Exposures to Fine Particulate Matter and Ozone Above USA Standards are Associated with Auditory Brainstem Dysmorphology and Delayed Auditory Brainstem Evoked Potentials in Healthy Dogs

Partha S. Mukherjee
Boise State University

Exposures to fine particulate matter and ozone above USA standards are associated with auditory brainstem dysmorphology and delayed auditory brainstem evoked potentials in healthy dogs.

Lilian Calderón-Garcidueñas[1,2], Luis O. González-González[3], Randy J. Kulesza [4], Tatiana M. Fech[4], Gabriela Pérez-Guillé[3] , Miguel Angel Jiménez-Bravo Luna [3], Rosa Eugenia Soriano-Rosales[3], Edelmira Solorio[3], José de Jesús Miramontes-Higuera[2] , Aline Gómez-Maqueo Chew[2], Alexia F. Bernal-Morúa[2], Partha S. Mukherjee[5], Ricardo Torres-Jardón[6], Paul C. Mills[7], Wayne J. Wilson[7], Beatriz Pérez-Guillé[3], Amedeo D'Angiulli[8].

[1] The University of Montana, Missoula, MT, USA 59812

[2] Universidad del Valle de México, México

[3] Instituto Nacional de Pediatría, Mexico City, Mexico 04530

[4] Auditory Research Center, Lake Erie College of Osteopathic Medicine, Erie, PA 16509, USA

[5] Mathematics Department, Boise State University, Boise, Idaho, USA

[6] Centro de Ciencias de la Atmósfera, Universidad Nacional Autónoma de México, Mexico City, Mexico 04510

[7] The University of Queensland, QLD 4072, Australia

[8] Department of Psychology, Carleton University, Ottawa, Ontario K1S 5B6, Canada

KEY WORDS: air pollution, ozone, PM $_{2.5}$, brainstem auditory nuclei, ventral cochlear nuclei, medial superior olive, dysmorphology, neurodegeneration, BAEPs, dogs, Mexico City

[§] **Corresponding author:** Lilian Calderón-Garcidueñas MA, MD, PhD, The University of Montana, 32 Campus Drive, 287 Skaggs Building, Missoula, MT 59812, USA

E mail address: lilian.calderon-garciduenas@umontana.edu

Abstract

Background: Delayed central conduction times in the auditory brainstem have been observed in Mexico City (MC) healthy children breathing fine particulate matter ($PM_{2.5}$) and ozone (O_3) above the current United States Environmental Protection Agency (US-EPA) standards. MC children have α synuclein brainstem accumulation and medial superior olivary complex (MSO) dysmorphology. The present study used a dog model to further investigate the potential effects of air pollution on the function and morphology of the auditory brainstem.

Methodology: Twenty-four dogs living in clean air v MC, average age 37.1 ± 26.3 months, underwent brainstem auditory evoked potential (BAEP) measurements. Eight dogs (4 MC, 4 Controls) were analysed for auditory brainstem morphology and histopathology.

Results: MC dogs showed ventral cochlear nuclei hypotrophy and MSO dysmorphology with a significant decrease in cell body size, with many cell bodies $< 100 \, \mu m^2$, a significant decrease in neuronal packing density with many regions in the nucleus devoid of neurons and marked gliosis. MC dogs showed significant delayed BAEP absolute wave I, III and V latencies compared to controls.

Conclusions: Auditory nuclei dysmorphology and BAEPs consistent with an alteration of the generator sites of the auditory brainstem response waveform are a common denominator for dogs and children in highly polluted MC. This study puts forward the usefulness of BAEPs to study auditory brainstem neurodegenerative changes associated with air pollution in dogs and its potential use in young urbanites as a proxy for an evolving neurodegenerative process towards Alzheimer Disease. Recognition of the role of non-invasive BAEPs in urban dogs is warranted to elucidate novel neurodegenerative pathways link to air pollution and may be a promising early diagnostic strategy for AD.

Background

Air pollution is responsible for over 5.5 million premature deaths and 141.5 million years of disability-adjusted life years in 188 countries (Forouzanfar et al., 2015). Environmental pollutants, toxic industrial chemicals, pesticides, heavy metals, and hazardous wastes have detrimental, sometimes irreversible impact upon the developing central nervous system in children (Calderón-Garcidueñas et al., 2016a; Miller et al., 2016). Traffic-related air pollution has increasingly been identified as an important contributor to adverse health effects of air pollution and its impact extends from pregnancy to adult life (Kolosnjaj-Tabi et al., 2015; Stieb et al., 2016; Basagaña et al., 2016;Wu et al., 2016; Porta et al., 2016; Chen et al., 2017). Air quality in Mexico City (CDMX) has been recognized as among the worst in the world (Bravo and Torres 2002; Parrish et al., 2011; WHO, 2016). Despite a number of actions and restrictions imposed to curtail the air pollution during the past two decades, levels of $PM_{2.5}$ and O_3 (ozone) have stayed significantly above the US EPA standards (US EPA 2014), leading to continuously high exposures of today's children and teenagers, starting *in utero (*Bravo- Alvarez and Torres-Jardón, 2002; De Vizcaya-Ruiz et al., 2006; Querol et al., 2008; Davis, 2017). Brainstem Auditory Evoked Potentials (BAEPs) represent a reliable means of evaluating the integrity of the auditory nerve and brainstem (Strain et al., 1991; Wilson and Mills, 2005; Wilson et al., 2006, 2011) and providing information of the neurologic and audiologic status of healthy and high risk populations, including residents in highly polluted megacities (Scaioli et al., 2009;Calderón-Garcidueñas et al., 2011). For example we have reported that BAEPs in clinically healthy children (8.05±1.4 year old) in Southwest Mexico City (SWMC) had significant delays in wave III (t(50)=17.038; p<0.0001) and wave V (t(50)=19.730; p<0.0001), and also significantly longer inter-wave intervals for waves I-III, III-V, and I-V (all t(50)>7.501; p<0.0001) versus clean air controls (Calderón-Garcidueñas et al., 2011). These findings are

consistent with delayed central conduction time of brainstem neural transmission. These SWMC children also exhibited significant evidence of neuroinflammation, accumulation of α synuclein in auditory and vestibular nuclei, significant dysmorphology in medial superior olive (MSO) neurons, and alpha-synuclein neuronal aggregation (the early neuropathological hallmark of sporadic Parkinson's disease) (Calderón-Garcidueñas et al., 2011). The observed BAEPs abnormalities in SWMC children were interpreted as a sign of auditory neuronal injury associated with both neuroinflammation and neurodegeneration. This was thought to place these children at high risk for auditory and vestibular impairment, particularly since none of these abnormalities were present in low pollution matched control children (Calderón-Garcidueñas et al., 2011).

