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World Trade Center Dust Induces Nasal and Neurological Tissue Injury While Propagating Reduced Olfaction Capabilities and Increased Anxiety Behaviors

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Abstract

Previous *in vitro* and *in vivo* World Trade Center particulate matter (WTC_{PM}) exposure studies have provided evidence of exposure-driven oxidative/nitrative stress and inflammation on respiratory tract and aortic tissues. What remains to be fully understood are secondary organ impacts due to WTC_{PM} exposure. This study was designed to test if WTC particle-induced nasal and neurologic tissue injury may result in unforeseen functional and behavioral outcomes. Namely, WTC_{PM} exposure was found to incite neurologic injury and olfaction latency in intranasally (IN) exposed mice. Single high-dose and repeat low-dose nasal cavity insults from WTC_{PM} dust resulted in significant olfaction delays and enduring olfaction deficits. Anxiety-dependent behaviors also occurred in mice experiencing olfaction loss including significant body weight loss, increased incidence and time spent in hind stretch postures, as well as increased stationary time and decreased exploratory time. Additionally, WTC_{PM} exposure resulted in increased whole brain wet/dry ratios and wet whole brain to body mass ratios that were correlated with exposure and increased exposure dose ($p < 0.05$). The potential molecular drivers of WTC_{PM}-driven tissue injury and olfaction latency may be linked to oxidative/nitrative stress and inflammatory cascades in both upper respiratory nasal and brain tissues. Cumulatively, these data provide evidence of WTC_{PM} exposure in relation to tissue damage related to oxidative stress-driven inflammation identified in the nasal cavity, propagated to olfactory bulb tissues and, potentially, over extended periods, to other CNS tissues.

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World Trade Center dust; particulate matter exposure; central nervous system; intranasal instillation

Background

Epidemiological studies have identified strong correlations between particulate matter (PM) exposure and neurologic dysfunction, linking exposure to PM with molecular and phenotypical modifications within the brain (Block & Calderón-Garcidueñas, 2009; Calderón-Garcidueñas *et al.*, 2002; Mateen & Brook, 2011). Studies investigating interactions involving the nose-to-brain interface as the primary entry route for particle neuro-translocation indicate this emerging field to be a vital public health topic. During oral and/or nasal inhalation, larger particles deposit in the upper airways while smaller particles deposit in the lower airway. Smaller particles can translocate from either region to secondary targets including the central nervous system, in addition to entering systemic circulation. This especially holds true for WTC-exposed cohorts who were exposed to high concentrations of debris and inhalable PM resulting in significant deposition on upper airway epithelia throughout the nasal cavity. This is evidenced by increased incidence of rhinosinusitis in first responders who arrived on-scene immediately following the towers collapse, but also in those who arrived downtown weeks to months after the towers collapsed (Wisnivesky *et al.*, 2011; de la Hoz *et al.*, 2010).

Hernandez *et al.* (2020) previously identified intranasal exposure to WTC_{PM} to be associated with significant increases in upper- and lower respiratory inflammation markers, as well as temporal deviations in serum NO₂⁻ and myographic aortic dysfunction in mice. Collectively, those findings highlight the tight interplay between chronically altered inflammatory events, homeostatic balances and diseased pathogenesis. Studies have identified ultrafine PM (<100 nm) may translocate across the olfactory epithelium and into olfactory bulbs of children and young adults in Mexico City coinciding with upregulation of cyclooxygenase-2 in the olfactory bulbs and other brain regions (Calderón-Garcidueñas *et al.*, 2008; Calderón-Garcidueñas *et al.*, 2004). Following WTC_{PM}-induced epithelial injury, as well as respiratory and neurological barrier breaching, subjects may have the potential to become predisposed to inflammatory cascades, thus making them more susceptible to developing pulmonary and neurological disorders including increased risk and vulnerability to subsequent ambient exposures. Intact epithelial linings have been found to be effective barriers against foreign substance exposures, while damaged epithelial linings have been found to result in decreased barrier protection (Herbert *et al.*, 2012; Kobayashi & Costanzo, 2009). The recognition of these preliminary links between ambient PM exposure and potential neurologic dysfunction underscores the importance in determining if exposure to WTC_{PM} would cause similar outcomes.

The primary olfactory pathway (located at the nasal-neurological interface) has been studied extensively in terms of pathogen invasion into CNS tissues; however, literature based in exposure sciences on PM influence in this region remains sparse. In the context of WTC

exposed cohorts, WTC_{PM} exposure was not a single exposure event, but a multiple exposure event, given continuous exposures through rescue and recovery operations, working on the WTC pile, and the year-long outdoor clean-up phases as well as indoor clean-ups which are less well documented. Due to the unique exposure scenarios encountered by first responders at the WTC pile - both in regard to the unprecedented atmospheric conditions (i.e., continual large particle resuspension of highly alkaline dusts) but also as a result of the belabored exercise-induced mouth-breathing coupled with inconsistent personal protective equipment usage, these cohorts were repeatedly exposed to extremely high concentrations of super-coarse alkaline dust (Lippmann *et al.*, 2015). Therefore, by nature of its deposition trajectory, WTC_{PM} could significantly impact the oronasal and oropharyngeal cavities, and has been identified to adversely impact the upper- and lower- respiratory tracts (Hernandez *et al.*, 2020).

Symptom surveys of first responders suggest on-site arrival correlation with the onset/ diagnosis of spirometric abnormalities and exposure duration with respect to post-traumatic stress disorder (PTSD) development and is evidenced by extensive co-morbidities within- and between- physical and mental health disorders (Pietrzak *et al.*, 2014; Wisnivesky *et al.*, 2011; Perrin *et al.*, 2007). Although mechanisms propagating air pollution-induced central nervous system (CNS) pathologies are not well understood, microglial activation has been suggested as a key component in understanding ambient PM and potentially WTC_{PM} induced neuroinflammation. Intertwined are effects of exposure-related neuro-inflammatory mechanisms, neurological exposure-related post-traumatic stress disorder and panic disorder outcomes, all of which are associated with neuro-endocrine disruption (Daskalakis *et al.*, 2013). Overall, our hypothesis is centered around understanding if WTC_{PM} exposure disrupts nasal and olfactory epithelial integrity and thus results in decreased olfaction capabilities and increased anxiety-like behaviors. The present study aims to advance our understanding of the biological mechanisms by which WTC_{PM} elicits proinflammatory responses and injurious outcomes via the nasal-olfactory interface, illuminating potential long-term health issues associated with PM exposures.

Methods

Animals:

Pathogen-free, 8 to 10-week-old male C57BL/6 inbred mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Prior to use in any experiments, all animals were housed in an approved facility at NYUSOM and acclimated for 1–2 weeks under controlled temperature ($22 \pm 2^\circ\text{C}$) and relative humidity (30–50%) with a 12-hr light/ dark cycle. Mice were provided *ad libitum* access to standard laboratory chow and filtered water. All protocols were approved by the NYU Grossman School of Medicine IACUC.

WTC_{PM} Samples:

Particle sampling, sizing and characterization have previously been described (Gavett, 2003; Lioy *et al.*, 2002a; McGee *et al.*, 2003). Mass median aerodynamic diameter of WTC_{PM<53} was found to be $23\mu\text{m}$. Particle size distributions of 10– $53\mu\text{m}$, 2.5– $1\mu\text{m}$, or $2.5\mu\text{m}$ comprised 42%, 0.5%, and 1.5% of the total mass, respectively (Vaughan *et al.*, 2014).

Dust stocks were collected on September 12th and 13th, 2001, and suspended in sterile DPBS just prior to use for *in vivo* exposures. All samples were sonicated for approximately 1 hour before use and were vortexed before administration to produce an as homogenous as possible mixture.

