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Air pollution is associated with brainstem auditory nuclei pathology and delayed brainstem auditory evoked potentials

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Abstract

We assessed brainstem inflammation in children exposed to air pollutants by comparing brainstem auditory evoked potentials (BAEPs) and blood inflammatory markers in children age 96.3 ± 8.5 months from highly polluted ($n=34$) versus a low polluted city ($n=17$). The brainstems of nine children with accidental deaths were also examined. Children from the highly polluted environment had significant delays in wave III ($t(50)=17.038$; $p<0.0001$) and wave V ($t(50)=19.730$; $p<0.0001$) but no delay in wave I ($p=0.548$). They also had significantly longer latencies than controls for interwave intervals I–III, III–V, and I–V (all $t(50)>7.501$; $p<0.0001$), consisting with delayed central conduction time of brainstem neural transmission. Highly exposed children showed significant evidence of inflammatory markers and their auditory and vestibular nuclei accumulated α synuclein and/or β amyloid_{1–42}. Medial superior olive neurons, critically involved in BAEPs, displayed significant pathology. Children's exposure to urban air pollution increases their risk for auditory and vestibular impairment.

Keywords

Air pollution; alpha synuclein; auditory nuclei; brainstem inflammation; brainstem evoked auditory potentials; beta amyloid; children; neuroinflammation

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1. Introduction

Environmental pollutants, chemicals, metals and drugs have a detrimental, sometimes irreversible impact upon the developing central nervous system in children (Danzer, 2008). Air quality in Mexico City (MC) has been recognized as among the worst in the world (Bravo-Alvarez and Torres-Jardón, 2002; Molina et al., 2007). Since pollution levels in MC have been sustained or worsened in the last 20 years, exposures of today's children begins in utero and impacts neurological function for their entire life (Bravo-Alvarez and Torres-Jardón, 2002). MC children are frequently exposed all year long, at least several hours per day, to a significant burden of air pollutants, including concentrations above the current USA standards for ozone (O₃), and particulate matter < 2.5 µm in diameter (PM_{2.5}), as well as lipopolysaccharides-associated with PM (PM-LPS) (Bonner et al., 1998; Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008).

In a cohort of MC residents, including children aged 2–17 years, we found brainstem vascular pathology with significant immunoreactivity (IR) of endothelial cells to vascular cell adhesion molecule-1 (VCAM-1) and strongly stained mononuclear perivascular cells and microglia-like cells with CD163, CD68, and HLA-DR (Calderón-Garcidueñas et al., 2008a). Alpha-synuclein neuronal aggregation – the early neuropathological hallmark of sporadic Parkinson's disease – and accumulation of 3 nitro-tyrosine and 8-hydroxydeoxyguanosine (8-OHdG)-evidence of oxidative stress - were also detected in MC children's brainstem nuclei (Calderón-Garcidueñas et al., 2008a).

We hypothesized that brainstem nuclei involved in our previous pathology study could be investigated through brainstem auditory evoked potentials (BAEPs), a non-invasive technique to record scalp electrical potentials which reflect the electrophysiological activity of the brain pathways of the auditory tract (inner ear to the auditory cortex). Importantly, BAEPs provide an objective measure of the correlates of environmental pollution in the brain (Rothenberg et al., 2000; Murata et al., 2004). Delayed BAEPs latencies have been described in children exposed to lead and methylmercury compounds, and in association with neonatal hyperbilirubinemia, iron-deficiency anemia, and protein energy malnutrition (Rothenberg et al., 2000; Murata et al., 2004; Sarici et al., 2001; Kürekçi et al., 2006; Shapiro, 2005; Vandana, 2006). Experimental exposures to environmental tobacco and induced bovine spongiform encephalopathy also produce alterations in BAEPs (Golub et al., 2007; Arai et al., 2009).

In the present study, we tested the hypothesis that, as compared to children residing in an urban area with concentrations of main criteria air pollutants below the current USA standards, clinically healthy MC children would have altered BAEPs as a manifestation of neuroinflammation associated with severe exposure to air pollutants (Bonner et al., 1998; Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008; Estrada et al., 2004).

Thus, the primary objective of this study was to measure latencies of BAEPs in children from MC and a control urban area with consistently low levels of air pollution. The second objective was to characterize the brainstem pathology that may correspond to the altered BAEPs in a cohort of matched MC and control children that were clinically healthy until their sudden accidental deaths. Given that the superior olivary complex (SOC) is one of the major structures involved in BAEP generation, we selected to study the morphology of the medial superior olive (MSO), the largest and most conspicuous of the SOC cell groups. The MSO plays an essential role in localization of sound sources and processing the temporal features of low frequency sounds. Further, neuronal cell bodies and dendritic arbors of the human MSO are arranged in a geometrically precise fashion (Kulesza 2007). Thus, we

regarded morphology of MSO neurons as a gauge of brainstem injury (Kulesza and Mangunay, 2008).

2. Materials and Methods

2.1. Design

This prospective protocol was approved by the review boards and ethics committees at each institution, written consent was obtained from parents and verbal consent from children involved in the clinical study. Examination of autopsy materials was approved by the Institutional IRB. The geographic areas selected for this study were: Southwest MC (SWMC) and Polotitlán. SWMC has had concentrations of O₃ and PM_{2.5} above the USA National Ambient Air Quality Standard (NAAQS) for several years (Bonner et al., 1998; Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008; Estrada et al., 2004). The selection of children from SWMC was based on three factors: i. the SW location of the base Mexico City pediatric hospital, ii. children living and attending school close to the hospital, iii. the location of the two closest atmospheric monitoring stations: Pedregal and Coyoacán. Polotitlán is located 114 km NW of MC at 2300 m above sea level, and its selection as a control city was based on 5 key factors: i. concentrations for all major air pollutants below the current USA standards, ii. access to a children's healthy population, iii. previous clinical studies with the Polotitlán cohort that indicated that children had no evidence of air pollution-related health issues, (Calderón-Garcidueñas et al., 2007a, 2008b, 2010), iv. an altitude above sea level similar to that of MC, and relative proximity to MC to facilitate the follow-up of the control cohorts.

