diacetate (H₂DCFDA) (Thermo Fisher #D399). The compound was resuspended in 100 % ethanol to a final stock concentration of 10 mM H₂DCFDA. After exposure to Aroclor 1254 (PCB, concentration noted in figure legend) or methanol (VEH, matched v/v with 50 μM PCB dose) for 24 h, astrocytes (3×10^4 cells/well) were washed in serum-free astrocyte medium and incubated with H₂DCFDA at 10 μM for 30 m in the dark and read on a fluorescent microplate reader (Molecular Devices SpectraMax iD3) at E_x/E_m 490/530.

4.6. Glutathione measurement

Glutathione quantification was completed using a reduced glutathione (GSH) and oxidized glutathione (GSSG) ratio detection assay (Abcam, ab138881) per the protocol described by the manufacturer. Astrocytes (2.5×10^5 cells/well) were plated on a 12-well plate and exposed to PCB or vehicle as described above. After PCB or vehicle exposure, cells were washed twice in PBS then lysed in 0.5 % NP-40 in PBS. Following lysis and collection, deproteinization was completed using trichloroacetic acid and 1 M NaHCO₃ was used to adjust the pH to 5. Deproteinization was confirmed by the DC Protein Assay (Bio-Rad #500011). After accounting for dilution during deproteinization, GSSG was calculated using the following formula: (total glutathione − GSH)/2.

4.7. Viability analysis

Viability analysis was performed by formazan quantification in a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche, #11465007001). Primary astrocytes were grown in a 96 well plate (3×10^4 cells/well). Prior to treatment the cells were washed with serum-free astrocyte media (MEM supplemented with 100 μg/mL P/S, 1 mM L-glutamine), and then incubated in serum-free astrocyte media for 1 h. Then the serum-free media was removed and the appropriate treatment (Aroclor 1254 [concentration noted in figure], methanol [matched to highest concentration of Aroclor 1254] or hydrogen peroxide [100 μM]) for 24 h, treatment media removed and replaced with 100 μL serum-free astrocyte media. MTT labelling reagent was added to the well at a final concentration of 0.5 mg/mL, after which the cells were incubated for 4 h in a humidified incubator (37 °C, 5% CO₂), then 100 μL of solubilization solution was added to each well and the plate was incubated overnight. Formazan quantification was completed using a microplate reader at 550 nm wavelength. LDH release was analyzed to determine plasma membrane damage using Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific #88953) as per the manufacturer's instruction. Data shown is 490 nm – 680 nm absorbance reading. Cells were exposed to PCBs or methanol as described above and a volume-matched vehicle (methanol) group was included for each (10, 25, and 50 μM) PCB treatment group. TNF-α was included as a positive control for LDH release at a concentration of 60 ng/mL.

4.8. qRT-PCR

Following PCB or vehicle treatment for 24 h, astrocyte RNA (300 ng) was prepared using RNeasy mini kit (Qiagen #74104) and reverse transcribed in a 60 μL reaction volume using a High-Capacity RNA-to-cDNA kit (Thermo Fisher #4387406). To quantify gene expression changes, quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) to quantify gene expression changes was performed in a 384-well plate format using SsoAdvanced Universal Probes Supermix (Biorad, 1725281) and Taqman primer/probe pairs. Taqman primers/probes as follows: *AQP4 (Mm00802131_m1), SLC10A6* (Mm00512730_m1), *NOX1* (Mm00549170_m1), *C3 (Mm01232779_m1), SOD1 (Mm01344233_g1), AMIGO2 (Mm00662105_s1), GFAP* (Mm01253030_1), *GSTA2* (Mm00833353_mH), *PRDX1* (*Mm01621996_s1), 18 s* rRNA (HS999999_s1). The small eukaryotic ribosomal subunit (18S) was used as an endogenous control and 18S rRNA expression level was not statistically different between vehicle and

treatment conditions. ABI Prism 7900 H T Sequence Detection System (Life Technologies) was used to perform qRT-PCR reactions. Data were analyzed using the $2^{-\Delta\Delta Ct}$ relative quantification of gene expression method which consists, in brief, of normalizing gene of interest expression to the endogenous control (18S rRNA), followed by comparison to biological control (vehicle) and calculation of fold change utilizing $2^{-(\Delta\Delta Ct)}$.