Intranasal (IN) Instillation:

WTC_{PM} particle preparation was previously described by Hernandez *et al.*, 2020 with dosing categories per mouse ranging from low (63 µg), medium (125 µg), or high (1000 µg). Table 1 lists the assays used in this study including dosing amount and frequency, sacrifice or assay timepoints, and inhalable human equivalent dosing (HED; mg/m³). Mice were anesthetized with 1–3% isoflurane in oxygen (Butler Schein, Dublin, OH) and affixed at a 45° angle. Top and bottom incisors were secured and a WTC_{PM} suspension was instilled intranasally (IN) in a volume of 50 µl divided equally into each nostril. DPBS was delivered as a negative control for all studies and a 1% Triton-X 100 solution (TX; Sigma-Aldrich®, St. Louis, MO) was delivered as a positive control in olfaction studies and is a standard methodology used to elicit chemical lesioning of epithelial cells and olfactory sensory neurons (OSN). In olfaction studies, WTC_{PM} was administered 5 days post-TX IN irrigation as OSN tissue recovery is known to begin 11–14 days post-TX exposure (Nathan *et al.*, 2005).

Animal Processing Post-Exposure:

Mice were euthanized via intraperitoneal injection (pentobarbital; 0.36 mg/g). Nasal epithelia, olfactory mucosa, olfactory bulbs, and whole brain samples were collected and stored at –80°C. For histopathologic evaluations, whole brains were fixed using 10% formalin *in situ*.

Histopathology:

Olfactory bulb and frontal cortex tissues were evaluated for inflammation (H&E staining), microglial activation (immunohistochemical staining using an anti-CD68 marker), and histochemical evidence of amyloid deposition (Congo Red staining). All tissues were semi-quantitatively evaluated by a certified histopathologist (Mass Histology Associates, Inc.; Worcester, MA), and graded accordingly: N/0= Normal; 1= Minimal; 2= Mild; 3= Moderate; and 4=Severe.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR):

Total RNA was isolated from the olfactory mucosa, olfactory bulbs and the prefrontal cortex (RNeasy® Lipid Tissue MiniKit; Qiagen, Valencia, CA). A high capacity cDNA Reverse Transcription Kit (Applied Biosystems) was used for cDNA synthesis and Taqman™ Gene Expression Assay (Life Technologies/Thermo Fisher Scientific) for qRT-PCR. Relative expression of HO1, SOD2, Txnrd2, Prdx6, TNFα and IBA-1 mRNA was determined using Ct with GAPDH serving as the endogenous reference gene.

Inductively coupled plasma mass spectrometry (ICP-MS):

Nasal septum and whole brains were subjected to wet/dry weight ratio and trace elemental analysis (Perkin Elmer NexION 350D) using standardized drying and digestion protocols (Titan MPS™ Microwave) for tissue-specific programs. Sc, In and TB served as internal standards and results given in µg/g of dried tissue calculated by ICP-MS Syngistix™ V1.1 software.

Olfaction Functional Assay:

Single IN exposure (1000 µg) and multi-exposure studies (630 µg of WTC_{PM} over the course of three weeks; 10 IN exposures of 63 µg of WTC_{PM}) conducted in mice were assayed for olfaction latency twice: at the end of the first round of exposure and olfaction latency testing, as well as after a 10-day recovery period following the first round of olfaction latency testing. Odor familiarization and latency testing were performed according to protocols described by Yang & Crawley (2009) in the buried food test. Prior to olfaction testing, mice were odor familiarized to Annie's Bunny Grahams (Honey Whole Grain Graham Snacks) for 4 hrs and returned to their respective groups with free access to standard chow and water. Overnight fasting (18 hr.) took place the same evening with food replaced at the conclusion of testing. Acclimation and testing for latency were performed with stationary video surveillance during the light phase between 10 am and 2 pm. Each mouse was considered to have uncovered the cookie when it began to hold the cookie in its forepaws and eat it.

Anxiety Behavioral Assay:

Behavioral testing for the following endpoints were performed by video examination noting exploratory/stationary time, freezing, grooming incidence, grooming time, rearing incidence, rearing time, hind stretching frequency, and hind stretching time for each individual mouse (Schellinck *et al.*, 2010; Sousa *et al.*, 2006). Behavioral indices and visualized representations of the listed behaviors are listed in Table 2. All data are expressed as percentages based upon each mouse's individual total time and time spent in each behavior.

Griess Reagent System:

Cell-free mouse NLF were assayed using the Griess Reagent System (Promega, Madison, WI) and prepared according to manufacturer protocol. Concentrations of total nitrite were calculated from a standard curve established with serial dilutions of sodium nitrite starting at 100 µM and ending at 0.39 µM, with a limit of detection of 2.5 µM. Colorimetric optical density was read at 535 nm.

Statistical Analyses:

Statistical Analyses were performed using GraphPad Prism® software (Version 5.0, GraphPad Software Inc.) or Microsoft Excel. All data are expressed as mean ± SEM. An unpaired t-test was used to determine the level of statistical significance of differences within treatment groups with respect to the various intranasal treatments and control treatments. A one-way analysis of variance (ANOVA) with a Student-Newman-Keul's post-hoc analysis was used to determine significant differences associated with multiple

IN exposure groups as well as the control group. Dixon and Grubbs analyses were used to screen for outliers. Differences were interpreted as statistically significant when p-values were below the threshold of 0.05. For anxiety behavioral testing, statistical analysis, as previously described by Luo *et al.*, was performed using a two-tailed nonparametric Mann-Whitney U-test for mean differences between control and exposed groups (Luo *et al.*, 2002).

Results

WTC_{PM} exposure in vivo induces neurologic injury and olfaction latency

The data presented in Figure 1 attest to the overall indices of CNS injury, with increased (n=3; p<0.05; 10–16%) wet/dry whole brain ratios of WTC_{PM} exposed mice 24 hours post-final IN treatment (Figure 1A). Increases in WTC_{PM} IN exposure concentrations were found to be correlated with body weight loss (r=0.8) and with whole brain mass decreases (p=0.01; r=0.99) (Figure 1B). Figures 1C and D indicate brain to body mass ratios to be increased and positively correlated (r=0.8) with increased WTC_{PM} IN exposure concentrations and not by changes in body weight. CNS tissues collected 30 days post-final WTC_{PM} IN treatment revealed the olfactory bulb to be intact, with only one mouse's brain exhibiting inflammation in the olfactory bulb (Figures 2A and B) and prefrontal cortex (Figures 2C and D). Microglial activation was found to be minimal across groups and contained no amyloid deposits (immunohistochemical anti-CD68 marker staining and Congo Red staining not shown).

Figures 3A–C demonstrate olfaction latency in mice treated once with TX (positive control for olfaction latency), WTC_{PM} only (1mg), or both TX and WTC_{PM}. Compared to IN control mice, IN WTC_{PM} alone resulted in a 50% increase in olfaction latency (+10 sec) and TX alone resulted in an 80% increase in olfaction latency (+16 sec; Figures 3b and C). The most robust and significant change in olfaction latency was found when WTC_{PM} was introduced intranasally 5 days-post TX treatment (during the olfactory sensing neuron recovery period), resulting in a 143.3% increase in olfaction latency (+28.7 sec; p<0.05) as compared to control mice (Figure 3C). Repeat-low dose treatment with IN WTC_{PM} alone (10 exposures of 63 µg; 630 µg total dose) over the course of 24 days revealed mouse olfaction deficit increases of approximately 136% (+37.1 sec) compared to control group (Figure 4A). The same group of WTC_{PM} treated mice who were given a 10-day recovery period and retested for olfaction recovery revealed an olfaction deficit of 33%, similar to the deficit observed in mice in which the nasal epithelia was disrupted with TX (Figure 4A).