2.2. Participants

The general characteristics of the study clinical populations are seen in Table 2. Children's clinical inclusion criteria were: negative smoking history and environmental tobacco exposure, lifelong residency in MC or in the control city, residency within 5 miles of the city monitoring stations, full term birth, unremarkable clinical histories, including no hearing impairments, negative history of hospitalizations for respiratory illnesses, negative histories of repeated upper respiratory or ear infections, personal and familial histories of deafness, febrile episodes or vaccinations in the previous 3 months. Cohorts were matched by socioeconomic status and parent educational level. Participants were from middle class families living in single-family homes with no indoor pets, used natural gas for cooking and kitchens were separated from the living and sleeping areas. Children slept in bedrooms with no carpeting and had open windows for ventilation. Outdoor daily exposures were recorded, including the transit time to and from school, the time spend in recess and physical education during school, the outdoor playing time and while engaging in other activities. Since children's activities change during the weekend, the outdoor times were averaged for the 7 days.

2.3. Data collection

Mexico City and control children had complete physical exams and within a week underwent both an audiologic and oto-neurologic full clinical examination, that included a complete ear examination with an otoscope, tonal audiometry, logaudiometry and tympanometry with Jerger scale in the hands of a Board Certified Pediatric Otoneurologist and Audiologist with significant expertise. Additionally, brainstem auditory evoked potentials (BAEPs), and fasting blood samples were collected between March 2 and 31, 2009.

Successively, we also studied the autopsy materials from five MC clinically healthy children (11y F, 13y F, 14y F, 15y F, 19y M) from non-smoking households (average age 14.4±2.96

SD) at the time of their sudden accidental deaths not involving brain injury. Four children (2y F and 3 17y old males) residents in low polluted cities served as controls from a previous work, (Calderón-Garcidueñas et al., 2008a). One 14 year old girl was added to complete the control group (average age 13.4 ± 6.5 SD). Autopsy subjects had a negative toxicology screening panel including drug alkaline and acid/neutral screen, amphetamines, benzodiazepines, cocaine/opiates, alcohol, volatiles and cannabinoids.

2.4. Audiometry examination

Audiometry was carried out using Interacoustics Diagnostic Audiometer AD229 with a peltor H7A headphone in a sound proof room with a constant 20° C temperature. The subject-controlled Highson-Westlake procedure was used in accordance with ISO 8253-1. A threshold was defined as 2/3 correct responses in a procedure with 5dB increases and 10dB decreases. Pure-tone air-conduction hearing thresholds were measured at 125, 250, 500, 700, 1000, 1500, 2000, 3000, 4000, 6000 and 8000 Hz.

2.5. BAEPs

The BAEPs were recorded using an adapted version of the standard clicks procedure (Thivierge and Côté, 1987), with an Eosate Biomedica System. Disc electrodes were attached to the mastoid processes (M_{1-2} , reference electrode), vertex (C_z active electrode) and midline forehead (Fp_z , ground). Impedance was kept below 5 K Ohms. Stimulation was delivered by model ER-3A tube earphone. The contralateral ear was masked by white noise of 40 dB during stimulation. The auditory stimuli were 100 μ s monoaural unfiltered rarefaction clicks at a rate of 11.4/s. The threshold for the clicks was determined from the pure tones at 4000 Hz (see procedure described in the audiometry examination section above). The clicks were given at intensities of 80 dB hearing level, 5 blocks of 200 repetitions were recorded for each ear. Responses were amplified and averaged 1,000 times for each ear, respectively (thus, 2,000 times in total). Bandpass filters were set at 300 Hz (Low) and 3000 Hz (High). Absolute latency for waves I, III, V and interpeak latency I–III, III–V and I–V were recorded. Because no significant within-subjects differences were detected between the two sides, the latency obtained from left and right ears were averaged to represent each subject by one value (see BAEPs results for more details).

2.6 Peripheral blood analysis

Blood samples were taken for a complete blood count (CBC) with differential and custom made human multianalyte Elisa cytokine arrays. We followed the manufacturer's instructions for Custom Human Multi-Analyte ELISArray Kits, SABiosciences, Frederick, MD 21703, USA. The following cytokines and chemokines were included in our panel: Interleukin-1 β (IL1 β), Interleukin-6 (IL6), Interleukin-8 (IL8), Interleukin-10 (IL10), Interleukin-12 (IL12), Interleukin-17 (IL17), Tumor necrosis alpha (TNF α), Interferon gamma (IFN γ), Transforming growth factor beta (TGF β), Macrophage-derived chemokine (MDC) and Monocyte chemoattractant protein-1 (MCP-1).

2.7 Autopsies and tissue preparation

Autopsies were performed 3.7 ± 1.7 h after death. Cases were consecutive and included unrelated children with no pathological findings at the general autopsy other than the acute cause of death. The brainstem was removed and immersed in 10% neutral formaldehyde, fixed for 48–72 h, and transferred to 70% alcohol. The brainstem was sectioned from the midbrain at the level of the superior colliculi to the lower medulla. An average of 13 blocks were obtained from each brainstem and 56 ± 11 slides were examined per block. Paraffin sections 8 μ m thick were cut and routinely stained with hematoxylin and eosin (HE) and luxol fast blue (LFB). Immunohistochemistry (IHC) was performed on brainstem serial

sections. The sections were deparaffinized, and immunostained as described previously (Calderón-Garcidueñas et al., 2008a). Antibodies included: β amyloid 17–24, 4G8 and β amyloid 1–16, 6E10 (Signet, Covance, Emeryville, CA); α -synuclein, (LB509 InVitrogen, Carlsbad, CA); CD163 (SeroTec, Raleigh, NC 27604), 8OHdG (Northwest, Life Sciences Specialities, Vancouver, WA 98683), Iba-1, HLA-DR (Ab20181), and GFAP (Abcam, Cambridge, Mass). To confirm the positive synuclein immunoreactivity (Beach et al., 2008) (IR) sections were repeated a minimum of three times. Sections were read blindly by two senior neuropathologists and an anatomist. Immunoreactivity was evaluated as minimal (+), moderate (++) and strong (+++).