Indications that air pollution could affect brain function in children warrant further investigation using models that allow for greater integration of BAEP, morphological and histopathological data. One such model is the dog, an animal on which there is an established history of BAEP research (Sims, 1988; Fischer and Obermaier, 1994; Wilson and Mills, 2005; Wilson et al., 2006, 2011; Schmutz, 2014). The use of a dog model would allow researchers to study dog cohorts with complete medical and air pollution exposure data. This allows for the study of associations amongst BAEP results, auditory brainstem morphology and pathology, $PM_{2.5}$ and O_3 exposures. Our previous work revealed significant dysmorphology in the human medial superior olive (MSO), an essential auditory brainstem center (Calderón-Garcidueñas et al., 2011). Further, this nucleus appears to be susceptible to neurodevelopmental disorders (Kulesza and Mangunay, 2008; Kulesza et al., 2011; Lukose et al., 2015). Because of the abnormalities in auditory brainstem response observed in these animals, we therefore choose to examine the morphology of the MSO.

In the present study, we aimed to determine if: 1. Dogs living in polluted air showed signs of abnormal auditory brainstem morphology and/or histopathology

compared with dogs in clean air cities. 2. Dogs living in polluted air showed abnormal BAEP waveforms compared to dogs living in clean air.

A key question in the setting of air pollution impacting dogs and humans alike has to be asked: Since non-invasive BAEPs abnormal studies in dogs are associated with brainstem nuclei dysmorphology and high exposures to PM $_{2.5}$ and ozone are the common denominator for similar findings in young urbanites, the key question is: Could BAEPs abnormalities in young urbanites be a proxy for an evolving neurodegenerative process towards AD? We argue the answer is yes.

Methods

Study areas

Two study regions were used in this study: Mexico City Metropolitan Area (MCMA) and Tlaxcala, Tlaxcala, a control city. Mexico City Metropolitan Area (MCMA) is the largest urban center in North America and is a good example of extreme urban growth and massive involuntary exposure of 24 million people to concentrations of O_3 and PM $_{2.5}$ above the Environmental Protection Agency (EPA) Standards (Bravo and Torres 2002; Parrish et al., 2011; WHO, 2016, Calderón-Segura et al., 2004; Dzepina et al., 2007; Valle-Hernández et al., 2010; Mugica-Álvarez et al., 2012; US EPA, 2014; Davis, 2017).

Its metropolitan area of over 2,000 square kilometers lies in an elevated basin 2,240 m above sea level surrounded on three sides by mountain ridges, a broad opening to the north, and a narrower gap to the south-southwest. Dogs (and humans) living in Southern Mexico City have been exposed to significant concentrations of O_3 and aerosols resulting from typical diurnal wind transportation of air masses rich in these secondary pollutants towards smog receptor sites in southern MCMA. In addition, they have high exposures to secondary tracers (NO_3^-) and particles with lipopolysaccharides (PM-LPS); volatile organic compounds (VOCs); $PM_{2.5}$ and its constituent organic and elemental carbon including polycyclic aromatic hydrocarbons; secondary

inorganic aerosols (SO_4^{2-}, NO_3^-, NH_4^+); and metals (Zn, Cu, Pb, Ti, Mn, Cr, V), (Figures 1-3) (Osornio-Vargas et al., 2003; Dzepina et al., 2007; Querol et al., 2008; Valle-Hernández et al., 2010; Vega et al., 2010). Finally, they also have high exposures to total PAHs including benzo [a] pyrene (Marr et al., 2006). These semi-volatile compounds are emitted mainly by uncontrolled sources such as diesel motor vehicle, biomass and garbage burning (Figure 4). The control city has relatively few contributing emission sources from industry and cars and good ventilation conditions due to regional winds (Calderón-Garcidueñas et al., 2001, 2002, 2003).

Experimental Animals

The study protocol was approved by the Institutional Animal Care and Use Committee at the Instituto Nacional de Pediatría (INP). Procedures used were in accordance with the guidelines of the INP on the Use and Care of the Animals, the regulations of the NOM-062-ZOO-1999 Official Mexican Standard and the Guide for the Care and Use of Experimental Animals and the standard in the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23). The INP provided full veterinary daily care of the dogs included in this study. All of the dogs had been raised by their dam for 6 weeks, before weaning onto a commercially-available puppy food (Puppy, Pedigree®, MN, US). The food was changed to an adult formula (Pedigree®, MN, US) when they reached 12 months of age and water was provided ad libitum. The dogs were housed in outdoor-indoor kennels and husbandry was in compliance with the American Association of Laboratory Animal Certification Standards. All dogs had current vaccination status and underwent daily veterinary observation, regular anti-helmintic treatment for internal worms, daily clinical examinations, and weekly neurological examinations. Otoscopic examinations were performed by our veterinarians to rule out any disease of the external ear and/or eardrum that could interfere with hearing.

The dogs living in polluted air were 12 healthy dogs bred and raised at the INP animal facility located in Southwest MC. The control dogs living in clean-air were 12 dogs whelped and housed in an outdoor-indoor kennel located in the control city. Clean air controls were called CNTL.

Necropsies and tissue preparation

Four Mexico City dogs (32.2±17.6 months) and 4 controls (56.7±49.7 months) were selected for brain histopathological studies. Animals were euthanized with an overdose of sodium pentobarbital (Pisabental PISA, Mexico). Brains were removed from the skull within 3 minutes after death, and brains were trimmed to include the cochlear nuclei and SOC and placed in cryoprotectant (30% sucrose and 4% paraformaldehyde in 0.1 M phosphate buffer [PB], pH 7.2) until they were saturated. Tissue blocks were sectioned on a freezing stage microtome at a thickness of 40 µm and free floating sections were collected in 3 wells. Sections from the first well were collected in PB and mounted from cresyl gelatin in series and stained for Nïssl substance with Giemsa. Sections from the second well were collected in distilled water and stained for myelin and Nïssl substance. After staining of slides from wells one and two, the slides were passed through ascending alcohols, cleared and sealed under coverslips. Giemsa-stained tissue sections were examined with an Olympus BX45 microscope. The nuclei of the dog superior olive were identified based on the delineations described previously (Goldberg and Brown, 1968). Cell body profiles of MSO neurons were traced at a final magnification of 1000X and tracings were quantified using Image J (2.0; Fiji). The software was calibrated to a standard scale bar and for all cell body profiles, an index of circularity was calculated using the following equation: Circularity = $[4\pi*Area/Perimeter^2]$. For morphometric analysis, classification of cell body morphology was made using objective criteria. Specifically, neuronal profiles were classified as fusiform if the major axis/minor axis was >3. If the circularity measure for a given

profile was >0.6, the profile was classified as ''round''; all remaining profiles were classified as stellate. We have previously correlated each of these criteria with distinct cell body morphologies in the human MSO (Beebe et al., 2014; Kulesza, 2014; Lukose et al., 2015).