Repeated exposure to WTC_{PM} propagates anxiety behaviors in vivo

In the same testing group as Figure 4A, marked changes in temporal weight loss/limited weight gain were noted, where control mice experienced higher weight gains overall as compared to WTC_{PM} exposed mice (630 µg WTC_{PM}; Figure 4B). Moreover, WTC_{PM} exposed mice experienced a weight gain plateau between days 17 and 22 and while not statistically significant, weight gain differences between groups were found to increase over time (Figure 4C). Behavioral assessments were performed in both single-dose high dose (1 mg) and repeat-dose low dose (630 µg total) exposed WTC_{PM} groups evaluating time

spent exploring, stationary time, hind stretching frequency and time spent hind stretching (Figure 5). TX and single-dose WTC_{PM} exposed groups showed no significant changes in any behavioral endpoints assessed (data not shown) but a similar comparison did reveal markedly decreased exploration times ($-4\% \pm 3.8$ for TX group, $-5.8\% \pm 3.0$ for WTC_{PM} group, and $-6.7\% \pm 4.1$ for TX+WTC_{PM} group) as well as increased stationary times ($+77\% \pm 3.8$ for TX group, $+112.5\% \pm 3.0$ for WTC_{PM} group, and $+129.3\% \pm 4.1$ for TX+WTC_{PM} group) as compared to the corresponding values in control groups, although this difference did not meet the $p < 0.05$ criterion for statistical significance (Figures 5A and B). When evaluating exploratory and stationary parameters within the repeat-low dose WTC_{PM} group, mice experienced an overall 25% reduction in exploration time ($78.8\% \pm 5.76$ for control group and $59.86\% \pm 6.1$ for WTC_{PM} group) and a 100% increase in stationary time ($21.2\% \pm 5.76$ for control group and $40.1\% \pm 6.1$ for WTC_{PM} group), as compared to control values (Figures 5C and D). Interestingly, the same mice exhibited significant increases in hindlimb stretching frequency ($+470\%$; 1.9 ± 0.4 for control group and 10.6 ± 1.9 for WTC_{PM} group; Figure 5E) as well as time spent stretching ($+301\%$; $3.4 \text{ sec} \pm 0.9$ for control group and 13.6 ± 1.9 for WTC_{PM} group; Figure 5F). These behaviors were abrogated and reflected those of control group when given a 10-day recovery period.

WTC_{PM} exposure in vivo solicits nitritative and oxidative stress in upper respiratory and CNS tissues

Figure 6A illustrates a 24-hour time course comparison of NO₂⁻ production in NLF. Total NO₂⁻ production values at 1 hour ($10.6 \mu\text{M} \pm 2.5$) and 6 hours ($8.1 \mu\text{M} \pm 1.8$) post-WTC_{PM} exposure were near control values ($8.4 \mu\text{M} \pm 1.6$) and peaked 24 hours post-exposure ($25 \mu\text{M} \pm 3.2$), triple the control value ($p < 0.05$). Olfactory mucosa tissues (olfactory mucosal lining and epithelia) assayed for antioxidant gene transcription revealed mRNA HO1 transcripts remained unchanged in all treated groups (single-dose, multiple-dose, and multiple-dose + 90-day recovery; Figure 6B). Conversely, SOD2 mRNA transcripts remained significantly downregulated across single-dose and repeat-dose treatment groups and remained significantly downregulated after a 90-day recovery period in the highest dose group (Figure 6C). Olfactory mucosal changes in CCL2, ICAM1, and VCAM were not significant (data not shown). Antioxidant Txnrd2 (intracellular/mitochondrial) and Prdx6 (cytoplasmic) temporal mRNA transcripts were significantly elevated in mouse olfactory bulbs exposed to a single 1 mg dose of WTC_{PM} (Figures 7A and B). The highest fold-changes in mRNA expression levels for Txnrd2 and Prdx6 occurred 24- and 168- hours post-treatment with ~5-fold and ~4-fold changes, respectively, from control olfactory bulb levels. Additionally, mRNA expression levels of both Txnrd6 and Prdx6 in both the frontal cortices and hippocampi were unremarkable when compared to control values (data not shown). Figure 7C illustrates long-term significantly upregulated antioxidant (HO1) and inflammation-associated (TNF α , IBA-1) mRNA transcripts in mouse olfactory bulbs 90 days post-exposure.

Discussion

The novel technique in which suspended WTC_{PM} was delivered through IN instillation and its exposure-related study limitations have been previously published about particle delivery

and particle size considerations (Hernandez *et al.*, 2020). Additionally, initial attempts to deliver dry intact WTC_{PM} with the use of a nasal insufflator as well as a pump-syringe delivery mechanism failed to deliver the full dose of WTC_{PM} due to the large particle size and abrasive nature of the dust. Thus, liquid particle suspensions were used instead to allow for consistent particle distribution throughout the nasal cavity. In contrast with an inhalation delivery mechanism, only 10–12% of an inhaled airstream passes over the olfactory lined ethmoturbinates in rodents, with a similar pattern to that found in humans (Kimbell & Subramaniam, 2001; Keyhani *et al.*, 1995; Hahn *et al.*, 1993). As an overwhelming amount of WTC_{PM} was deposited in the nasal cavities of humans caught in the WTC plume, the use of suspended particles may be the most optimal delivery method when evaluating nasal-neurologic outcomes. Although steps were taken to homogenize the suspended particle mixture, challenges regarding use of a non-homogenous large particle dust mixture include lack of traditional dose-response and/or time course outcomes for single- or repeat-dose exposure groups, as every dose batch and individual exposure dose contains compositional variances which may account for non-standard plot curves. The only recent historical high exposure event that could feasibly correlate to the WTC_{PM} exposure event in terms of dust plume volume would be volcanic ash exposure from the Mount Saint Helens eruption in 1980. With all things considered, these exposure events are not comparable regarding human exposure dose and human health outcomes as there are limited data on the human health impacts resulting from the Mount Saint Helens eruption, as well as compositional differences between the volcanic dust and WTC dust.

To date, a handful of studies have investigated the capacity of WTC_{PM} to elicit physiologic changes in humans (Rom *et al.*, 2010; Weiden *et al.*, 2010; Wu *et al.*, 2010; Reibman *et al.*, 2005) and rodents (Hernandez *et al.*, 2020; Cohen *et al.*, 2014; Vaughan *et al.*, 2014; Gavett, 2003). Cumulatively, these studies have demonstrated disease development to be moderately linked to the oxidative and inflammatory potential of the WTC_{PM}. Other WTC_{PM} epidemiologic studies have found similar associations between WTC_{PM} exposure and new onset disease development including pulmonary, cardiovascular, and mental health outcomes (Kwon *et al.*, 2016; Wisnivesky *et al.*, 2011; Adams *et al.*, 2006; Reibman *et al.*, 2005). Mechanistic understanding to assist in determining pathogenic originations have been ill-defined to date. Typically, air pollution exposures do not occur as single pollutant exposure events, but occur as multi pollutant episodes which may further potentiate environmentally induced injuries caused by repetitive insult from ambient pollutants. The contribution of ambient PM and its ability to originate or advance disease in people exposed to WTC_{PM} still remains to be investigated, as long latency disease manifestations, specifically neurodegenerative diseases that may be induced by WTC_{PM} exposure, may not occur for years or decades due to the fact that as age increases, neuronal activity decreases. This underscores the need for more detailed research on this topic (Clouston *et al.*, 2021; de la Hoz *et al.*, 2010; Landrigan *et al.*, 2005).

Previously published evidence of both pulmonary and nasal tissue injury in mice intranasally exposed to WTC_{PM} revealed increased inflammatory proteins in both bronchoalveolar- and nasal lavage fluids in mice, as well as WTC_{PM} retention in tissues (Hernandez *et al.*, 2020). Due to their proximity to the nasal passages, olfactory bulbs and their olfactory sensing neurons and receptor cells which line the nasal epithelium may provide a direct link between

upper respiratory tissues and CNS tissues, whereby olfaction loss after acute or chronic exposure to several volatile chemicals have been reported. Odor molecules dissolved in the nasal mucus are detected by olfactory neuron and receptor cells within the epithelium, undergoing sensory transduction from the epithelium, through the cribriform plate, thus connecting the epithelia to the limbic system at the olfactory bulbs (Doty, 2015; Touhara, 2014).

More importantly, studies investigating exposure to diesel exhaust particles and O₃ in urban areas have shown evidence of adverse neurological changes including dopaminergic neuronal toxicity, and astrocyte, cerebellar, substantia nigra, and hippocampal damage (Block & Calderón-Garcidueñas, 2009; Calderón-Garcidueñas *et al.*, 2008; Block *et al.*, 2006; Calderón-Garcidueñas *et al.*, 2004; Calderón-Garcidueñas *et al.*, 2002). These adverse changes have been shown to largely be a result of oxidative stress and pro-inflammatory induction. Additionally, olfaction decline and/ or loss has been identified as a hallmark of neurodegenerative diseases (Godoy *et al.*, 2015). While the full molecular basis of these alterations remains largely unknown, a leading hypothesis to these changes may be a result of metal content and/or particle translocation leading to downstream alterations in gene expression and homeostatic dysfunction.