To analyze the morphology of MSO neurons, sections were stained for Nissl substance with neutral red according to a protocol optimized for human tissue (Schmidt et al., 2010). For morphometric analyses, sections were randomly (but systematically) selected and only neurons with visible nucleoli and cell bodies that were completely within the tissue section were included. Cell bodies were traced while focusing (to most accurately determine the cell body contour) with the aid of a camera lucida attachment (Olympus). Gray scale tracings were analyzed using ImageJ software (calibrated to a standard scale bar). An index of circularity was calculated for each soma using the following equation: $\text{Circularity} = [4\pi \times \text{Area}/\text{Perimeter}^2]$ that yields an estimate of soma shape independent of size (Yin and Chan, 1990). Using this formula a perfectly circular soma yields a value of “1”, while increasingly irregular or elliptical profiles yield decreasing values. Measurements were made of the orientation (angle) of the long axis of individual neurons using Image J (<http://rsbweb.nih.gov/ij/>). Tissue sections were examined with an Olympus BX45 microscope.

2.8 Data analyses

To compare differences between cohorts, we used either planned independent samples t-tests or nonparametric Wilcoxon or Mann-Whitney tests (with $\alpha = 0.05$, two-tailed). The morphology of medial superior olive neurons was compared with ANOVA.

3 Results

3.6 Air pollution levels

Mexico City children in this study were exposed to significant concentrations of ozone (O_3), and particulate matter (PM) for their entire life (Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008). The climatic conditions in MC are relatively stable, thus, pollutant concentrations are consistent year after year (Bravo-Alvarez and Torres-Jardón, 2002). The annual arithmetic mean of $\text{PM}_{2.5}$ 24 hour average concentrations in Southwest Mexico City (SWMC) (March 2008–March 2009) was $24.6 \mu\text{g}/\text{m}^3$ (the US EPA $\text{PM}_{2.5}$ annual arithmetic mean standard stands for $15 \mu\text{g}/\text{m}^3$). During the first trimester of 2009 that includes the study period, $\text{PM}_{2.5}$ 24 hour average concentrations in SWMC were $29 \pm 10.5 \mu\text{g}/\text{m}^3$. The median of $\text{PM}_{2.5}$ daily concentrations during March 2009 was $27.6 \mu\text{g}/\text{m}^3$ with a maximum of $49.8 \mu\text{g}/\text{m}^3$ and a 75th percentile of $36.7 \mu\text{g}/\text{m}^3$ (the US EPA $\text{PM}_{2.5}$ 24-hour standard is $35 \mu\text{g}/\text{m}^3$). In March 2009, $\text{PM}_{2.5}$ 1 hour average concentrations reached values as high as $90 \mu\text{g}/\text{m}^3$ during the mid-morning school recess time. Table 1 shows the statistics of $\text{PM}_{2.5}$ data in SWMC for different exposure terms between March 2008 and March 2009. Fine fraction particles composition in Mexico City are dominated by carbonaceous (organic and black carbon) species with the remaining fraction composed by inorganic and crustal species, suggesting widespread sources of precursors and efficient secondary aerosol formation (Molina et al., 2007; Querol et al., 2008). Motor vehicles have been found to be a significant source of polycyclic aromatic hydrocarbons (PAHs) one of the components of the carbonaceous species. In the control

city, due to the combination of: i. the relatively few contributing emission sources from industry and cars, ii. the partial orographic disconnection of this microregion with the atmospheric basins of the Mexico City and the Toluca metropolitan areas, and iii. the good ventilation conditions by the regional wind, concentrations for criteria air pollutants including: O₃, PM₁₀, PM_{2.5}, SO₂, NO₂ and CO levels are expected to be below the current US standards (Gobierno Estado de Mexico 2005; Gobierno Estado de Mexico 2008). Although there are no available data for the years 2008–2009, historical information from a past intensive air quality monitoring campaign in Polotitlán performed in 2004 and a recent monitoring period in 2010 that included July–October showed that levels of all of the above mentioned air pollutants were always below their respective National Ambient Air Quality Standard (NAAQS) (Gobierno Estado de Mexico 2005; Gobierno Estado de Mexico 2008, unpublished 2010 data). The arithmetic mean of PM₁₀ 24 hour averages collected between November and December 2004 was $78 \pm 14.9 \mu\text{g}/\text{m}^3$. PM_{2.5} levels were not measured in that monitoring campaign.

Recent studies about the typical fraction of PM_{2.5} in PM₁₀ in rural sites around Mexico City reporting similar PM₁₀ levels to those measured in the past in Polotitlán indicate that PM fine fraction represents ~ 40 of the PM₁₀ (Querol et al., 2008). Thus, it could be expected that for the control city, the PM fine fraction for the PM₁₀ data collected in the 2004 campaign would be approximately $31.2 \mu\text{g}/\text{m}^3$. Although the PM₁₀/PM_{2.5} reference value used to infer the fine fraction in the control site could be influenced by local sources and by abnormal meteorological conditions in central Mexico during those measurements (Querol et al., 2008), typical PM contributions in Polotitlán are mainly from resuspension dust from agricultural soil and non paved roads. It is well known that PM fine fraction from these types of PM emissions is very small and clearly dominated by crustal material (Chow 1995, Querol et al., 2008).

Considering the fact that the population growth rate and that occupational activities in Polotitlán have remained practically without changes in the last 5 years (Gobierno Estado de Mexico 2008), it is valid to assume that PM_{2.5} levels as well as concentrations for O₃, SO₂, NO₂ and CO have been kept below the respective air quality standards.

3.7 Anthropometric measurements, physical examination and outdoor exposures results

All children included were clinically healthy (i.e., *appeared* to be healthy and had no physical complaints). There were no significant differences in BMI's between controls and MC children (Table 2). Control children spend significantly more time outdoors than Mexico City children ($p=0.002$) (Table 2). Otoneurological exams showed unremarkable findings for the controls, while MC findings were atypical in phoneme recognition (18/34), finger to nose test dysmetria (13/34), gait deviation (13/34) and Romberg (positive in 4 children).