Brainstem auditory evoked potentials (BAEPs)

BAEPs assessments were performed under general anaesthesia using a mixture of xylazine (Rompun®) 7 mg/kg/IM, atropine sulfate (Atropisa®) 0.05 mg/kg/IM and tiletamine + zolazepam (Zoletil®) 10 mg/kg/IM. All BAEP assessments were completed in a sound attenuating room using the Biopac system®, Model MP100, ERS100. The average duration of the BAEP assessment was approximately 10 minutes per dog. The BAER protocols used were based on Kemper et al. (2013) and are described below. To elicit each BAEP, acoustic click stimuli (driven by 0.1 ms electrical square wave stimuli of rarefacting polarity were presented at 90 dB nHL (referenced to human hearing) and 11.4 clicks per second through model ER-3A insert earphones to the test ear. The non-test ear was masked during the recordings using white noise at 40 dB nHL. To record the BAEP, each dog was placed in the right lateral recumbency, and disposable stainless steel needle electrodes were placed subcutaneously at each dog's vertex (non-inverting) (equivalent to the Cz position using the international 10–20 system of electrode placement in humans), and rostral to the tragus of the test ear (inverting) and non-test ear (ground). Electrode impedances were maintained at <5 kΩ as measured by the Biopac system® ''impedance check'' function. A 300–3000 Hz bandpass filter was employed to reduce the presence of extraneous signals and 1000 thousand sweeps were amplified and averaged for each BAEP recording. Two BAEP waveforms (forming a ''pair'') were recorded from each ear of each dog. These waveforms were labelled ''raw'' BAEP waveforms. Raw BAER waveforms for each ear of each dog were averaged to give one BAER waveform for each ear of each dog.

BAER waveforms were analysed for the absolute latencies of waves I, III and V and the interwave intervals of I-III, III-V and I-V.

Statistical analyses

BAEP data were analyzed using IBM SPSS Statistics version 20 statistical package. Two sets of dependent variables were used: 1) three BAEP peak latencies (I, III, and V) measured for the right and left ear; 2) The BEAP peak-to-peak intervals, I-III, III-V, and I-V, for the right and left side. Given the sample size, we compared the effects of dog group membership on the BAEP latencies by using separate planned non parametric tests (Mann-Whitney U) examining the pairwise mean differences for each BAEP peak and interval (as defined above). To buffer the effects of multiple comparisons we adjusted the significance threshold at $p < 0.03$, using the Simes-Bonferroni procedure. Data sets that met a normal distribution were compared using parametric tests (i.e. t-test) and results are presented in the text and figures as mean ± standard deviation (SD). Morphometric brainstem data sets were examined for normality using the D'Agostino & Pearson normality test in GraphPad Prism 7.0 (La Jolla, CA). Data sets that failed to meet a normal distribution were compared using non-parametric tests (Kruskal-Wallis). Additionally, for the MSO a contingency table of neuronal morphology was constructed and the distribution of this population was compared using a Chi-square test.

Results

Air pollution levels

Southwest Mexico City dogs in this study have been exposed to concentrations of $PM_{2.5}$ and O_3, above the current EPA standards for their entire life (Figures 1-4). The climatic conditions in SWMC are relatively stable through the year thus pollutant concentrations are relatively uniform without strong variations. According to data from the government air quality monitoring

network, during the 2002-2012 period that includes the period the dogs have lived in SWMC, the PM$_{2.5}$ 3-year averages of annual average concentrations in the representative monitoring stations were always above the respective primary PM$_{2.5}$ US EPA annual standard of 12 µg/m^3. This standard is attained when the 3-year average of annual means is less than or equal to the above mentioned concentration. In addition, the four highest daily maximum eight-hour average concentrations for each of 3 consecutive years for O$_3$ in the same monitoring sites for the 2002-2012 period (Figure 3) were above the current 8-h average O$_3$ NAAQS of 75 ppb.

In the control city, previous studies in dogs have shown normal brain, respiratory and cardiovascular tissues (Calderón-Garcidueñas et al., 2001, 2002, 2003).

Clinical evaluations

Dogs in SWMC as well as dogs with lifetime exposures to clean air at no time showed any evidence of overt respiratory, cardiovascular, or neurological diseases. Their food and water intake was within normal limits.

Auditory Brainstem Morphometry and Pathology

In control animals, the MSO is a U-shaped nucleus composed mainly of neurons with round/oval cell bodies (Figure 5A). Control MSO cell bodies measured 261 µm^2 (95% CI = 251-270 µm^2). In the four Mexico City animals, MSO neuronal cell bodies were significantly smaller and the mean cell body size ranged in size from 106 to 158 µm^2. This difference in cell body size was statistically significant (Kruskal-Wallis, <.0001; Dunn's multiple comparisons test, <.0001 for all four animals). Indeed, at least 30% of the MSO neurons in each of the four dogs examined had cell body areas smaller than 100 µm^2 (Figure 5B).

In control animals, the MSO was composed of 72% round/oval neurons, 21% stellate and 7% fusiform neurons. There was no difference in the distribution of neuronal morphologies among the control animals (Chi square, p = .67). Moreover, the distribution of cell body morphologies

in the MSO from the both 17m animals was significantly different from controls (figure 5C; Chi square; p < .005). In the 17m animals, there were markedly more fusiform neurons.

Finally, in control animals, the neuronal density within the MSO measured 22.5 neurons per 0.1 mm^2 (SD = 6.7, 95% CI = 19.84 - 25.26). In Mexico City animals, the density of MSO neurons measured 11.03 neurons/0.1 mm^2 (SD = 5.1, 95% CI = 9.53 - 12.52) (Figure 5D). This difference in neuronal density was statistically significant (ANOVA, <.0001; Dunnett's multiple comparisons test; p<.0001, except for control vs 17m, p = .001). Notably, within each of the MSO there were large patches that were devoid of neurons (asterisks in figure 5E).

Hypotrophy of the ventral cochlear nuclei (VCN) was significant in MC dogs. In both divisions of the VCN, there was focal gliosis, a loss of cell body orientation (relative to incoming auditory nerve fibres) and significant loss of neurons. Additionally, there was a marked increase in very small fusiform neurons, which are typically rare in the VCN.

BAEPs

The subjects from the two groups, dogs from Mexico City (MXC) living in polluted air and control dogs (CNTL), did not have significant between-group variability in the distribution age (p = 0.843) or gender (p = 0.755) (Tables 1A,B,C). Since no morphological or amplitude anomalies were found, our analysis was focused on the most commonly reproducible BAEP components in healthy dogs with typical development (Eger and Lindsay, 1997; Wilson et al., 2005, 2011; Kemper et al., 2013).

Tables 2A and B show the statistics for the latencies of the BAEP peaks split by groups. The comparisons revealed that the MXC group showed significant delays in all the BAEP peaks examined as compared to the Control dogs from the less polluted city. Therefore, the time of occurrence or latency of all peaks showed a consistent delay shift.

We did not find any significant differences (range of nonsignificant p-values for all comparisons was: 0.16-1.0) in the effects of the groups on the BAEPs intervals (I-III, III-V, and I-V) (Tables 3A and B), indicating that the inter-peak time distances remained similar in all groups.