Brain to body weight ratios have been a valuable tool used in deciphering and/or detecting organ toxicities (Bailey *et al.*, 2004). Findings from whole brain wet/dry ratios and brain/body weight ratios identified whole brains which have experienced edematous increases with respect to a single WTC_{PM} exposure at increasing doses, with ratios returning to baseline after a 30-day recovery period. External factors influencing whole brain mass/volumetric changes could be due in part to inflammatory and oxidative stress cascades as evidenced by increased neutrophils, inflammatory proteins and nitrate stress outcomes in previously published datasets (Hernandez *et al.*, 2020), as well as increased NLF NO₂⁻ and downregulated olfactory mucosal SOD2 gene transcripts and upregulated olfactory bulb oxidative and inflammatory gene transcripts reported herein. Fixed whole brain tissues revealed potential tissue injury, increased cellular response, and activated microglia in WTC_{PM} exposed mice. Excessive inflammatory responses have been shown to induce progressive microglial initiated neuronal death via microglial activation (direct/indirect), propagation, and amplification, all hallmarks of neurodegenerative disease outcomes (Gandy *et al.*, 2013; Gandy and Heppner, 2013; Block *et al.*, 2006; Polazzi & Contestabile, 2002).

In WTC_{PM} exposed mice, TNF α was found to be significantly upregulated 24 hours and 90-days post-exposure. IBA-1, a marker for activated microglial was also found to be upregulated 90-days post exposure and is suggestive of continued inflammatory consequences in olfactory bulb tissues long after exposure had concluded. These inflammatory incidents may be driven in part by thiol-specific antioxidant proteins involved in the reduction of oxide radicals, as supported by exacerbations in olfactory mucosal mRNA transcripts of Txnrd2 (intracellular/mitochondrial) and Prdx6 (cytoplasmic), both 24 hours and 7 days post WTC_{PM} exposure (Wang *et al.*, 2019). ROS/RNS events within the nasal-neurologic interface may also be driven in part, by particles, more specifically, metal particles that may settle within the nasal cavity or be taken up through olfactory sensing neurons within the olfactory epithelial layer (Oberdorster *et al.*, 2004). Although

there were no statistical increases in wet/dry ratios or trace elements found in septum samples (Supplemental Figures 1–3), whole brain sample levels offer some insight on particle transport into CNS tissues with increased levels of Al and Cr (Supplemental Figures 4A and B). Mechanism of transport is beyond the scope of this study but could be due to olfactory sensing neuron uptake or through pulmonary to blood translocation.

With evidence of acute and prolonged oxidative and inflammatory responses within the upper airways and CNS tissues, increases in TNF α mRNA expression and protein production have provided a glimpse into how inflammatory responses may lead to or alter homeostatic balances associated with neurodegenerative diseases (Frankola *et al.*, 2011). TNF α induction within the nasal cavity by nasal and olfactory epithelial cells has been shown to interfere with olfactory sensing neuron cell regeneration, resulting in loss of smell (Chen *et al.*, 2017; Turner *et al.*, 2010). While PM and its impact on the olfactory system has not been heavily studied in the particulate exposure sciences field, olfaction decline and/or loss has been identified as a hallmark of neurodegenerative diseases (Godoy *et al.*, 2015). Odor molecules dissolved in the nasal mucus are detected by olfactory neuron and receptor cells within the epithelium, undergoing sensory transduction from the epithelium, through the cribriform plate, thus connecting the epithelia to the limbic system at the olfactory bulbs (Doty, 2015; Touhara, 2014).

Dysregulation of intracellular calcium homeostasis or aberrant calcium signaling has been implicated in CNS dysfunction, affecting both neuronal and non-neuronal cells (Magi *et al.*, 2016; Chakroborty & Stutzmann, 2011). Aspartate Beta-Hydroxylase (Asph) gene involvement in calcium homeostasis has been greatly detailed throughout molecular literature but has not been extensively researched in the exposure sciences (Yang *et al.*, 2010; Dinchuk *et al.*, 2000). Preliminary evidence for calcium dysregulation has been presented with Asph mRNA transcript upregulation (15–20%) 90-days post-exposure in mice (Supplemental Figure 5). Equally, oxidative stress had also been implicated in early AD pathologies, linked to metal homeostatic imbalances (Bayer *et al.*, 2006; Miranda *et al.*, 2000). Despite unremarkable SOD2 mRNA transcripts at 90 days post-WTC_{PM} exposure, SOD2 data are informative in terms of functional pathogenesis with respect to neurological disease manifestations. SOD2 is a major mitochondrial antioxidant defense enzyme involved in free radical detoxification with critical implications regarding calcium homeostasis maintenance in neuronal cells (Zhao *et al.*, 2019).

Molecular studies tend to contain biological data which inform on the occurrence of significant biological changes. However, these studies are often deficient, in that many of the observed molecular changes may or may not be directly related to functional changes at a whole- tissue or organism level. Given the complex dynamic of molecular pathophysiology, it is important to question - if exposure-related molecular changes are observed, do phenotypic evaluations exist that could inform on disease pathogenesis or overt disease progression? Within the nasal passages, olfactory information is processed in olfactory epithelial cells lining the upper regions of the nasal cavity. The remaining nasal cavity is lined with neuron-lacking respiratory epithelia which serve as a protective surface. Within the olfactory epithelia, olfactory sensing neurons/receptor neurons are responsible for transmitting olfactory information back to the CNS. Of utmost importance are olfactory

sensing neurons- the only CNS tissue with direct links to the external world, which contain unique stem cells that give rise to new olfactory neurons throughout adult life, with capacity to replace olfactory receptor neurons after damage to the olfactory nerve. Olfactory receptor neuron turnover is critical and key considering it is the only CNS tissue to also regenerate (Slotnick *et al.*, 2010; Suzuki *et al.*, 2000).

Studies herein investigated the role of WTC_{PM} exposure and olfactory outcomes via olfactory sensing neuron damage and olfaction latency evaluations. Overall, WTC_{PM} intranasal exposure alone was found to induce olfaction latency, whether from a single large exposure or multiple smaller concentrations of exposures over time. Most interestingly, when WTC_{PM} exposure occurred during the olfactory sensing neuron recovery period in TX treated mice, olfaction latencies increased by 143.3%, suggesting that ability to smell was directly linked to peripheral olfaction damage. Additionally, WTC_{PM} exposure was found to inhibit injured olfaction tissue recovery processes. Olfaction latencies were also found to be mostly, but not fully abrogated after 10 days of recovery, suggesting olfactory sensing epithelia and neurons had begun the unique process of regeneration (Nathan *et al.*, 2005). Changes in body weight of WTC_{PM} exposed mice who gained less weight and gained it more slowly may be partially due to appetite loss and has been indicative of depression/anxiety in studies involving chronic mild stress models (Monteiro *et al.*, 2015; Krishnan & Nestler, 2011). Behavior analyses herein indicate WTC_{PM} exposed mice exhibited increased anxiety behaviors including attenuated hindlimb stretching frequency as well as time spent in an attenuated hindlimb stretch position and is representative of increased risk assessment/anxiety related behaviors. Most notable, WTC_{PM} exposed mice spent significantly less time exploring their cages and spent more time being stationary. WTC mental health studies have found increased incidence of depression and anxiety in numerous cohorts (Jordan *et al.*, 2019; North *et al.*, 2015; Adams & Boscarino, 2011).