4.9. Seahorse extracellular flux analyzer experiments

Primary murine cortical astrocytes were plated at 3×10^4 cells/well in astrocyte media (MEM supplemented with 100 μg/mL P/S, 1 mM L-glutamine, and 10 % FBS) and allowed to rest for 3 days. Cells were then treated in serum-free astrocyte media with Aroclor 1254 or methanol, at the concentration noted in the figure legend. The mitochondrial stress test protocol was followed as per the manufacturer's instructions (Agilent). Briefly, the medium was replaced with DMEM supplemented with 1 mM pyruvate, 2 mM glutaMAX (Thermo Fisher #35050079) and 10 mM glucose (pH of 7.2–7.4). A sensor cartridge was equilibrated with Seahorse XF Calibrant at 37 °C in a CO_2-free incubator overnight. The analysis protocol was comprised of three consecutive measurements to determine oxygen consumption rate (OCR) prior to the injection of each compound; oligomycin (final concentration 0.5 μM), FCCP (2 μM), and rotenone/antimycin (0.5 μM each). Data shown is combined across three independent experiments and was analyzed using a general linear model.

4.10. Glucose uptake

Glucose uptake was quantified using Glucose Uptake-Glo™Assay (Promega J1341). Astrocytes were plated on a 96-well plate (30,000 cells/well) and treated as described for other assays (in brief, for 24 h following 1 h serum starvation). The cells were incubated with the 2DG6P detection reagent for 1 h at room temperature and luminescence was detected on a microplate Molecular Devices SpectraMax iD). Data shown is from three independent experiments.

4.11. Curated interaction and gene ontology analysis

All of the curated gene, compound and disease comparisons in the Comparative Toxicogenomics Database (CTD) are publicly available (Davis et al., 2021). The comparisons shown here were conducted with data downloaded on February 7, 2019. For analysis of gene-disease and gene-gene ontology association (Fig. 2A), the following Medical Subject Heading (MeSH) IDs were used: Parkinson's disease (D010300) and PCBs (D011078). The gene ontology (GO) name for antioxidant activity used was 16209. A dataset was created including the inferred gene associations were for D010300 (PD) and D011078 (PCBs) and the curated gene associations for antioxidant activity (GO: 16209). This dataset of genes was then analyzed using the MyVenn tool to identify overlapping genes, resulting in a list of 54 genes with known antioxidant activity and inferred associations with PD and PCBs.

Gene ontology analysis was performed on these 54 genes to identify the most enriched biological processes in *Homo sapiens* using the Gene Ontology Consortium's GO enrichment analysis (http://geneontology.org) (Ashburner et al., 2000; Mi et al., 2019; Gene Ontology Consortium, 2021) to determine the specific oxidant species and antioxidant processes associated with both PD and PCBs. These data were analyzed using a Fisher's Exact test with Bonferroni correction. The p-values for the most enriched (>100 fold enrichment) processes are: removal of superoxide radicals ($p = 2.50 \times 10^{-10}$), cellular oxidant detoxification ($p = 9.73 \times 10^{-97}$), cellular response to superoxide ($p = 5.47 \times 10^{-10}$), response to superoxide ($p = 1.53 \times 10^{-9}$), cellular response to oxygen radical ($p = 5.4 \times 10^{-10}$), response to oxygen radical ($p = 1.51 \times 10^{-11}$), hydrogen peroxide catabolic process ($p = 8.63 \times 10^{-19}$), glutathione derivative metabolic process ($p = 2.74 \times 10^{-13}$), cellular detoxification

($p = 1.34 \times 10^{-94}$), detoxification ($p = 3.69 \times 10^{-91}$), and the response to lipid hydroperoxides ($p = 2.55 \times 10^{-3}$). To identify curated gene-disease associations, the MeSH IDs for PCBs (D011078), trichloroethylene (D014241), and dieldrin (D004026) were analyzed (Supplemental Fig. S2).

4.12. Statistical analyses

Data were analyzed using unpaired *t* test, one-way analysis of variance or two-way analysis of variance (ANOVA) (as noted). All data are reported at mean $\pm$ *SEM* with significance set at $p \leq 0.05$. Specific replicate values for each experiment and assay are as noted in the figure legend. Statistical analyses were conducted on biological replicates using GraphPad Prism version 9.0.1 for Mac, GraphPad Software, San Diego, California USA, www.graphpad.com and R (R Core Team, 2019).

CRediT authorship contribution statement

All authors participated in the design of a subset of experiments and data analyses/interpretation. **Mondona S. McCann & Harvey R. Fernandez:** performed the Seahorse extracellular flux analyzer experiments; **Mondona S. McCann:** performed the remaining experiments and wrote the first draft of the paper. All authors edited the paper.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.neuro.2021.07.001.

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