Discussion

Mexico City air pollution is a serious health problem for 24 million human residents and millions of their dog companions. The results of the morphometric analysis of auditory brainstem showed severe damage to key auditory nuclei in healthy young animal facility dogs with lifelong exposures to concentrations of $PM_{2.5}$ and O_3 above the current US EPA standards. This was seen in the hypotrophy of the ventral cochlear nuclei (VCN), severe dysmorphology and decreased neuronal packing density in the MSO and gliosis. The potential functional effects of this damage are serious, as the cochlear nuclei receive all input from the cochleae for distribution to the rest of the central auditory system (Godfrey et al., 2016), while MSO neurons specialize in temporal processing of low-frequency sounds for binaural hearing and precise geometric organization of the nucleus is essential for detection of timing differences between the two ears (Kulesza, 2007; Kulesza and Mangunay, 2008; Beebe et al., 2014; Nothwang, 2016). The MSO dysmorphology observed in Mexico City dogs is similar to the changes described in SWMC children with sudden accidental deaths, and no risk factors for diseases involving auditory nuclei (Calderón-Garcidueñas et al., 2011). Similar changes have been observed in post-mortem brainstem samples from autistic individuals with striking disruption in the morphology of MSO neurons involving cell body shape and orientation (Kulesza and Mangunay, 2008).

The importance of our current results and previous MC children's studies goes beyond the auditory impact, first it will give us the opportunity to execute early, noninvasive screening

for auditory performance leading to identification, diagnosis, and management of hearing impairment (Hall, 2015; Jerger and Hayes, 1976) and it will allow us to define the clinical relationship between auditory brainstem damage, cognitive deficits, and academic performance associated to environmental exposures in urban children: *This is the crucial impact.*

We have shown that young MC residents exhibit frontal tau hyperphosphorylation and amyloid-β (Aβ)1‑42 diffuse plaques, aggregated and hyperphosphorylated α-synuclein in olfactory nerves and key brainstem nuclei(Calderón-Garcidueñas et al., 2008, 2010,2013,2014, 2016, 2017). Epidemiological data and experimental animal studies support our thesis that highly exposed Mexico City children with synergistic risk factors such as insulin-metabolic derangements, metabolic syndrome, obesity, T2DM, female sex, and APOE4 carrier status are at significantly increased risk for developing neurodegeneration, particularly AD (Calderón-Garcidueñas 2016). Further, high exposures to endotoxins, metals, polycyclic aromatic hydrocarbons (PAH), and high-temperature, combustion-derived iron-rich magnetite in high concentrations measured in the brains of young MC residents likely contribute to the cascade of events conducing to neuroinflammation and neurodegeneration (Calderón-Garcidueñas 2011, 2016,2017; Maher et al., 2016). *We will be in the position to define if the auditory brainstem abnormalities are a proxy for an evolving neurodegenerative process towards Alzheimer disease.* The brainstem is key for the regulation of the sleep/waking cycle and normal sleep architectonics and for a number of auditory, vestibular, autonomic, oculomotor, somatomotor and somatosensory functions (Rüb et al., 2016). Key ascending dopaminergic, cholinergic, noradrenergic, serotonergic systems are located in the brainstem (Rüb et al., 2016). The auditory brainstem system (i.e. inferior colliculus, superior olive, dorsal cochlear nucleus) is not exempt from Alzheimer pathology: Ohm and Braak (1989) described patients with AD showing amyloid plaque formation in the central nucleus, dorsomedial nucleus of the inferior colliculus and in the deep layers of the dorsal cortex of the inferior colliculus. Neurofibrillary

tangles were rare but seen in the dorsal cochlear nucleus, the periolivary region, the ventral nucleus of the lateral lemniscus, and in the central nucleus of the inferior colliculus. Although loss of differentiated cortical profiles of auditory location and pitch processing are described in AD patients, i.e., processing auditory spatial variation in posterior cingulate cortex and interactions of pitch and spatial variation in posterior insula, the very early occurrence of AD-related cytoskeletal pathology in targeted brainstem nuclei suggests that exploration of affected nuclei may be a searching probe of this interface and could give light to network failure in the early stages of AD (Ohm and Braak, 1989; Albers et al., 2015; Golden et al., 2015, 2016; Rüb et al., 2016).

The results of the BAEP assessments showed evidence of significant delayed BAEP absolute wave latencies compared to controls, presumably resulting from damage to the generator sites of the auditory brainstem response waveform (as seen in the morphometric and histopathology studies), *but not the connecting pathways* since the effects of the groups on the BAEPs intervals (I-III, III-V, and I-V) is not significant, an indicator the inter-peak time distances remained similar regardless of exposure. A major difference between MC children and dogs was the delayed absolute latency for wave I originating from the peripheral portion of the VIII cranial nerve adjacent to the cochlea, suggesting a cochlear lesion in dogs not subjected at any time in their lifetime to noise levels affecting this sense organ. The middle ear lesions were not contemplated in the differential diagnosis given the thorough normal otoscopy examination of the dogs. Cochlear damage associated with hearing loss is generally caused by irreversible damage to the sensorineural tissues (Wong and Ryan, 2015), particularly by oxidative stress and inflammatory pathways (Fetoni et al., 2016; Tan et al., 2016). In the daily clinical practice, several intracellular mechanisms could also contribute to sensorineural cochlear damage, including common pathologies such as middle ear inflammation with fluid accumulation and

disruption of cochlear homeostasis (Zhang et al., 2015). Zhang and coworkers have shown lipopolysaccharide (LPS)-evoked middle ear infection disrupts inner ear fluid balance, with a significant impact upon the intra-strial fluid-blood barrier, essential for cochlear homeostasis (Zhang et al., 2015). Of importance for our work, systemic LPS-induced inflammation produced significant increases in cochlear expression of several powerful inflammatory cytokines: interleukin-1α, interleukin-6, monocyte chemotactic protein-1, macrophage inflammatory protein-1α, and RANTES (Quintanilla-Dieck et al., 2013). Children from SWMC (aged 9.7 ±1.2 years) exhibited systemic inflammation, increased numbers of mCD14+ monocytes ($p < .001$) and an endotoxin tolerance-like state (Calderón-Garcidueñas et al., 2009b) due to high exposure to PM-LPS. A similar finding was reported using a mouse model, with the study authors suggesting high exposure to LPS, such as may occur during environmental or occupational conditions, may induce cochlear damage in dogs and humans (Quintanilla-Dieck et al., 2013). Thus, the potential mechanisms for cochlear damage including oxidative stress, inflammation, high systemic production of inflammatory cytokines and LPS-induced inflammation are all present in SWMC residents (Calderón-Garcidueñas et al., 2009b, 2012b). It remains to be seen the extent and characteristics of the cochlear pathology in Mexico City dogs, a gap requiring extensive functional studies in both animal facility and pet dogs. Factors such as hypoxia, the functional role of Toll-like receptor 4, the extent of the damage to the sensory inner hair cells, IHC ribbon synapses or spiral ganglion neurons, the central plasticity and the impact of well known neurotoxics such as organic solvents and heavy metals present in the environment need to be carefully studied in Mexico City dogs (Sliwinska-Kowalska, 2015; Fan et al., 2016; Vethanayagam et al., 2016; Moser and Starr, 2016; Chambers et al., 2016). An intriguing paper from Kumar and Tandon (1996) describes "*significant prolongation of latencies of wave I and III indicating that conductivity of sensory impulse along acoustic nerve and pons is affected in chronic smokers which may be attributed to adverse effect on myelination by nicotine and*

toluene present in tobacco smoke". This is a key paper because in fact the fine and ultrafine particulate exposure in Mexico City residents is equivalent to smoking one package of cigarettes per day and in Beijing 40 cigarettes/day, thus megacity non-smoking residents have a huge involuntary particle exposures (Dailymail, UK February 9, 2017).