Overall, weight loss and increased anxiety behavior data from these studies may help to illuminate potential mental health impacts from WTC_{PM} exposure alone, considering the increased occurrence of PTSD experienced by many groups exposed to the dust which includes first responders, those attempting to escape the wreckage and various ground zero cleanup crews. What continues to remain unknown are the mental health consequences from WTC_{PM} exposure alone as well as the propagation of WTC_{PM} mental health outcomes in combination with PTSD symptoms. Use of neurodegenerative transgenic mice or knockout mice including *Nrf2*^{-/-}, *ApoE*^{-/-}, or human APP knock-in mice would almost certainly yield greater information regarding oxidative stressed states as well as implications in neurologic recovery. Lastly, none of these suggestions would be fruitful without the use of long-term studies, considering the time course required for pulmonary and neurologic diseases to develop. This approach is identical to that applied by the NIH Accelerating Medicines Partnership in Alzheimer's Disease (AMP-AD) wherein computational analyses of multi-scale, multi-omic data from human postmortem brain from sporadic AD and aged controls are used to predict the presence of network pathology that implicates molecules as critical hubs and drivers of pathogenesis (Zhang *et al.*, 2013). These predicted hubs and drivers are then validated by knocking down or overexpressing the predicted hub and driver genes in specific brain cell types of mouse models of amyloidosis or tauopathy and analyzed according to sex and aging (Haure-Mirande *et al.*, 2019; Audrain *et al.*, 2019). AMP-AD

investigators have reported that this analysis predicts the existence of at least 5 subtypes of AD based on computational analysis of multi-scale, multi-omic network pathology (Neff et al., 2021) and that these can be recapitulated using the mouse model validation approach described above. Given the experience of the “AMP-AD approach” to reconstitute human network pathology in the brains of mouse models, this same approach could be used to elucidate how IN instillation of WTC_{PM} perturbs multi-scale multi-omic networks in brains of normal and neurodegenerative-disease-related mouse models. Such an approach is currently under development and could be especially valuable in this situation where data from postmortem brains of WTC responders are entirely lacking. Notably, multi-omic biofluid-based and peripheral blood monocyte-based studies, when analyzed by machine learning approaches that link peripheral markers with the extensive neuropsychological and neuroimaging datasets, may yet lead to an unraveling of the molecular pathogenesis of WTC responder brain syndromes (Clouston et al., 2021; Kuan et al., 2021).

Conclusion

Cumulatively, these data provide evidence of WTC_{PM} exposure in relation to oxidative stress-driven inflammation identified in the nasal cavity, propagated to olfactory bulb tissues and, potentially to other CNS tissues over extended time periods post-exposure. These are the first data on the acute and subacute responses to WTC_{PM} exposure at the nasal-neurologic interface, and this is a continuum study from previously published nasal-pulmonary studies. These studies support the necessity for continued investigation into mixed pollutant scenarios in conjunction with WTC_{PM} exposure to identify propagating factors of disease. The contribution of PM and its ability to originate or advance disease in humans exposed to WTC_{PM} still remains to be fully elucidated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Availability of data and material:

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Table of Abbreviations

Al	Aluminum
ANOVA	Analysis of Variance
As	Arsenic
Asph	Aspartate Beta-Hydroxylase
C57BL/6	C57BL/6 Inbred Mouse
Ca	Calcium
CNS	Central Nervous System
Cr	Chromium
H&E	Hematoxylin and Eosin
HO1	Heme Oxygenase 1
IACUC	Institutional Animal Care and Use Committee
IBA1	Ionized calcium binding adapter molecule 1
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
In	Indium ICP-MS Internal Standard
IN	Intranasal
IT	Intratracheal Instillation
K	Potassium
mRNA	Messenger Ribonucleic Acid
Na	Sodium
NLF	Nasal Lavage Fluid
NO₂⁻	Nitrite
PM	Particulate Matter
PMN	Polymorphonuclear Neutrophil
Prdx6	Peroxiredoxin 6
RNA	Ribonucleic Acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction

Sc	Scandium ICP-MS Internal Standard
SEM	Standard Error Mean
SOD2	Superoxide dismutase 2
TNFα	Tumor Necrosis Factor alpha
TX	Triton-X 100 (1%)
Txnrd2	Thioredoxin Reductase 2
WTC	World Trade Center
WTC_{PM}	World Trade Center Particulate Matter
WTC_{PM<53}	World Trade Center Particulate Matter <53 μ m

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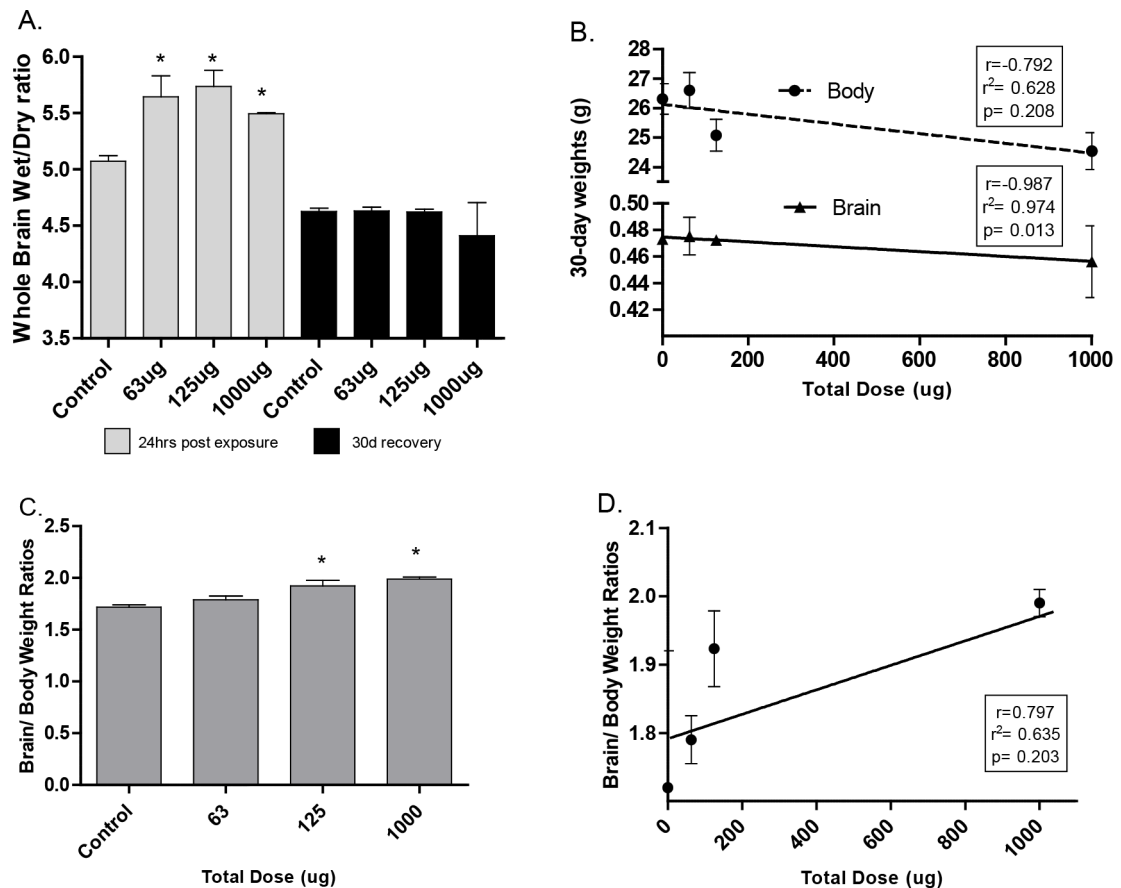


Figure 1. Markers of neurologic injury in mouse whole brains.

A) WTC_{PM} induced edema in whole brains 24 hours post-single exposure. B) Linear regression and Pearson correlation values of concentration vs body weight and whole brain weights in 30 day mice. C) Wet whole brain to weight ratios in 30 day mice. D) Linear regression and Pearson correlation values of concentration vs brain/ weight ratios. Reported values are averages of individually measured samples \pm SEM with n=3. Asterisks (*) indicate a statistically significant difference from control values ($p < 0.05$).