3.8 Laboratory findings

Peripheral blood results are shown in Table 3. MC children had significantly lower WBC counts ($p=0.01$), % of neutrophil counts ($p=0.0004$) and total neutrophil counts/ μL ($p=0.0003$) with neutrophils as low as $1550/\mu\text{L}$. Higher total lymphocytes ($p=0.0007$) were recorded in MC children. Total number of monocytes were also lower in MC children ($p=0.04$). Cytokine and chemokine data showed significantly higher concentrations in MC children of IL1 β ($p=0.02$), TNF α ($p=0.002$), TGF β_1 ($p=0.002$), MDC ($p=0.002$) and MCP-1 ($p=0.002$) (Table 3). While, IL8 was down-regulated in MC children ($p=0.002$) and no differences between cohorts were seen with IL6, IL10, IL12 and GM-CSF.

3.9 BAEPs

The absolute values of Waves I, III, and V for the two groups are shown in Table 4. Inspection of that data finds that the latencies of the control subjects are well within normal limits at 1.76, 3.80 and 5.62 ms (Møller 1994, Markand 1994). On the other hand, the children exposed to high levels of air pollution have clinically significant increases in waves III and V that occur at 4.26 and 6.30 msec while wave I is normal at 1.75 msec. On visual inspection, BAEP waves in MC children were prolonged on the left side in 48%, and 26% each on the right side and bilaterally, according to the current clinical standards (i.e., interwave I–V latency > 4.4 ms). In contrast, BAEP prolongation was observed in just one child from the control group. Since no morphological or amplitude anomalies were found, our analysis then focused on the most commonly reproducible BAEP components in children with typical development. Since there were no statistical differences within subject that reflected the qualitative assessment based on the clinical cut off, we focused our analysis on average latencies irrespective of lateralization effects.

Planned contrasts (assuming equal variances, since Levene's tests for equality of variances were not significant) revealed that, relative to their counterparts, the MC children had significant delays in wave III ($t(50) = 17.038$; $p < 0.0001$) and wave V ($t(50) = 19.730$; $p < 0.0001$) but no delay in wave I ($t(50) = 0.606$; $p = 0.522$) (Table 4). In addition, the MC children had significantly longer latencies than the comparison children for interwave intervals I–III ($t(50) = 13.603$; $p < 0.0001$), III–V ($t(50) = 7.501$; $p < 0.0001$), and I–V ($t(50) = 17.962$; $p < 0.0001$), thereby indicating delayed central conduction (Table 4).

3.10 Brainstem neuropathology

Gross brain examination was unremarkable in both controls and Mexico City subjects. Microscopic examination of the medial superior olive, a prominent nucleus within the superior olivary complex (SOC), revealed the typical fusiform cell bodies in a control case (Figure 1A) with prominent medial and lateral dendrites contrasting with smaller rounded neuronal bodies and loss of large calibre primary dendrites in MC children ($n = 4$) (Figure 1B). The SOC in all MC children exhibited astrocytic cells with abundant cytoplasm and neuronal strong (+++) immunoreactivity (IR) for 8 hydroxy-deoxyguanosine and beta amyloid IR to the 6E10 antibody (Figures 1C, D, E). Control SOC neurons were negative for 8OHdG and β amyloid. Morphometric analysis of the medial superior olivary nucleus in control versus MC children (Table 5) revealed statistically significant differences in cell body area, perimeter, major axis and circularity compared to controls ($p < 0.0001$).

Microscopic examination of all sections was done without access to the identification code and all slides were run together with each immunohistochemistry protocol and for the H&E and LFB stains. Sections from the control brains were blinded and independently read as having no pathological diagnosis. Most of the pathology in MC children was observed in the lower sections of the brainstem, including the medulla oblongata and all exposed children exhibited abnormal findings. The dorsal cochlear nucleus showed scattered positive α synuclein neurons (Figure 2A), increased microglial activity (Figure 2B), and βA_{1-42} neuronal IR with the 6E10 antibody (Figure 2C). Sections of the medulla oblongata in all MC cases examined showed the presence of neuronal α synuclein cytoplasmic aggregates, and IR threads and dots within the spinal trigeminal nucleus, nucleus ambiguus, medial vestibular nucleus, inferior vestibular nucleus, dorsal motor nucleus of the vagus, spinal lemniscus neurons, lateral reticular nucleus, central tegmental tract, arcuate nucleus, raphe midline, extra-raphé medial and lateral tegmentum neurons, and ventrolateral medulla (Figures 2D,E). Positive alpha synuclein neurons were mostly isolated or in small clusters (Figures 2D, E). Perivascular mononuclear cells were seen in the dorsal tegmentum of the medulla, these cells were positive for microglia stains including Iba-1, HLA-DR (Figures

2F, G, H), and CD163. Significant microglial activity was present in the area postrema, while the cuneate fasciculus had significantly less activity than the gracile fasciculus with CD163, a marker of perivascular macrophages (Figures 3A, B, C). The spinal trigeminal nucleus, the medial vestibular nuclei and the dorsal cochlear nucleus showed clusters of microglia Iba1 + in 4/5 MC children (Figures 2G, 3E). The dorsal motor nucleus of the vagus exhibited strong IR to α synuclein in 4/5 MC children (Figure 3D), along with reactive astrocytes (Figure 3F). The solitary nucleus and tract exhibited increased microglia activity (Figure 3G). The nucleus of the solitary tract amyloid stains were negative, however there were scattered positive α synuclein neurons. Neurons in the arcuate nucleus were also positive for α synuclein in 2/5 MC children (Figure 3H). Amyloid stains showed positive staining in the Golgi region in neurons of the inferior olivary complex with the 4G8 Ab.

Pontine levels showed neuronal positive α synuclein clusters as well as isolated cells in the pigmented nucleus of the superior cerebellar peduncle, the nucleus reticularis tegmenti pontis, and the dorsal and ventral tegmental nuclei. Discrete perivascular inflammatory cells were seen in relation to the locus coeruleus in one 11 year-old MC girl, along with scattered macrophages with neuromelanin around pigmented neurons. Extensive sampling of the locus coeruleus failed to show α synuclein or amyloid IR. Positive IR for 4G8 was seen in the abducens nuclei. Significant microglial activity in pontine nuclei was demonstrated with Iba-1 in all MC children. The ventral nucleus of the lateral lemniscus exhibited microglial activity and blood vessels show hyperplastic endothelial cells.