The key differences in BAEPs results between dogs and humans include the delayed absolute latency for wave I and the inter-peak time distances (I-III, III-V, and I-V) similar in controls versus MC dogs. Factors such as the size of the generator nuclei, axon sizes and numbers, integrity of axons and synaptic connections, metabolic variables, responses to oxidative stress and protective systems at work, etc., might account for no differences with conduction times between stations. Preliminary studies in control dogs indicate that the axons in the trapezoid body (arising from the cochlear nucleus) are larger than have been observed in cats and even humans. We propose that the axons in the dog auditory system are so large and fast that even a significant drop in the number of axons does not impact relay speed.

Limitations, perspectives, and future directions

We acknowledge our main limitations include the relatively small group of dogs with detailed pathology and morphometric evaluation. Both cochlear and extensive brainstem histopathological studies are warranted in exposed dogs and clean air controls. The early occurrence of damage to central auditory structures points to a key role of the auditory measures for early detection of the damaging effects of air pollution. Further research into the relationships between morphological abnormalities as shown on morphometric analysis, and functional abnormalities as shown on BAEP analysis, is warranted to determine if the relatively easy to conduct, non-invasive BAEP can serve as a functional tool for the early detection of these targeted effects. Further research is also needed into the use of animal models to define the relationships between auditory function using non-invasive methodology and air pollution. Such

research could lead to the use of pet dogs as sentinels for monitoring the potential effects of air pollution on human populations in megacities.

Conclusions

In summary, adding to the already accumulating evidence of detrimental neural effects of air pollutants, we have shown data which document damage to the brainstem auditory system in Mexico City dogs chronically exposed to high concentrations of $PM_{2.5}$ and O_3, endotoxins, PAHs, etc. The present findings support the view that highly exposed dogs and children are at a higher risk for developing auditory impairments and since neurodegenerative diseases such as Alzheimer disease target auditory brainstem nuclei, identifying early determinants of risk trajectories would greatly facilitate multidisciplinary prevention efforts for potentially modifying disease course at early stages. We propose that neuroprotection of young MCMA residents should be a public health priority.

Competing interests

The authors declare they have no competing financial interests

Grant Support

References

Albers MW, Gilmore GC, Kaye J, Murphy C, Wingfield A, Bennett DA, Boxer AL, Buchman AS, Cruickshanks KJ, Devanand DP, Duffy CJ, Gall CM, Gates GA, Granholm AC, Hensch T, Holtzer R, Hyman BT, Lin FR, McKee AC, Morris JC, Petersen RC, Silbert LC, Struble

RG, Trojanowski JQ, Verghese J, Wilson DA, Xu S, Zhang LI. (2016).At the interface of sensory and motor dysfunctions and Alzheimer's disease. *Alzheimers Dement.* 2015; 11:70-98.

Basagaña X, Esnaola M, Rivas I, Amato F, Alvarez-Pedrerol M, Forns J, Sunyer J. (2016). Neurodevelopmental Deceleration by Urban Fine Particles from Different Emission Sources: A Longitudinal Observational Study. *Environmental Health Perspectives*, **124:**1630-1636

Beebe K, Wang Y, Kulesza R. (2014). Distribution of fragile X mental retardation protein in the human auditory brainstem. *Neuroscience*. **25,** 273:79-91.

Bravo- Alvarez HR, Torres-Jardón R (2002). Air pollution levels and trends in the México City metropolitan area. In: *Urban Air Pollution and Forest: Resources at Risk in the MC Air Basin.* Edited by Fenn M, de Bauer L, Hernández T. Ecological Studies, Vol. 156, Springer-Verlag, New York, 121-159.

Calderón-Garcidueñas L, Mora-Tiscareño A, Fordham LA, Chung CJ, García R, Osnaya N, Gambling TM, Acuña H, Monroy S, Villarreal-Calderón A, Carson J, Koren HS, Devlin RB. (2001). Canines as sentinel species for assessing chronic exposures to air pollutants. Respiratory and Cardiac Pathology**.** *Toxicol Sci*; **61**:342-367

Calderón-Garcidueñas L, Azzarelli B, Acuña H, Garcia R, Gambling TM, Monroy S, Del Tizapantzi MR, Carson JL, Villarreal-Calderon A, Rewcastle B (2002). Air pollution and brain damage. *Toxicol Pathol* **30**, 373-389

Calderón-Garcidueñas L, Maronpot RR, Torres-Jardon R, Henriquez-Roldan C, Schoonhoven R, Acuña -Ayala H, Villarreal-Calderon A, Nakamura J, Fernando R, Reed W, Azzarelli B, Swenberg JA (2003). Air pollution and brain damage. *Toxicol Pathol* **31**,524-538

Calderón-Garcidueñas L, Mora-Tiscareño A, Gómez-Garza G, Broadway J, Chapman S, Valencia-Salazar G, Jewells V, Maronpot RR, Henríquez-Roldán C, Pérez-Guillé B, Torres-

Jardón R, Herrit L, Brooks D, Osnaya-Brizuela N, Monroy ME, González-Maciel A, Reynoso-Robles R, Villarreal-Calderon R, Solt AC, Engle RW(2008). Air pollution, cognitive deficits and brain abnormalities: A pilot study with children and dogs. *Brain and Cognition* **68**,117-127.

Calderón-Garcidueñas L, Solt AC, Henríquez-Roldán C, Torres-Jardón R, Nuse B, Villarreal-Calderón R, Osnaya N, Stone I, García R, Brooks DM, González-Maciel A, Reynoso-Robles R, Delgado-Chávez R, Reed W (2008). Long-term air pollution exposure is associated with neuroinflammation, an altered innate immune response, disruption of the blood-brain-barrier, ultrafine particulate deposition, and accumulation of amyloid beta-42 and alpha-synuclein in children and young adults. *Toxicol Pathol* **36,** 289-310.

Calderón-Garcidueñas L, Franco-Lira M, Henríquez-Roldán C, González-Maciel A, Reynoso-Robles R, Villarreal-Calderon R, Herritt L, Brooks D, Keefe S, Palacios-Moreno J, Villarreal-Calderon R, Torres-Jardón R, Medina-Cortina H, Delgado-Chávez R, Aiello-Mora M, Maronpot RR, Doty RL(2010). Urban air pollution: influences on olfactory function and pathology in exposed children and young adults. *Exp Toxicol Pathol* **62,** 91-102.

Calderón-Garcidueñas L, D'Angiulli A, Kulesza RJ, Torres-Jardón R, Romero L, Keefe S, Herritt L, Brooks DM, Avila-Ramirez J, Delgado-Chávez R, Medina-Cortina H, González-González LO (2011) Air pollution is associated with brainstem auditory nuclei pathology and delayed brainstem auditory evoked potentials. *Int J Dev Neurosci* **29**, 365-375.