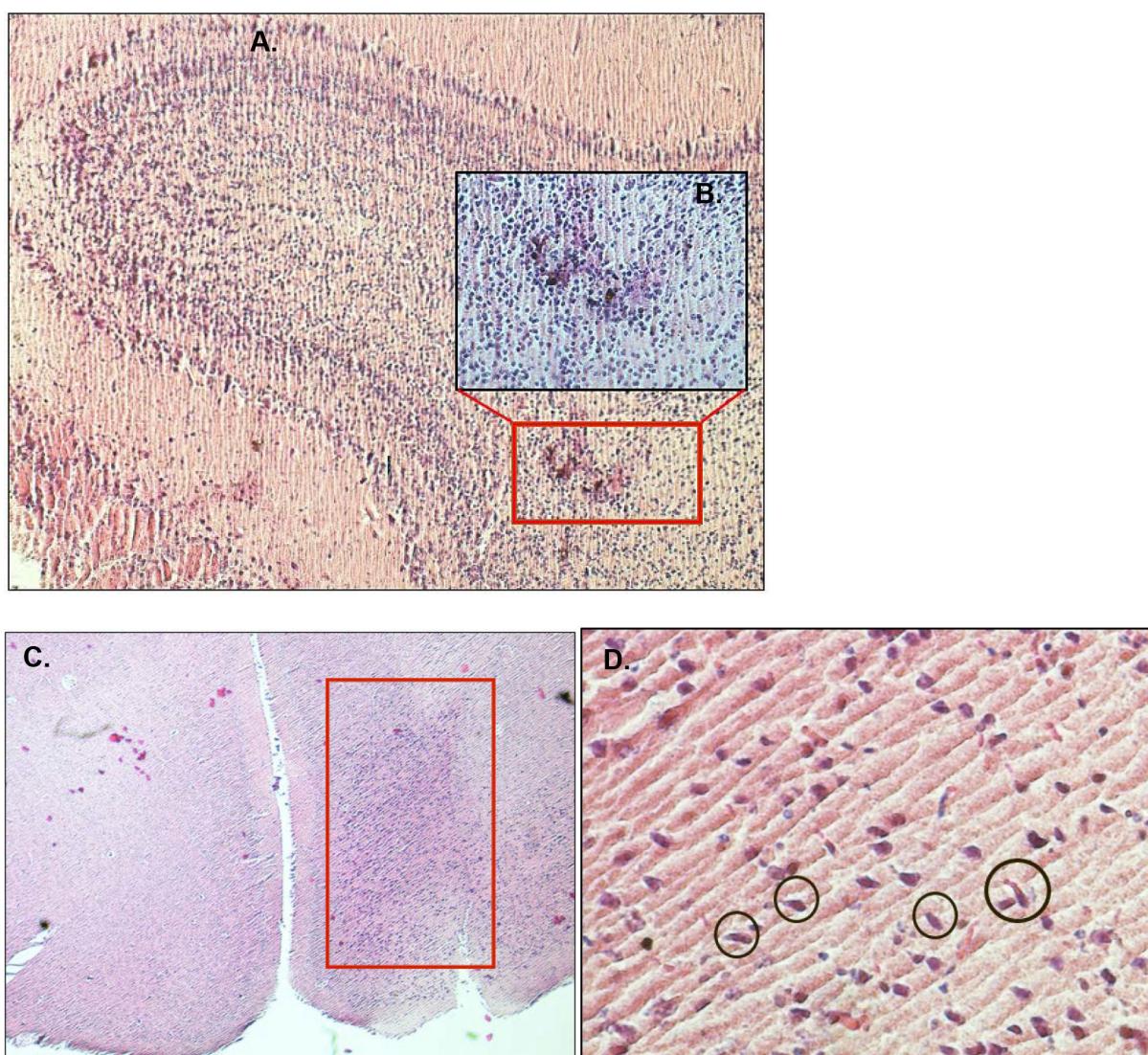


Figure 2. Gross histopathological examination of olfactory bulb and prefrontal cortex in single-dose (125 µg) WTCpM mice.

A & B) Lesion formation and increased cellular response in whole olfactory bulb. H&E staining x100 magnification. C) Increased cellular response in right prefrontal cortex tissue. D) Magnified region (C) of increased cellular response and amoeboid shaped microglia. H&E staining; x40 and x400 magnification.

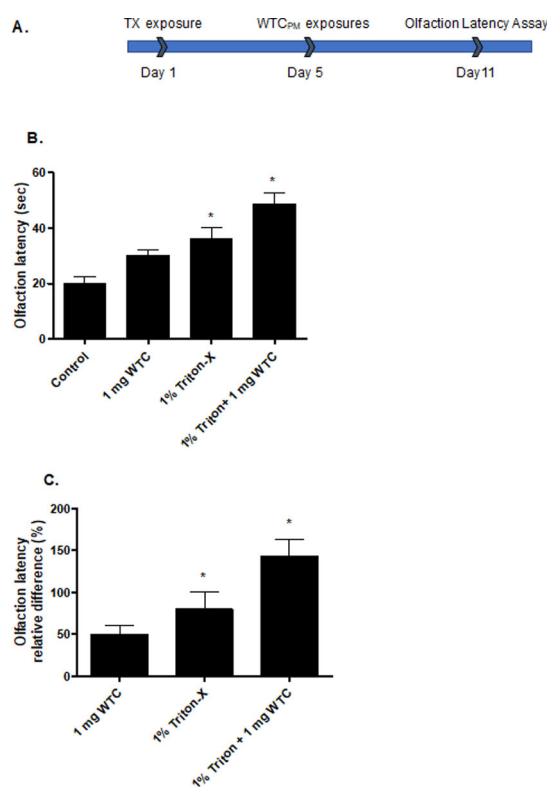


Figure 3. Single-dose WTC_{PM} exposure: Buried pellet latency assay

A) Exposure temporal matrix parameters. B) Time (sec) to uncover pellet among differentially exposed groups. C) Percentage (%) differences among treatment groups in relation to latency. Reported values are averages of individually measured latency times \pm SEM with $n=5/6$. Asterisks (*) indicate a statistically significant difference from control values ($p<0.05$) compared with WT (Mann–Whitney U-test).

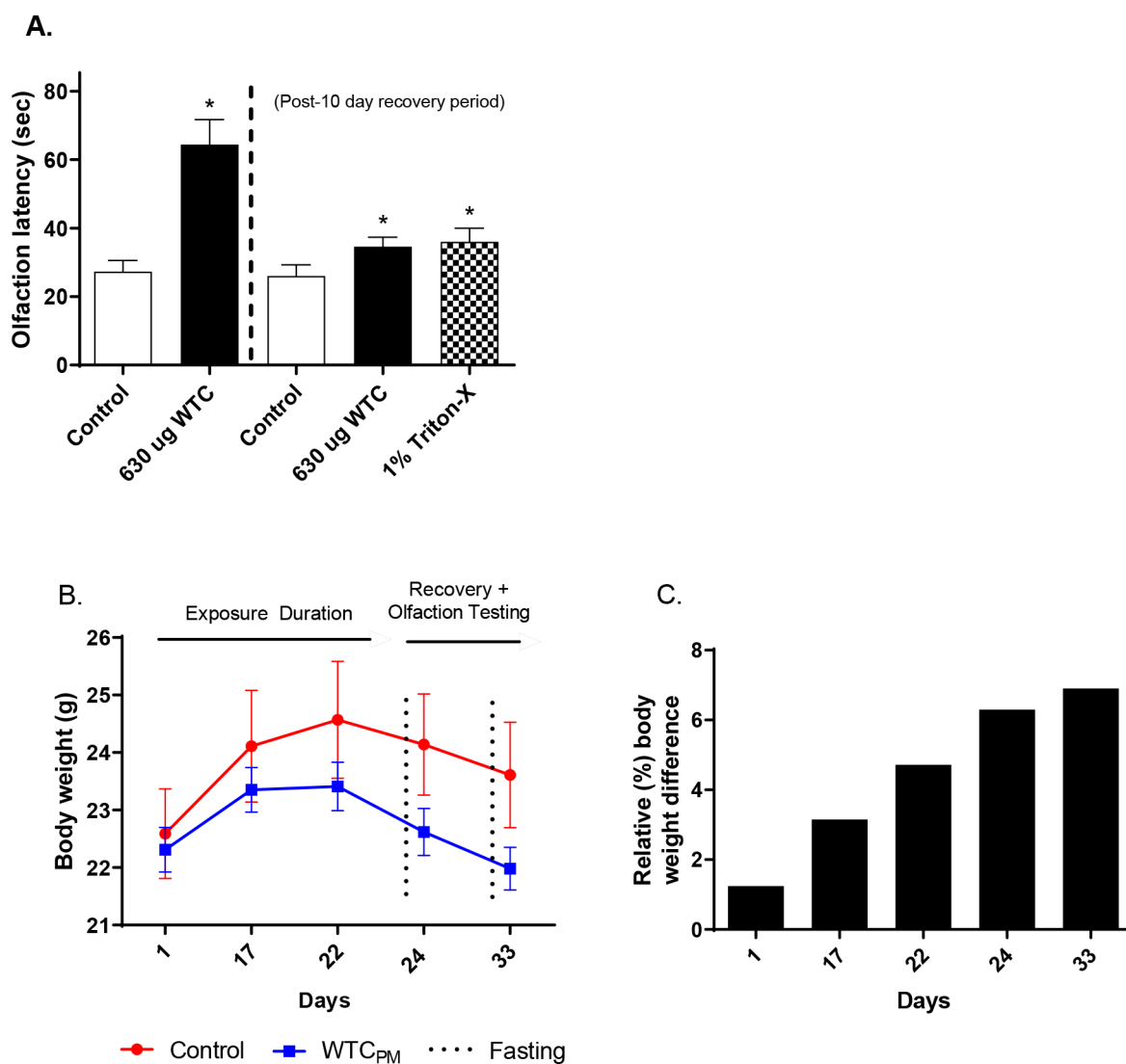


Figure 4. Repeat-low dose WTC_{PM} exposure in relation to olfaction latency and body weight changes in mice.