In midbrain levels, for 2/5 MC children the substantia nigrae pars compacta showed scattered reactive astrocytes and polymorphonuclear and mononuclear cells were seen attached to the adjacent capillary endothelial cells (Figures 4A, B). Microglial stains showed a significant increase in activated Iba-1 cells in 3/5 MC children, including the two children with IR to alpha synuclein (Figure 4C). In contrast, the substantia nigrae pars compacta in control children exhibited unremarkable pigmented neurons and very few perivascular Iba-1 cells (Figure 4D). Isolated pigmented neurons from the substantia nigrae pars compacta showed positive alpha synuclein (Figure 4E). A few pigmented neurons in the pars compacta were surrounded by macrophages containing neuromelanin (pigment incontinence) (Figure 4A). Amyloid stains showed positive staining in neurons of oculomotor nucleus and the Edinger-Westphal nucleus with the 4G8 Ab, while no positive staining was seen with the 6E10 antibody.

4 Discussion

Clinically healthy children with life long exposures to severe urban air pollution exhibited significant delays in waves III and V in their auditory brainstem evoked potentials when compared to age matched controls residing in a low pollution area. The significantly longer latencies for interwave intervals I–III, III–V, and I–V in Mexico City children are consistent with delayed central conduction time of neural transmission through the brainstem. The findings indicate that transmission through the cochlea and auditory nerve are normal in both groups, but that transmission through the cochlear nuclei (Wave III), superior olivary complex (wave IV), and the lateral lemniscus (wave V) pathways are abnormal in the exposed children. Thus, several of the brainstem nuclei and pathways that contribute to auditory processing are being involved, including the formation of the lateral lemniscus containing axons originating from cells in the dorsal and ventral cochlear nuclei and in the superior olivary nucleus. A major finding in this study was the involvement of the medial superior olive neurons, critically involved in BAEPs. The abnormal BAEPs, the dysmetria, gait deviation and positive Romberg test in the highly exposed children likely reflect the brainstem diffuse pathology involving the cochlear and vestibular nuclei and cerebellum

connections (afferents from vestibular nuclei) described in Mexico City children's autopsies but absent in control children.

4.1 Systemic inflammation and peripheral blood findings

The finding in MC children of significant decreases in peripheral blood neutrophils and evidence of systemic inflammation have been consistently described in MC versus control children by our laboratory (Calderón-Garcidueñas et al., 2007a; Calderón-Garcidueñas et al., 2009). The sequestration of neutrophils in CNS and extra CNS capillary beds reflects endothelial activation, a feature of systemic inflammation associated with increases in pro-inflammatory cytokines i.e., TNF α and potent vasoconstrictors, i.e., endothelin-1 (Calderón-Garcidueñas et al., 2007a,b; Calderón-Garcidueñas et al., 2009; Bröske et al., 2010; López et al., 1993; József et al., 2002). The systemic response in MC children involves increased concentrations of pro-inflammatory cytokines such as tumour necrosis factor TNF- α , IL1 β , macrophage-derived chemokines i.e., MDC and MCP-1, and the concomitant induction of anti-inflammatory mediators such as transforming growth factor (TGF)- β .

Since brain blood vessels express receptors for TNF- α and IL1 β (Nadeau and Rivest 1999), and TNF- α and IL1 β can evoke expression of inflammatory mediator genes such as cyclooxygenase-2 (COX2) (Rivest 2001) within the brain capillary endothelium, our brainstem neuroinflammatory findings in this work and previous ones, suggest that systemic cytokines may be key early CNS aggressors (Calderón-Garcidueñas et al., 2008a,b; Calderón-Garcidueñas et al., 2009, 2010; Block and Calderón-Garcidueñas, 2009). The alteration in the systemic concentrations of chemokines i.e., MCP-1 is of particular interest in children exposed to air pollution given that an alteration in the expression of this chemokine might lead to the persistence of an inflammatory reaction and the establishment of a chronic inflammatory process (Yadav et al., 2010). MCP-1 is one of the major chemokines participating in CNS inflammatory processes and there is a strong evidence of its key role in the recruitment of monocytes/macrophages and activated lymphocytes into the CNS and its interaction with tight junction proteins (Yadav et al., 2010; Stamotovic et al., 2003). However, our evidence is strictly correlative and further research is needed to support these conjectures.

In keeping with an expected anti-inflammatory response, MC children have significant concentrations of Transforming growth factor- β 1, a neuroprotective and a key immune system modulator of inflammation and neuronal survival; its signaling has been shown to play a pivotal role in down-regulating chemokines and chemokine receptors (Doyle et al., 2010). Regulatory T cells inhibitory cytokines such as IFN γ -a known induced microglial inflammatory mediator- was also increased in MC children. Type I interferons are crucial in host defense but are also implicated as causative factors for neurologic disease (Hofer et al., 2010; Kim et al., 2010; González-Pérez et al., 2010).

This work and our previous studies confirm the significant systemic inflammation and the immunodysregulation documented in highly exposed children (Calderón-Garcidueñas et al., 2007a,b; Calderón-Garcidueñas et al., 2009; Block and Calderón-Garcidueñas, 2009).

4.2. Brainstem neuropathology

In agreement with our previous neuropathology brainstem studies (Calderón-Garcidueñas et al., 2008a; Villarreal-Calderon et al., 2010), the brainstem in MC children exhibited an inflammatory component with trafficking of inflammatory cells including perivascular cells IR for CD163. This is an important finding in this work and previous ones (Calderón-Garcidueñas et al., 2008a), in view of the nature of the CD 163 immunoreactivity: CD163 is a marker of perivascular macrophages expressed in microglia and probably activated as a

