# Molecular Changes in Rat Brain Due to Air Nano Pollution

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## ABSTRACT

Air pollution has been found to cause neurodegeneration, deposition of heavy metals & beta amyloid plaques in the brain. Air pollution consists of nanopollutant of different sizes that can enter the system and cause diseases. To assess the effect of nanopolluants, we exposed rats to different particle sizes for 0.5, 1, 3 & 10 months, and analyzed brain tissues. Spectroscopy revealed accumulation of certain heavy metals in the rat brain. The uptake was highest at early exposure time. Microarray data showed changes in pituitary hormone genes and these changes were evaluated further by qPCR. We found significant alteration of pituitary hormones including glycoprotein hormones, prolactin and growth hormones in rats exposed to air pollution.

***Keywords***: air nano pollution, qPCR, heavy metals

## 1 INTRODUCTION

Air pollution is a global problem that has been correlated with cardiovascular and respiratory-related morbidity [1]. Individuals living in urban areas may be exposed to nanoscopic pollution from automobile exhaust and various other industrial byproducts. However, the detrimental effects of air pollution may extend beyond the respiratory and cardiovascular systems. Studies in both animals and humans have shown that high levels of air pollution are associated with chronic neuroinflammation, as well as signs of neurodegeneration. For example, premature accumulation of heavy metals and β-amyloid has been found in the brains of humans and dogs living in highly polluted cities [2, 3]. Thus, air pollution may in fact be a factor contributing to the pathogenesis of common neurodegenerative diseases and possibly tumor formation.

Industrial facilities, waste incinerating plants, and fossil fuel burning are considered the main sources of anthropogenic heavy metal emissions. It is well known that the particulate matter has considerably high amount of heavy metals as well as rare earth elements. Air in industrial and metropolitan areas is highly contaminated with heavy metals compared to air in the rural areas. Close to highways, motor vehicles are the largest emission source. Metals such as As, Cd, Co, Ni, Pb, Sb, V, Zn, and the platinum group elements Pt, Pd, and Rh can be characterized as being road-specific heavy metals. They are mainly derived from combustion residues and losses from fuels and engine and transmission oils, abrasion from tires, brake linings, exhaust catalysts, and road pavement, and corrosion of galvanized protection barriers.

The heavy metals are present in particulate matter of all sizes. Fine particles (diameter <10 $\mu$m) represent the main fraction of airborne aerosols (about 80%). These particles can be inhaled, and thus pose a high risk to human health. Carcinogenic substances such as As and Cd and their compounds occur in airborne dust in particle fractions with an aerodynamic diameter of <1 $\mu$m, whereas Ni can be found in concentrations of up to 30% in more coarse dust fractions, >10 $\mu$m. Fine dust particles (0.1-2 $\mu$m) normally have long residence times in the atmosphere and can penetrate deeply into the body upon inhalation. Although exposure to ultrafine dust particles containing heavy metals is an important consideration in assessing the risks of airborne pollutants, their ambient concentrations have not yet been investigated sufficiently.

A wide range of minor and trace metals are known to play important roles in biological processes including the activation or inhibition of enzymatic reactions, competition amongst elements and metalloproteinases for binding sites and modification of the permeability of cell membranes. It would seem reasonable, therefore, to assume that the trace metals might influence brain pathology. Several studies have focused on the relationship between trace metals and cancer in humans. Published data revealed that an enhanced concentration of Cd may result in prostate, renal and lung cancers. [4-8] Lead has been linked with the cancers of stomach, small intestine, large intestine, ovary, kidney, lung, myeloma, all lymphomas and all leukemias [9-11]. Some studies on experimental animals, such as mice,

correlated higher concentrations of chromium and zinc with accelerated tumor growth [12-16]. Significant correlations have been reported between high levels of nickel, and cobalt in lung and nasal cancer patients [17, 18].

The purpose of this study is to analyze the accumulation of heavy metals and altered gene expression, whose relation with illness has been reported, in the brain of rats exposed to polluted air. The rats were exposed through the breathing air to concentrated particulate matter of different sizes and several time points during one year.

# 2 MATERIAL AND METHODS

## 2.1 Particle preparation

A special vehicle was designed and built for this experiment. This vehicle holds the rat cages inside and devices to collect, concentrate, separate and deliver the particles of different sizes into the rat cages. A schematic presentation is shown in Figure 1. Polluted air was collected from the highway (Riverside, CA, USA, at the intersection of US highways 91 and 215) and humidified to promote particle aggregation. The particles were conducted into a cyclone separator [19] and were separated according to their size, the particles were either ultrafine (PM<0.15 μm in diameter), fine (PM<2.5 μm in diameter), or coarse (PM 2.5-10 μm in diameter). After the separation, the air containing particles of specific size was conducted into the sealed rat cages.