A) Olfaction latency among WTC_{PM} (630 μ g total dose) exposed mice post-final exposure and 10 days post-recovery. B) Olfaction testing schematic of repeat-low dose WTC_{PM} exposed mice and associated weight changes in control vs WTC_{PM} exposed mice. C) Relative weight gain differences between control and repeat-low dose WTC_{PM} exposed mice. Reported values are averages of individually measured latency times \pm SEM with $n=5/6$. Asterisks (*) indicate a statistically significant difference from control values ($p<0.05$) compared with WT (Mann–Whitney U-test).

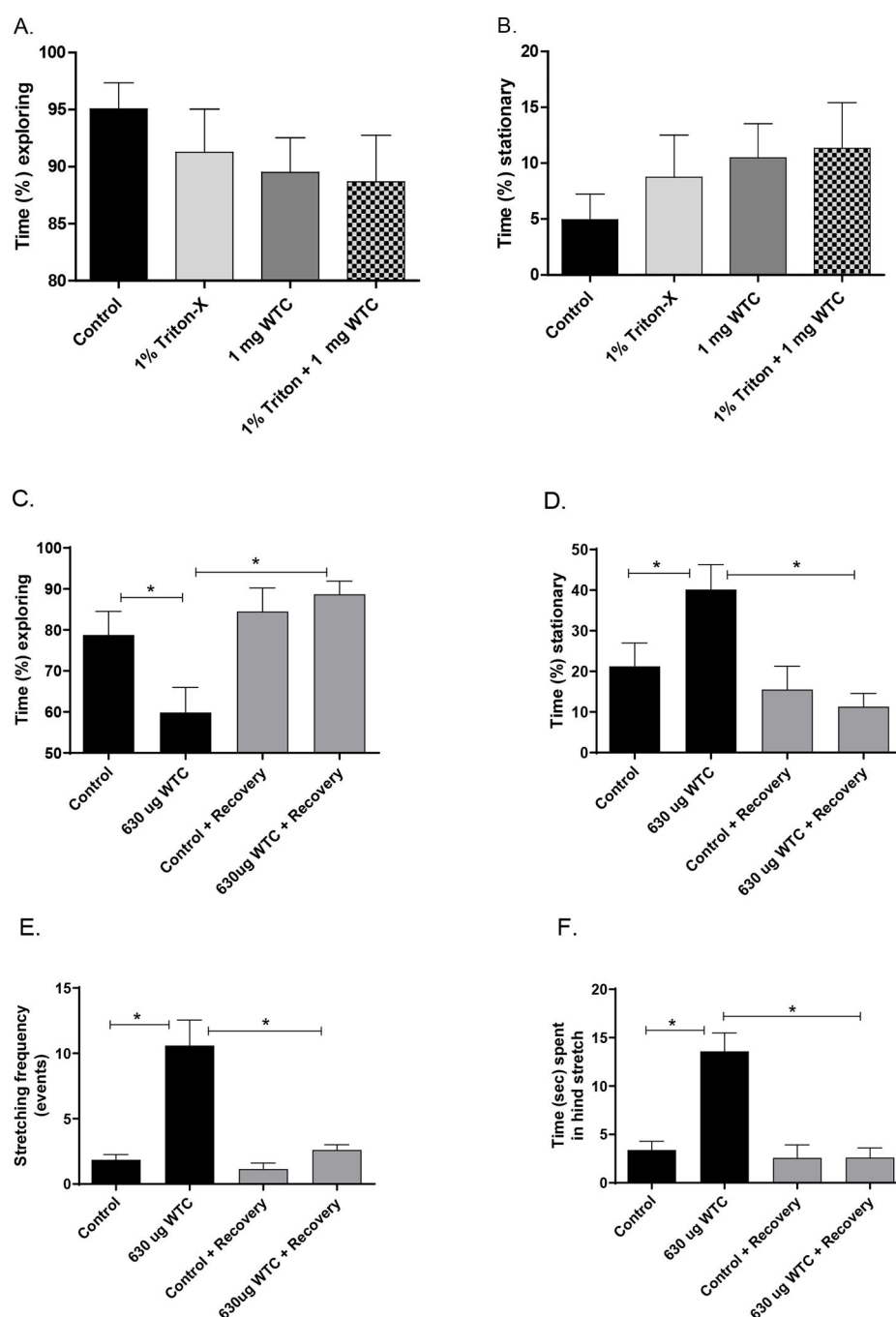


Figure 5. Behavioral assessments in single (A&B) and multi-dose (C-F) WTC_{PM} treated mice. Reported values are averages of individually measured samples \pm SEM with $n=5$ or 6 . Asterisks (*) indicate a statistically significant difference from control values ($p<0.05$) compared with WT (Mann–Whitney U-test).