result of vascular compromise (Borda et al., 2008). The macrophage scavenger receptor functions as an innate immune sensor for bacteria and has been identified as an endocytic receptor for haemoglobin-haptoglobin complexes, offering protection against haemoglobin-mediated oxidative tissue damage (Fabriek et al., 2009; Kristiansen et al., 2001; Graversen et al., 2002). Moreover, CD163 macrophages occupy a unique position at the blood-brain-barrier (BBB) and up-regulate prostaglandin-synthesizing enzymes in response to systemic inflammation (Galea et al., 2008). The general tendency of anti-inflammatory signals to induce CD 163 synthesis and the triggering of signalling cascades resulting in the production of anti-inflammatory molecules (Van Gorp et al., 2010) makes CD 163, a potential marker in the setting of air pollution exposure. Inflammation along with oxidative stress have been identified as the common and basic mechanisms through which air pollution causes damage (Block and Calderón-Garcidueñas, 2009), and accumulation of α synuclein and β amyloid is associated with neurodegenerative conformational diseases (Surguchev and Surguchov, 2010). Major alterations in the architecture of the SOC and significant statistical differences in morphometry were present in highly exposed children. The brunt of the inflammatory changes involved the medulla oblongata with accumulation of β amyloid and alpha synuclein in key auditory nuclei, the dorsal motor nucleus of the vagus, the nucleus of the solitary tract, arcuate nucleus, raphe midline, extra-raphé medial and lateral tegmentum neurons. Thus, the BAEPs abnormalities identified in clinically healthy Mexico City children could be interpreted as a sign of auditory neuronal injury and consequent dysfunction. None of these changes were seen in control children.

4.3. Brainstem inflammation and air pollution

The brainstem inflammatory findings are important in the context of air pollution for several reasons: 1. In the last two decades, Mexico City residents have been chronically exposed to significant concentrations of fine particulate matter (Bravo-Alvarez and Torres-Jardón, 2002; Molina et al., 2007). The largest source contributors include gasoline-powered and diesel engine exhaust, paved road dust, plus emissions from food cooking, tire dust, fecal material, and cigarette smoke (Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008; Estrada-García et al., 2004). A considerable fraction of the PM consists of organic compounds including biologic components from bacteria and fungi, and transition metals (Bravo-Alvarez and Torres-Jardón, 2002; Rosas-Pérez et al., 2007; Molina et al., 2007; Querol et al., 2008; Estrada-García et al., 2004). Particulate matter could reach the supratentorial brain and the brainstem by uptake through olfactory neurons and cranial nerves, such as the trigeminal (nasal, oral cavity) and vagus (respiratory, cardiovascular and gastrointestinal tracts) pathways, trafficking of macrophage-like cells loaded with PM from the lung capillary bed to the systemic circulation, and by a direct transfer of ultrafine particles (particulate matter with aerodynamic diameters <100 nm) from the systemic circulation and/or red blood cells to brain endothelial cells (Calderón-Garcidueñas et al., 2001, 2007a,b, 2008b, 2010, 2008a; Block and Calderón-Garcidueñas, 2009). 2. Under experimental conditions, nanoparticles (NPs) (Mailänder and Landfester, 2009) are uniformly distributed throughout the nasal passages (García and Kimbell, 2009), brain oxidative stress is seen in mice exposed to TiO₂ NPs delivered to the abdominal cavity (Ma et al., 2010), while intravascular delivery deposits the NPs in the brain (Sarlo et al., 2009), thus supporting the natural exposure pathways described in highly exposed humans, 3. The dorsal vagal complex (DVC) is an air pollutant target with pivotal connections between the central nervous system and peripheral viscera (Villarreal-Calderon et al., 2010). The area postrema lacking a blood-brain-barrier, plays a role in controlling the entry of blood or CSF borne substances to DVC neurons (Maolood and Meister, 2009). Ultrafine and fine PM could have direct access to the DVC and/or could travel transsynaptically in the vagus or trigeminal nerves and reach the medulla (Maolood and Meister, 2009; Standish et al., 1995). The transneural vagal transport has been clearly shown with herpes virus where

the virus is identified in vagal pre-ganglionic neurons of the dorsal motor nucleus of the vagus, nucleus ambiguus, and reticular formation after cardiac viral injections (Standish et al., 1995; Kyrkanides et al., 2009), 4. An association between gastrointestinal inflammation and brainstem pathology has been well established (Al-Chaer et al., 1997; Ammori et al., 2008; Villaran et al., 2010). Such evidence is particularly relevant in this context because the gastrointestinal tract is a preferred extra-pulmonary translocation site for PM deposition upon inhalation (Bennett et al., 1997). Thus, the transneural PM transport from the GI tract to the brainstem is a plausible inflammatory pathway in highly exposed MC residents (Miwa et al., 2006; Asan et al., 2004). The aggregation of α synuclein and the Iba-1 microglia activation in the dorsal motor nucleus of the vagus upon the experimental administration of intragastric proteasome inhibitors (Miwa et al., 2006) brings a crucial issue: the transport of ubiquitin-proteasome natural inhibitors associated with PM, since Actinomycetes-the source of powerful proteasome inhibitors- are present in urban air (Asan et al., 2004). Finally, the sustained inflammation of the brainstem, including the DVC is a risk factor for the development of Parkinson's disease (Hirsch and Hunot, 2009).

4.4. Clinical implications and limitations

Our current findings of pontine-mesencephalic inflammation would suggest that neurons on the ascending monoaminergic brainstem systems with important connections with the prefrontal cortex (PFC) could also be involved. The pontine-mesencephalic inflammation and the PFC structural lesions we have previously described in the same Mexico City cohorts could be participating factors for the working memory deficits reported in MC children (Calderón-Garcidueñas et al., 2008b).

The sustained systemic and CNS inflammation are key consistent findings in Mexico City residents when compared to controls, and circulating cytokines including TNF α and IL1 β are well known to cause neuroinflammation, neurotoxicity, and cerebrovascular damage (Block and Calderón-Garcidueñas, 2009). Thus, the long term effects of the sustained CNS and systemic inflammation in children ought to include perturbations in the trajectory of cerebral development during childhood and higher risk for cognitive deficits. The size of the study populations have to be increased and longitudinal children studies focusing in brainstem functions ought to be included in the agendas of institutes interested in the well-being of children exposed to urban air pollution.