Calderón-Garcidueñas L, Kavanaugh M, Block ML, D'Angiulli A, Delgado-Chávez R, Torres-Jardón R, González-Maciel A, Reynoso-Robles R, Villarreal-Calderón R, Guo R, Hua Z, Zhu H, Perry G, Diaz P (2012). Neuroinflammation, hyperphosphorylated tau, diffuse amyloid plaques and down- regulation of the cellular prion protein in air pollution exposed children and adults. *J Alzheimers Dis* **28**, 93-107.

Calderón-Garcidueñas L, Mora-Tiscareño A, Styner M, Gómez-Garza G, Zhu H, Torres-Jardón R, Carlos E, Solorio-López E, Medina-Cortina H, Kavanaugh M, D'Angiulli A (2012). White matter hyperintensities, systemic inflammation, brain growth and cognitive functions in children exposed to air pollution. *J Alzheimers Dis* **31**, 183-191.

Calderón-Garcidueñas L, Franco-Lira M, Mora-Tiscareño A, Medina-Cortina H, Torres-Jardón R, Kavanaugh M. (2013). Early Alzheimer's and Parkinson's disease pathology in urban children: Friend versus Foe responses-it is time to face the evidence. *Biomed Res Int* doi: 10.1155/2013/161687.

Calderón-Garcidueñas L, Torres-Jardón R,Kulesza RJ, Park SB, D'Angiulli A (2014). Air pollution and detrimental effects on children's brain. The need for a multidisciplinary approach to the issue complexity and challenges. *Front Hum Neurosci* Aug 12;8:613. doi: 10.3389/fnhum.2014.00613.

Calderón-Garcidueñas L, Franco-Lira M, D'Angiulli A, Rodríguez-Díaz J, Blaurock-Busch E, Busch Y, Chao CK, Thompson C, Mukherjee PS, Torres-Jardón R, Perry G (2015). Mexico City normal weight children exposed to high concentrations of ambient PM2.5 show high blood leptin and endothelin-1, vitamin D deficiency, and food reward hormone dysregulation versus low pollution controls. Relevance for obesity and Alzheimer disease. *Environ Res* **140**,579-592.

Calderón-Garcidueñas L, Mora-Tiscareño A, Melo-Sánchez G, Rodríguez-Díaz J, Torres-Jardón R, Styner M, Mukherjee PS, Lin W, Jewells V (2015). A critical Proton MR Spectroscopy marker of Alzheimer's disease early neurodegenerative change: Low hippocampal NAA/Cr ratio impacts APOE ε4 Mexico City children and their parents. *J Alzheimers Dis* **48,** 1065-1075.

Calderón-Garcidueñas L, Jewells V, Galaz-Montoya C, van Zundert B, Pérez-Calatayud A, Ascencio-Ferrel E, Valencia-Salazar G, Sandoval-Cano M, Carlos E, Solorio E, Acuña-Ayala H, Torres-Jardón R, D'Angiulli A (2016). Interactive and additive influences of gender, BMI and Apolipoprotein 4 on cognition in children chronically exposed to high concentrations of PM $_{2.5}$ and ozone. APOE4 females are at highest risk in Mexico City. *Environ Res* **150**, 411-422.

Calderón-Garcidueñas L, Reynoso-Robles R, Vargas-Martínez J, Gómez-Maqueo-Chew A, Pérez-Guille B, Mukjerhee PS, Torres-Jardón R, Perry G, González-Maciel A (2016).Prefrontal White matter pathology in air pollution exposed Mexico City Young urbanites and their potential impact on neurovascular unit dysfunction and the development of Alzheimer's disease. *Environ Res* **146**, 404-417

Calderón-Garcidueñas L, Avila-Ramírez J, Calderón-Garcidueñas A, González-Heredia T, Acuña-Ayala H, Chao CK, Thompson C, Ruiz-Ramos R, Cortés-González V, Martínez-Martínez L, García-Pérez MA, Reis J, Mukherjee PS, Torres-Jardón R, Lachmann I (2016). Cerebrospinal Fluid Biomarkers in Highly Exposed PM $_{2.5}$ Urbanites: The Risk of Alzheimer's and Parkinson's Diseases in Young Mexico City Residents. *J Alzheimers Dis* **54**,597-613

Calderón-Garcidueñas L, Leray E, Heydarpour P, Torres-Jardón R, Reis J. (2016). Air pollution, a rising environmental risk factor for cognition, neuroinflammation and neurodegeneration: The clinical impact on children and beyond. *Rev Neurol (Paris)* **172**, 69-80

Calderón-Garcidueñas L, Villarreal-Rios R (2017).Living close to heavy traffic roads, air pollution and dementia. Lancet. 2017 Jan 4. pii: S0140-6736(16)32596-X. doi: 10.1016/S0140-6736(16)32596-X.

Calderón-Segura ME, Gómez-Arroyo S, Villalobos-Pietrini R, Butterworth FM, Amador-Muñoz O (2004). The effects of seasonal weather on the genotoxicity, cytokinetic properties, cytotoxicity and organochemical content of extracts of airborne particulates in Mexico City. *Mut Res* **558**, 7-17.

Chambers AR, Resnik J, Yuan Y, Whitton JP, Edge AS, Liberman MC, Polley DB (2016). Central gain restores auditory processing following near-complete cochlear denervation. *Neuron* **89**, 867-879

Chen H, Kwong JC, Copes R, Tu K, Villeneuve PJ, van Donkelaar A, Hystad P, Martin RV, Murray BJ, Jessiman B, Wilton AS, Kopp A, Burnett RT (2017). Living near major roads and the incidence of dementia, Parkinson's disease, and multiple sclerosis: a population-based cohort study. *Lancet.* pii: S0140-6736(16)32399-6. doi: 10.1016/S0140-6736(16)32399-6.

Davis LW (2017). Saturday Driving Restrictions Fail to Improve Air Quality in Mexico City. *Sci. Rep.* **7**, 41652; doi: 10.1038/srep41652

http://www.dailymail.co.uk/sciencetech/article-3201954/Breathing-Beijing-s-air-equivalent-smoking-FORTY-cigarettes-day-Smog-map-China-reveals-shocking-extent-pollution.html

De Vizcaya-Ruiz A, Gutiérrez-Castillo ME, Uribe-Ramirez M, Cebrián ME, Múgica-Alvarez, V, Sepúlveda J, Rosas I, Salinas E, Garcia-Cuellar C, Martinez F, Alfaro-Moreno, E. Torres-Flores V, Osornio-Vargas A, Sioutas C, Fine PM, Singh M, Geller MD, Kuhn T, Miguel AH, Eiguren-Fernandez A, Schiestl RH, Reliene R, Froines J (2006). Characterization and in vitro biological effects of concentrated particulate matter from Mexico City. *Atmospheric Environment* **40**, 583-592

Dzepina K, Arey J, Marr L, Worsnop DR, Salcedo D, Zhang Q (2007). Detection of particle-phase polycyclic aromatic hydrocarbons in Mexico City using an aerosol mass spectrometer. *International Journal of Mass Spectrometry* **263**, 152–170.