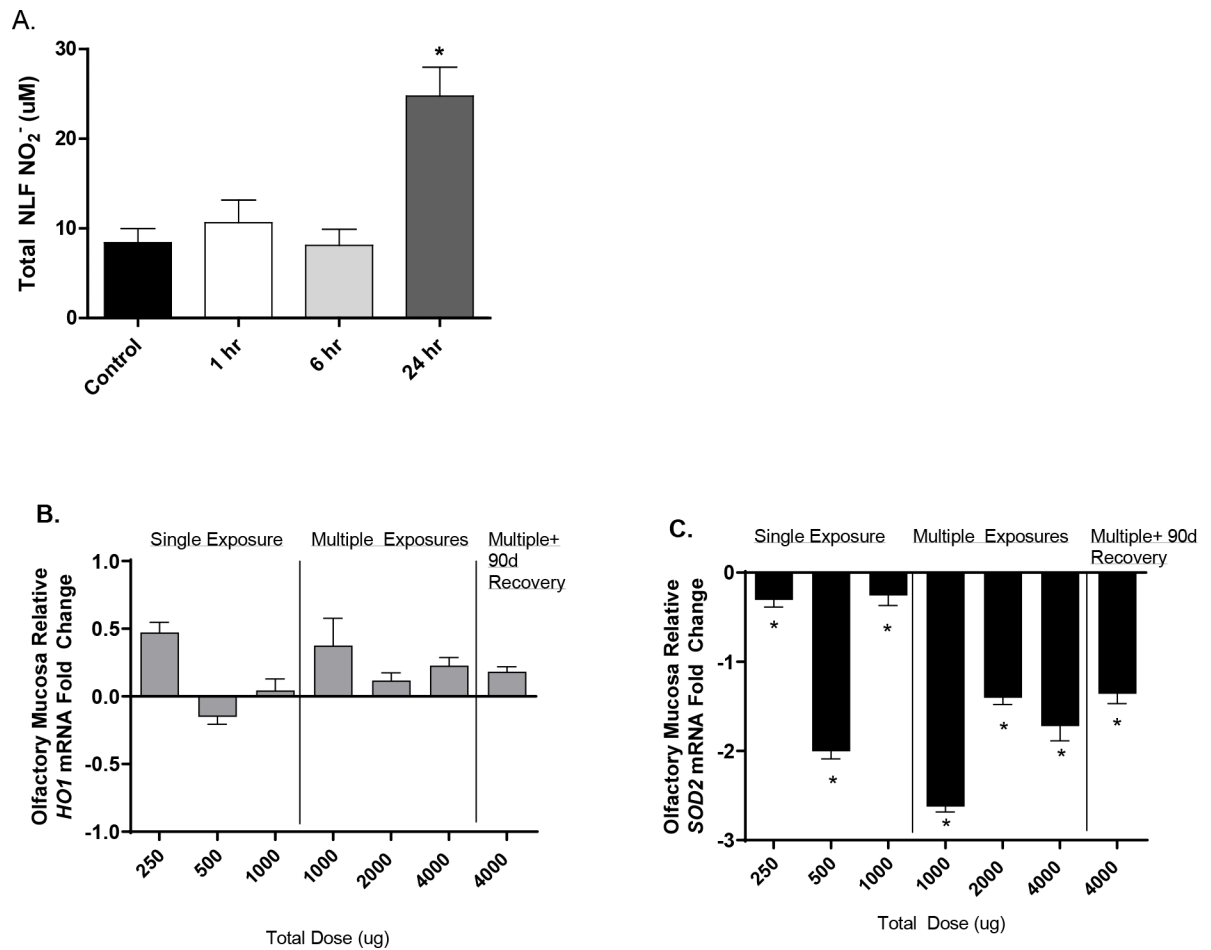


Figure 6. Upper respiratory nitrate and oxidative stress parameters in WTC_{PM} exposed mice.

A) Time course of NLF NO₂⁻ in mice exposed to a single dose of 125μg WTC_{PM}.

Reported values are averages of individually measured NLF samples ±SEM with n=3.

B & C) Olfactory mucosal HO1 and SOD2 mRNA transcript changes in single and multi-dose WTC_{PM} exposed mice, relative to DPBS controls. Reported values are averages of individually calculated CT values ±SEM; n=4–6. Asterisks (*) indicate a statistically significant difference from control values (p<0.05)

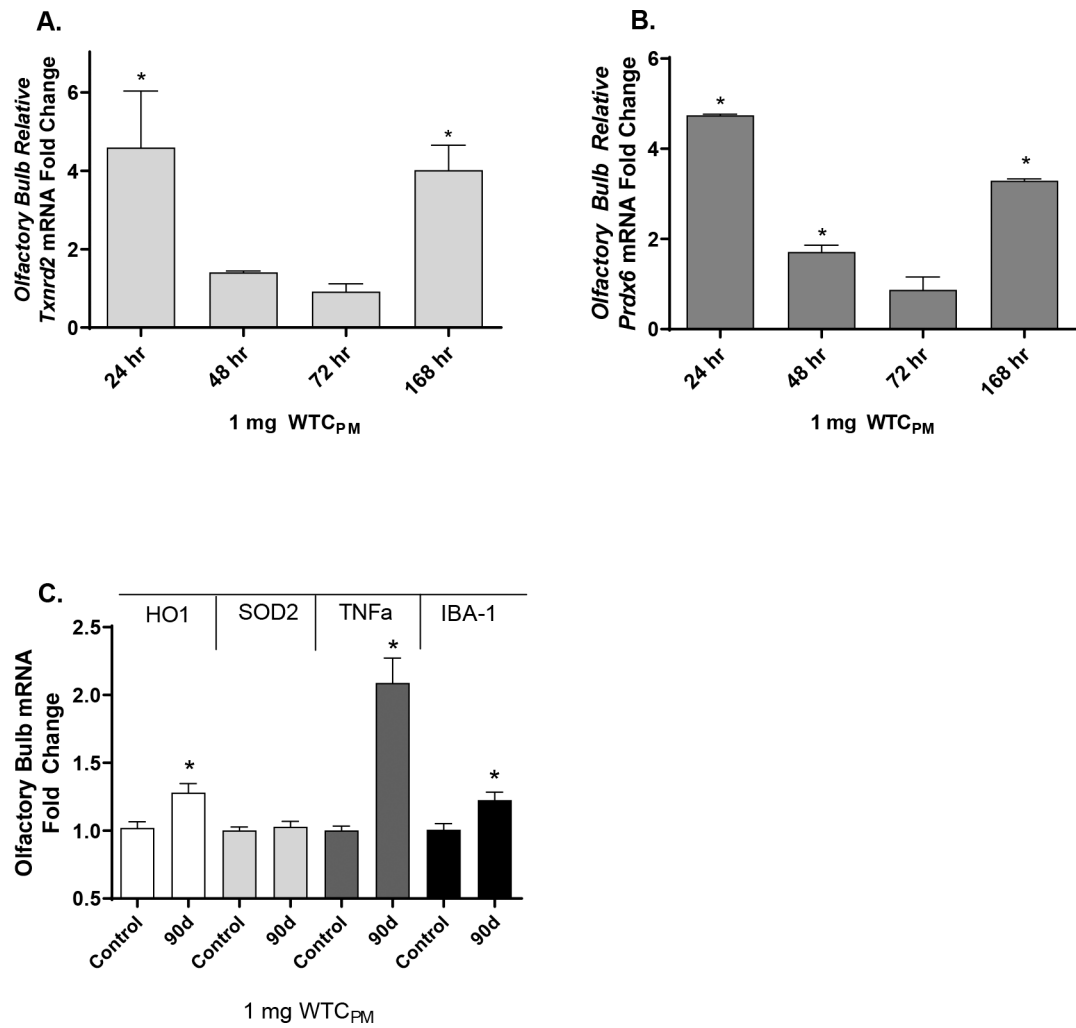


Figure 7. Temporal gene expression: oxidative stress and inflammation mRNA changes in single-dose WTC_{PM} exposed mouse olfactory bulbs.

A & B) Temporal mRNA oxidative gene transcripts of *Txnrd2* and *Prdx6* in mouse olfactory bulbs. C) Oxidative (HO1 and SOD2) and inflammatory-associated (TNFα and IBA-1) gene transcripts in mouse olfactory bulbs 90 days post-WTC_{PM} exposure. Reported values are averages of individually calculated CT values ±SEM; n=3–4. Asterisks (*) indicates a statistically significant difference from control values (p<0.05).

Table 1.

Exposure assay matrices and human equivalent dosing (HED).

Assay	Dosing and Dosing Frequency	Inhalable WTC _{PM} HED (mg/m ³)	Sacrifice or Assay Timepoint
Neurologic injury in whole brains	- Single IN dose of 63, 125 or 1000 µg	- 2, 4, and 31 mg/m ³	- Sacrifice: 24 hrs. or 30 days post-exposure
Buried pellet latency	- Day 1: single IN TX dose - Day 5: single IN 1000 µg WTC _{PM} dose	- 31 mg/m ³	- Assay: Day 11
Behavioral assessment	- Single dose: IN 1000 µg WTC _{PM} - Multi-dose: 63 µg repeat IN dose (10 IN exposures of 63 µg of WTC _{PM})	- 31 mg/m ³ - 20 mg/m ³	- Assay: 24 hours post-final exposure and 10 day recovery period (day 11) post-final exposure
Exploratory: Nitrate and oxidative stress endpoints	- Single exposure - Multi-dose exposure (four exposures over the course of 7 days; every other day). - Single exposure: time course	- 4, 31, 62 and 124 mg/m ³	- Sacrifice: 24 hrs. post-exposure - Sacrifice: 90 days post-final exposure - Time course sacrifice: 1, 6 or 24 hrs. post-exposure or 24, 48, 72 and 168 hrs. post-exposure

Mean HED (mg/m³) values are taken from Hernandez *et al.*, 2020 and are derived from regulatory allometric body weight scaling factors, assuming an average mouse weight of 0.02 mg and 50 kg or 70 kg for humans.

Table 2.

Defined mouse behavioral indices and associated behaviors (Sousa *et al.*, 2006, Schellinck *et al.*, 2010).

Behavior	Definition	Behavioral Indices
Exploratory	Time spent exploring the environment while in motion.	Anxiety
Freezing	Duration with which the mouse was completely stationary for any given amount of time.	Fear
Grooming	Incidence/duration in which the animal is spent time licking or scratching itself while stationary.	Anxiety
Stretch attenuated posture	Frequency with which the mouse demonstrates forward elongation of the head and shoulders followed by retraction to the original position.	Risk assessment/ anxiety