5 Conclusions

We have reported BAEP data that, together with converging brainstem pathology, show an association between exposure to severe air pollution and neural dysfunction of the auditory pathway. Since systemic inflammation and neuroinflammation could play an important role in the cascade of events leading to neuronal degeneration (Block and Calderón-Garcidueñas, 2009) the present findings support the view that these highly exposed children are at a higher risk for developing auditory impairments, and vestibular dysfunction. Based on our clinical studies we can suggest that the alterations are located in the auditory pathway and could be related to a variety of neuropathological lesions or even metabolic alterations. These lesions may include neuronal and axonal damage, accumulation of structurally abnormal proteins, as well as neuropil, glial and vascular damage. Studies in our laboratory are underway in experimental animals to clearly define the brainstem pathology, and in children to define whether we can detect brainstem metabolic alterations.

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Abbreviations

BAEPs	Brainstem auditory evoked potentials
8OHdG	8 hydroxi-deoxyguanosine
MC	Mexico City
MSO	Medial superior olive
SOC	Superior olivary complex
SWMC	Southwest Mexico City
O₃	Ozone
PM	Particulate matter
PM-LPS	Lipopolysaccharides-associated with PM
PM_{2.5}	Particulate matter less than 2.5µm in diameter
PAHs	Polycyclic aromatic hydrocarbons
PFC	Prefrontal cortex
NAAQS	National Ambient Air Quality Standard

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Figure 1.

Morphology of neurons in the superior olivary complex

Figure 1A and B

Shown in this figure are neurons from the medial superior olivary nucleus (MSO) from two specimens stained for Nissl substance (14 year old females, control in A and exposed in B). In A, MSO neurons display the typical fusiform cell bodies (arrows) and issue medial and lateral dendrites. Medial and lateral to the MSO cell column is a region with a high density of dendritic processes (asterisks). In B, MSO neurons have cell bodies that are generally smaller and more round (arrows) and do not appear to issue large caliber primary dendrites as in control child. The scale corresponds to 20 μ m.

C. MSO neurons from a 14y old MC girl showing the round abnormal morphology (arrow heads) surrounded by astrocyte-like cells with abundant cytoplasm (long arrow) and oligodendrocyte-like cells (short arrows). Hematoxylin and eosin stain $\times 100$

D. MSO neurons from an 11 y old Mexico City girl showing strong immunoreactivity (IR) for 8 hydroxydeoxyguanosin (brown product) indicative of oxidative stress.

Immunohistochemistry for 8OHdG 1: 100 $\times 20$

E. MSO neurons from a 13y old Mexico City girl showing strong IR for β amyloid with the 6E10 antibody (brown product) reactive to amino acid residue 1-16 of beta amyloid (the epitope lies within amino acids 3–8 of beta amyloid). Immunohistochemistry for β A 6E10 1: 2000 counterstained with hematoxilin $\times 10$

Figure 2.

Medulla oblongata at the level of the dorsal cochlear nucleus.

A. Medulla oblongata in an 11 year old MC female: the dorsal cochlear nucleus exhibits positive α synuclein IR in neurons, threads and dots. Immunohistochemistry for α synuclein 1: 500 red product $\times 20$

B. Same child as A, dorsal cochlear nucleus neurons surrounded by microglia positive for Iba-1. Immunohistochemistry for Iba-1 1:1000 brown product, counterstained with hematoxylin $\times 20$

C. Thirteen year old MC female dorsal cochlear nucleus neurons (arrow heads) showing strong IR for β amyloid with the 4G8 antibody reactive to amino acid residues 17-24 of the human beta amyloid peptide (epitope lies within amino acids 18–22 of the beta amyloid). Glial-like cells (arrows) with abundant IR are proximal to the neurons. Immunohistochemistry for β A 1: 2500, brown product $\times 40$

D. An isolated neuron IR for α synuclein in the tectospinal tract region. Eleven year old Mexico City girl. Immunohistochemistry for α synuclein 1: 500 red product $\times 40$

E. A cluster of positive α synuclein neurons, threads and dots located in the spinal lemniscus region. Same child as D. Immunohistochemistry for α synuclein 1: 500 red product $\times 40$

F. Positive Iba-1 perivascular mononuclear cells and microglia (brown product) in the region of the nucleus ambiguus. There is a significant variation in the number of mononuclear perivascular cells with IR to Iba-1 in medullary blood vessels as observed in the upper left

blood vessel compared to the right lower larger vessel. Eleven year old from MC.
Immunohistochemistry for Iba-1 1:1000 counterstained with hematoxylin $\times 20$
G. Medial vestibular nucleus with abundant positive Iba-1 activated microglia (brown product). Immunohistochemistry for Iba-1 1:1000 counterstained with hematoxylin $\times 20$
H. 15 year old Mexico City girl: cluster of perivascular cells positive for HLA-DR (brown product) in a blood vessel located in the region of the medial longitudinal fasciculus.
Immunohistochemistry for HLA-DR 1:1000 $\times 40$

Figure 3.

Medulla oblongata at mid-olivary level.

A. Area postrema in a 15 year old Mexico City girl showing a significant number of CD163 immunoreactive perivascular and microglia-like cells scattered in the region.

Immunohistochemistry for CD163 1:100 brown product $\times 10$

B. Cuneate fasciculus in a 19 year old Mexico City male showing immunoreactive CD163 perivascular and microglia-like scattered cells. Immunohistochemistry for CD163 1:100 brown product $\times 10$

C. Same subject as B, gracilis fasciculus with significant numbers of immunoreactive CD163 perivascular and microglia-like cells. Immunohistochemistry for CD163 1:100 brown product $\times 10$

D. The dorsal motor nucleus of the vagus in an 11y MC girl exhibits positive α synuclein IR in neurons, threads and dots. Immunohistochemistry for α synuclein 1: 500 red product $\times 20$

E. Same block as D stained with Iba-1 shows the dorsal motor nucleus of the vagus neurons surrounded by IR microglia. Immunohistochemistry for Iba-1 1:1000 brown product $\times 20$

F. Same dorsal motor nucleus of the vagus block as D and E stained with glial fibrillary acidic protein Ab GFAP to define reactive astrocytes (brown product) around neurons. Reactive astrocytes with abundant IR cytoplasm are identified with head arrows.