Eger CE, Lindsay P (1997). Effects of otitis on hearing in dogs characterized by brainstem auditory evoked response testing. *J Small Anim Pract* **38**, 380-386

Fan D, Ren H, Danzeng D, Li H, Wang P (2016). Influence of high-altitude hypoxic environments on the survival of cochlear hair cells and spiral ganglion neurons in rats. *Biomed Rep* **5**, 681-685

Fischer A, Obermaier G (1994). Brainstem auditory-evoked potentials and neuropathologic correlates in 26 dogs with brain tumors. *J Vet Intern Med* **8**, 363-368

Forouzanfar MH, Alexander L, Anderson HR, Bachman VF, Biryukov S, Brauer M, Delwiche K (2015). Global, regional, and national comparative risk assessment of 79 behavioural, environmental and occupational, and metabolic risks or clusters of risks in 188 countries, 1990–2013: A systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, **386**, 2287-2323

Godfrey DA, Lee AC, Hamilton WD, Benjamin LC 3rd, Viswanath S, Simo H, Godfrey LM, Mustapha AI, Heffner RS (2016). Volumes of cochlear nucleus regions in rodents. *Hear Res* doi: 10.1016/j.heares.2016.07.003

Goldberg JM, Brown PB (1968). Functional organization of the dog superior olivary complex: an anatomical and electrophysiological study. *J Neurophysiol.* **31,** 639-656.

Golden HL, Nicholas JM, Yong KX, Downey LE, Schott JM, Mummery CJ, Crutch SJ, Warren JD (2015). Auditory spatial processing in Alzheimer's disease. *Brain.* 138(Pt 1):189-202.

Golden HL, Agustus JL, Nicholas JM, Schott JM, Crutch SJ, Mancini L, Warren JD (2016). Functional neuroanatomy of spatial sound processing in Alzheimer's disease. *Neurobiol Aging*. **39**,154-64.

Hall JW 3rd (2016). Crosscheck Principle in Pediatric Audiology Today: A 40-Year Perspective. *J Audiol Otol* **20**, 59-67. Review.

Heneka MT, Carson MJ, El Khoury J, Landreth GE, Brosseron, F, Feinstein DL, Jacobs AH, Wyss-Coray T, Vitorica J, Ransohoff RM, Herrup K, Frautschy SA, Finsen B, Brown GC, Verkhratsky A, Yamanaka K, Koistinaho J, Latz E, Halle A, Petzold GC, Town T, Morgan D, Shinohara ML, Perry VH, Holmes C, Bazan NG, Brooks DJ, Hunot S, Joseph B, Deigendesch N, Garaschuk O, Boddeke E, Dinarello CA, Breitner JC, Cole GM, Golenbock DT, Kummer MP. (2015). Neuroinflammation in Alzheimer's disease. *The Lancet Neurology*, **14**, 388-405.

Jacobson MW, Delis DC, Bondi MW, Salmon DP (2015). Asymmetry in auditory and spatial attention span in normal elderly genetically at risk for Alzheimer's disease. *J Clin Exp Neuropsychol.* **27**, 240-253.

Jerger JF, Hayes D. The cross-check principle in pediatric audiometry. Arch Otolaryngol 1976;102:614-20.

 Kemper DL, Scheifele PM, Clark JG (2013). Canine brainstem auditory evoked responses are not clinically impacted by head size or breed. *Physiology & Behavior*, *110***,** 190-197

Kolosnjaj-Tabi J, Just J, Hartman KB, Laoudi Y, Boudjemaa S, Alloyeau D, Szwarc H, Wilson LJ, Moussa, F (2015). Anthropogenic carbon nanotubes found in the airways of Parisian children. *EBioMedicine*, **2**, 1697-1704.

Kulesza RJ Jr (2007) Cytoarchitecture of the human superior olivary complex: medial and lateral superior olive**.** *Hear Res* **225,** 80-90

Kulesza RJ, Mangunay K. (2008). Morphological features of the medial superior olive in autism. *Brain Res*. **20,**132-137.

Kulesza RJ Jr. (2014). Characterization of human auditory brainstem circuits by calcium-binding protein immunohistochemistry. *Neuroscience*. **31,** 318-331.

Kumar V, Tandon OP (1996). Brainstem auditory evoked potentials (BAEPs) in tobacco smokers. *Indian J Physiol Pharmacol*. **40**, 381-384.

Lukose R, Beebe K, Kulesza RJ Jr. (2015). Organization of the human superior olivary complex in 15q duplication syndromes and autism spectrum disorders. *Neuroscience*. **12**, 216-230.

Maher BA, Ahmed IA, Karloukovski V, MacLaren DA, Foulds PG, Allsop D, Mann DM, Torres-Jardón R, Calderón-Garcidueñas L. (2016). Magnetite pollution nanoparticles in the human brain. Proc Natl Acad Sci USA 113, 10797-10801

Marr LC, Dzepina K, Jimenez JL, Reisen F, Bethel HL, Arey J, Gaffney JS, Marley NA, Molina LT, Molina MJ (2006). Sources and transformations of particle-bound polycyclic aromatic hydrocarbons in Mexico City. *Atmospheric Chemistry and Physics 6*, 1733-1745.

Miller MD, Marty MA, Landrigan PJ (2016). Children's Environmental Health: Beyond National Boundaries. *Pediatric Clinics of North America*, **63**, 149-165.

Moser T, Starr A (2016). Auditory neuropathy, neural and synaptic mechanisms. *Nat Rev Neurol* **12**, 135-149

Mugica-Alvarez V, Figueroa-Lara J, Romero-Romo M, Sepúlveda-Sánchez J, López-Moreno T (2012) Concentrations and properties of airborne particles in the Mexico City subway system. *Atmos Environ* **49**, 284-293

Nothwang HG (2016). Evolution of mammalian sound localization circuits: A development perspective. *Prog Neurobiol* **141**, 1-24

Ohm TG, Braak H (1989). Auditory brainstem nuclei in Alzheimer's disease. *Neurosci Lett*. **96,** 60-63.

Parrish DD, Singh HB, Molina L, Madronich S (2011). Air quality progress in North American megacities: A review. *Atmospheric Environment* **45,** 7015-7025.

Porta D, Narduzzi S, Badaloni C, Bucci S, Cesaroni G, Colelli V, Davoli M, Sunyer J, Zirro E, Schwartz J, Forastiere F (2016). Air Pollution and Cognitive Development at Age 7 in a Prospective Italian Birth Cohort. *Epidemiology*, **27**, 228-236.

Querol X, Pey J, Minguillón MC, Pérez N, Alastuey A, Viana M, Vega, E. (2008). PM speciation and sources in Mexico during the MILAGRO-2006 Campaign. *Atmospheric Chemistry and Physics* **8**, 111-128

Rüb U, Stratmann K, Heinsen H, Turco DD, Seidel K, Dunnen Wd, Korf HW (2016). The Brainstem Tau Cytoskeletal Pathology of Alzheimer's Disease: A Brief Historical Overview and Description of its Anatomical Distribution Pattern, Evolutional Features, Pathogenetic and Clinical Relevance. *Curr Alzheimer Res.* **13,** 1178-1197.

Safdari BK, Sia TC, Wattchow DA, Smid SD (2016). Effects of pro-inflammatory cytokines, lipopolysaccharide and COX-2 mediators on human colonic neuromuscular function and epithelial permeability. *Cytokine*, **83**, 231-238

Scaioli V, Brinciotti M, Di Capua M, Lori S, Janes A, Pastorino G, Peruzzi C, Sergi P, Suppiej A. (2009). A multicentre database for normative brainstem auditory evoked potentials (BAEPs) in children: methodology for data collection and evaluation. *The Open Neurology Journal*, **3**, 72.

Schmutz SM (2014). An analysis of the inheritance pattern of an adult-onset hearing loss in Border Collie dogs. *Canine Genet Epidemiol* doi: 10.1186/2052-6687-1-6.

Sims MH (1988). Electrodiagnostic evaluation of auditory function. *Vet Clin North Am Small Animal Pract* **18**, 913-944

Sliwinska-Kowalska M (2015). Hearing. *Handb Clin Neurol* **131**, 341-363

Strain GM, Tedford BL, Jackson RM (1991). Postnatal development of the brainstem auditory-evoked potentials in dogs. *Am J Vet Res* **52**, 410-415

Stieb DM, Chen L, Hystad P, Beckerman BS, Jerrett M, Tjepkema M, Crouse DL, Omariba DW, Peters PA, van Donkelaar A, Martin RV, Burnett RT, Liu S, Smith-Doiron M, Dugandzic RM (2016). A national study of the association between traffic-related air pollution and adverse pregnancy outcomes in Canada, 1999–2008. *Environmental Research* **148**, 513-526.

Tan WJ, Thorne PR, Vlajkovic SM (2016). Characterisation of cochlear inflammation in mice following acute and chronic noise exposure. *Histochem Cell Biol* **146,** 219-30

Toutain PL, Cester CC, Haak T, Metge S(2001). Pharmacokinetic profile and in vitro selective cyclooxygenase-2 inhibition by nimesulide in the dog. *Journal of Veterinary Pharmacology and Therapeutics*, **24**, 35-42.

US EPA (2014) National Ambient Air Quality Standards. (NAAQS). Air and Radiation. U. S. Environmental Protection Agency. http://www.epa.gov/air/criteria.html.

Valle-Hernández BL, Mugica-Álvarez V, Salinas-Talavera E, Amador-Muñoz O. Murillo-Tovar MA, Villalobos-Pietrini R, De Vizcaya-Ruíz A (2010). Temporal variation of nitro-polycyclic aromatic hydrocarbons in PM 10 and PM 2.5 collected in Northern Mexico City. *Science of the Total Environment* **408**, 5429-5438.

Vega E, Eidels S, Ruiz H, López-Veneroni D, Sosa G, Gonzalez E, Gasca J, Mora V, Reyes E, Sánchez-Reyna G, Villaseñor R, Chow JC, Watson JG, Edgerto SA. (2010) Particulate air pollution in Mexico City: a detailed view. *Aerosol and Air Quality Research*, **10**, 193-211

Vethanayagam RR, Yang W, Dong Y, Hu BH (2016). Toll-like receptor 4 modulates the cochlear immune response to acoustic injury. *Cell Death Dis* **7**, e2245 doi: 10.1038/cddis.2016.156.

Wilson WJ, Mills PC (2005). Brainstem auditory-evoked response in dogs. *Am J Vet Res* **66**, 2177-2187

Wilson WJ, Bailey KL, Balke CL, D'Arbe CL, Hoddinott BR, Bradley AP, Mills PC (2006). On the dual structure of the auditory brainstem response in dogs. *Clin Neurophysiol* **117**, 2211-2230

Wilson WJ, Mills PC, Bradley AP, Petoe MA, Smith AW, Dzulkarnain AA (2011). Fast assessment of canine hearing using high click-rate BAER. *Vet J* **187**, 136-138

WHO (2006) WHO's Urban Ambient Air Pollution database - Update 2016. Version 0.2. Public Health, Social and Environmental Determinants of Health Department, World Health Organization, 1211 Geneva 27, Switzerland http://www.who.int/phe/health_topics/outdoorair/databases/AAP_database_methods_2016_v03.pdf.

Wu S, Yang D, Pan L, Shan J, Li H, Wei H, Wang B, Huang J, Bacarelli AA, Shima M, Deng F, Guo X, Deng F. (2016). Chemical constituents and sources of ambient particulate air pollution and biomarkers of endothelial function in a panel of healthy adults in Beijing, China. *Science of The Total Environment* **560**, 141-149.

FIGURES

Figure 1. Three year average $PM_{2.5}$ annual mean concentrations registered at the southwest Mexico City Metropolitan Area (MCMA) from 2002 to 2012. The dashed line represents the annual $PM_{2.5}$ US-EPA National Air Quality Standard. Since $PM_{2.5}$ measurements began in 2004, data previous to this year were obtained from the slope of the correlation PM_{10} v $PM_{2.5}$. Data concentrations were obtained from the Air Quality Monitoring Network of the Government of Mexico City.

Figure 2. PM$_{2.5}$ emissions inventory in MCMA for the year 2010. Diesel vehicles and industry are the main sources of highly toxic PM$_{2.5}$. Area sources include resuspension of dusts from paved streets and roads.

Figure 3. Four highest daily maximum eight-hour average ozone concentrations from 2002 through 2012 at the monitoring site of Pedregal, southwest Mexico City Metropolitan Area (MCMA).The dashed line represents the respective US-EPA National Air Quality Standard. Data from the government of Mexico City air quality monitoring network.

Figure 4. (A) Sum of the average concentrations of 12 PAHs, and, (B) the average concentrations of Benzo(a)pyrene in PM in several areas of North America and Europe. The sum of the PAHs include: phenanthrene, benzo(a)pyrene, benzo(b)fluoranthene, benzo(a)anthracene, fluorene, fluoranthene, pyrene, chrysene, benzo(k)fluoranthene, dibenzo(a,h)anthracene, indene(1,2,3-cd)pyrene, and Benzo(ghi)perylene. Data of PAHs are for the years: 2012 Canada sites; 2010 U.S.A. sites, 2005-2007 Mexico City sites, and July, 2004 France.

Figure 5. Dysmorphology in the MSO after pollution exposure. Shown in 5A is a reconstruction of the dog SOC in the rostral medulla. The SOC is situated posterior and lateral to the fibres of the pyramid (py) and medial to the abducens nerve (CN VI). The SOC includes the MSO, lateral superior olive (LSO) and the medial, ventral and lateral nuclei of the trapezoid body (MNTB, VNTB and LNTB, respectively). Shown in 5B is the distribution of soma sizes. These populations were not normally distributed: the horizontal lines represent the median, dots mark the mean, whiskers represent the 10th and 90th percentiles. MSO neurons in control animals were significantly larger than those from MXC animals. Shown in 5C are the distributions of the different cell body morphologies; these were dominated by round cells. Shown in 5D are the

neuronal densities from the MSO. The neuronal densities were significantly lower in MXC animals (whiskers represent the standard deviation).Shown in E are tracings of the MSO from each of the specimens. The position of MSO neurons are marked with circles and regions of neuronal loss are marked with asterisks. The scale bar in A and E both represent 1 mm. Orientation arrows denote posterior (p) and medial (m). Key to symbols: *** p < .001, **** p < .0001.