Immunohistochemistry for GFAP 1:100 counterstained with hematoxylin $\times 40$

G. Solitary nucleus in an 11y old MC girl stained with GFAP. Scattered IR astrocytes are seen. Immunohistochemistry for GFAP 1:100 brown product counterstained with hematoxylin $\times 20$

H. Arcuate nucleus cluster of IR α synuclein neurons. Same child as D.

Immunohistochemistry for α synuclein 1: 500 red product $\times 40$

Figure 4.

Mesencephalon at the level of the inferior colliculus

A. Pigmented neurons from the pars compacta substantia nigrae showing an astrocyte-like cell with abundant eosinophilic cytoplasm (arrow) and macrophage-like cells (head arrows). Hematoxylin-eosin $\times 100$

B. Same section as A to show two white blood cells attached to the endothelium of a capillary in the pars compacta. A polymorphonuclear cell (long arrow) and the mononuclear cell (head arrow) both are attached to the endothelial cells. An adjacent glial cell (short arrow) exhibits abundant cytoplasm. The pigmented neuron in the center of the picture is surrounded by glial-like cells. Hematoxylin-eosin $\times 100$

C. A blood vessel in the midst of the substantia nigrae pars compacta exhibit numerous Iba-1 IR cells and positive cells are seen surrounding the pigmented neurons. Immunohistochemistry for Iba-1 1:1000 red product, counterstained with hematoxylin $\times 40$

D. The substantia nigrae from a control 17 year old male shows numerous well pigmented neurons and few Iba-1 IR cells surrounding the pigmented neurons. Notice a few perivascular Iba-1 IR cells. Immunohistochemistry for Iba-1 1:1000 red product, counterstained with hematoxylin $\times 20$

E. Close up of a substantia nigrae pigmented neuron with IR for α synuclein in a 13y female contrast with the insert showing a pigmented negative neuron. Immunohistochemistry for α synuclein 1: 500 red product $\times 60$

Table 1

PM_{2.5} concentrations for Southwest Mexico City the target area for the clinical study. The clinical and laboratory studies including the brainstem auditory evoked potentials (BAEPs), and the collection of fasting blood samples were done between March 2 and 31, 2009.

Statistical descriptors PM _{2.5} *	March 2008–March 2009 24-hr averages	January–March 2009 24-hr averages	March 2009 24-hour averages	March 2009 1-hour averages
Arithmetic Mean	24.6	29.0	27.3	27.1
Standard Deviation	10.1	10.0	10.2	14.8
Percentile 50 th	24.0	25.9	26.1	24.0
Percentile 75 th	31.0	36.7	32.0	36.0
Percentile 90 th	38.4	39.3	42.2	48.8
Maximum	58.7	53.3	49.7	90.0

* PM_{2.5} concentrations are expressed as µg/m³

Table 2

General characteristics of the study clinical populations

Characteristic	Control	MC	p Value
Number	17	34	NA
Gender	7M, 10F	18M, 16F	NA
Age (months)	96.9±9.8 *	95.7±7.3	0.97
Weight (kg)	29.18±5.6	27.98±5.1	0.34
Height (m)	1.27±0.09	1.26±0.05	0.59
Body mass index	17.86±1.7	17.28±2.2	0.43
Outdoor hours per day	4.43±1.16	3.88±1.02	0.002

* Mean ± Standard Deviation

Table 3

Peripheral blood endpoints (Mean± SD) and their statistical significance in Control v MC children

Endpoints	Polotitlán Control Cohort	MC Cohort	p value
Hb g/dL	15.1±0.6	14.6±0.87	0.09
Ht%	43.3±2.4	42.3±1.8	0.05
WBC (×10 ³ /μL)	8.2±1.5	6.7±1.4	0.01
Neutrophils (%)	55.4±7.1	44.5±8.8	0.0004
Lymphocytes (%)	34.3±7	45.4±8.6	0.0007
Monocytes (%)	7.1±2.0	6.5±1.4	0.45
Neutrophils (μL)	4523±1046	3030±1126	0.0003
Monocytes (μL)	548±175	415±114	0.04
Interleukin-1β	0.13±0.14	0.94± 1.0	0.02
TNF α *	0.02±0.006	0.07±0.04	0.002
TGF β1	0.08±0.01	1.12±0.33	0.002
MDC	0.03±0.02	1.48±0.39	0.002
MCP-1	0.06±0.04	0.25±0.07	0.002
IL8	0.25±0.18	0.03±0.009	0.002
IFN γ	0.02±0.01	0.07±0.05	0.01
IL17	0.03±0.01	0.2±0.3	0.06
IL6	0.03±0.02	0.04±0.3	0.48
IL10	0.03±0.02	0.11 ±0.13	0.39
IL12	0.03±0.008	0.05 ±0.02	0.30

* All cytokines and chemokines values are in pg/ml

Table 4

Mean absolute latencies and relative mean interpeak differences of the replicable waveform components of the BAEPs in Mexico City and control group children.

BAEP waves	Groups	
	Mexico City ^a	Polotitlán ^b
I	1.75 (0.06) [§]	1.76 (0.05)
III	4.26 (0.08) *	3.80 (0.11)
V	6.30 (0.07) *	5.62 (0.18)
I-III	2.51 (0.11) *	2.04 (0.13)
III-V	2.04 (0.10) *	1.82 (0.09)
I-V	4.56 (0.08) *	3.86 (0.20)

Note. The values in parenthesis represent standard deviations; all values are in milliseconds.

^a
n = 35

^b
n = 17

p values:

[§]
p= 0.522,

*
<0.0001 compared to controls

Table 5
Morphometric Analysis of Medial Superior Olive Neurons control versus exposed children

MSO	Area (µm ²)	Perimeter (µm)	Major Axis (µm)	Minor Axis (µm)	Circularity
Control (n=66§)	275 ± 9	86 ± 2	33 ± 8	11 ± .4	.48 ± .01
Exposed (n=274§)	182 ± 5***	57 ± 1***	21 ± .4***	11 ± .2	.69 ± .01***

This table summarizes a morphometric analysis of the MSO from one control and four exposed specimens. Data are presented as average ± standard error.

The symbol § refers to the number of neurons counted.

The asterisks indicates statistical differences (ANOVA; *** = $p < 1 \times 10^{-15}$).