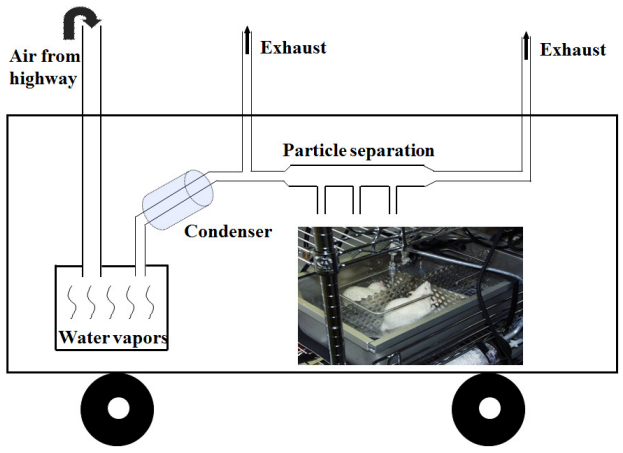


**Figure 1.** Process of collection, concentration, separation and delivery of the particles of different sizes, into the rat cages. Air pollutant were separated by a cyclone separator into Ultrafine (<0.15 μm in diameter), Fine (<2.5 μm in diameter) and Coarse (2.5 - 10 μm in diameter) fractions and captured on Teflon membranes.

## 2.2 Animal exposure to particles

Groups of five rats each were exposed through the breathing air to concentrated particulate matter. Control rats were exposed to purified air. Exposures were for 5 hours a day, 5 days a week for the following time points: 2 weeks, 1, 3, and 10 months. The rats were euthanized 24 hours after the final exposure, their brains harvested and half of the brain (0.1-0.5 g) was used for the heavy metal analysis in order to have a homogeneous sample [20]. The total number of rats used in the study was 100. Animals were monitored closely twice daily for clinical signs such as: recumbence, inability to access food and water, nonresponsive to stimuli, and seizures. Moribund animals displaying these symptoms were euthanized by $CO_2$ inhalation. Extra animals were added to each group of study, in case that unexpected mortality occurred.

## 2.3 Heavy metals analysis

Analysis of the content of the heavy metals of particles and tissue samples of rat brain tissues with/without exposure were done at a Good Laboratory Practice (GLP) certified laboratory (Exova, Santa Fe, CA, USA) using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) [21].

Representative samples of particles of each size were collected in a teflon membrane and analyzed. Teflon membranes were digested in 1 mL of nitric acid and 1 mL of hydrochloric acid for 1 hour on a heated plate set at 110°C. The samples were allowed to cool, and then 0.5 mL of 30% hydrogen peroxide was added. The digestion was then resumed for another 30 minutes. The digestates were cooled, internal standards were added, and then the digestates were diluted to a final mass of 20 grams. The membranes did not dissolve. A total of 11 heavy metals were analyzed in the particles.

## 2.4 Isolation of total RNA

RNA was isolated from frozen tissues with TRIZOL reagent (Invitrogen, Carlsbad, CA). To achieve higher purity, isolated RNA was treated with NucleoSpin RNA and Virus Purification kit (BD Biosciences, Palo Alto, CA) as per manufacturer's instructions and stored at -80$^0$C. Total RNA quality was assessed with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Palo Alto, CA), which provides information about the quality and overall yield of the samples, and with NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

## 2.5 Gene Array Analysis

Purified mRNA was used for gene chip analysis. Affymetrix GeneChip Rat Genome 230 2.0 microarray chips harboring probes for 28,000 genes were used at Cedars-Sinai Medical Center gene array core facility.

## 2.6 Quantitative RT-PCR.

Microarray results were confirmed using Q-RT-PCR both on the same samples used for microarray experiments

and on additional samples. 2µl (0.5µg) of RNA was reverse transcribed into first-strand cDNA using the Invitrogen High Capacity cDNA Reverse Transcriptase Kit (Life Technologies) using random hexamer primers. RT reaction mixtures contained the following: 10X RT buffer, 25x dNTP mix, 10X random primers, Multiscribe reverse transcriptase, RNase inhibitor and nuclease free water in a total volume of 20µl. Reactions were cycled on a GeneAmp PCR System 9700 (Applied Biosystems) as follows: 25$^0$C for 10 min, 37$^0$C for 120 min and followed by inactivation at 85$^0$C for 5 min. Real-time qPCR was carried out in MicroAmp Optical 96-well plates on the 7900HT Fast Real-Time PCR System (Applied Biosystems). Each well contains 25-100ng (depending on gene) of reverse-transcribed cDNA, TaqMan Fast Universal PCR Master Mix, and the corresponding primer/probe set (Applied Biosystems), as well as HPRT for an endogenous control, in a total volume of 25µl. All samples were run in triplicate. The thermal cycling conditions for real-time PCR were: a) 50$^0$C for 2 min, b) 95$^0$C for 10 min, and c) 40 cycles of melting (95$^0$C, 15 sec) and annealing/extension (60$^0$C, 60 sec). The $\Delta\Delta C_T$ method was used to determine the fold change between samples. Briefly, the $\Delta C_T$ for each condition was determined by subtracting the gene threshold ($C_T$) value for HPRT from the $C_T$ for target genes. The $\Delta\Delta C_T$ was calculated by subtracting the $\Delta C_T$ for each experimental sample from the $\Delta C_T$ of the air control sample, and thus the data were expressed as $2^{-\Delta\Delta C_T}$ relative to the control sample.

## 2.7 Statistical analysis

A total of three experimental groups (exposed to fine, ultrafine and coarse particles) and a control group exposed to purified air, were used in this study. Each group contained three to five animals. All data were expressed as means ±SD. Differences were considered statistically significant at $p<0.05$ (Student t test).

# 3 RESULTS

The heavy metal content of the rat brains was measured by Inductive Coupled Plasma Atomic Emission Spectroscopy (ICP-AES). Metals included As, Cd, Co, Ni, Pb, Sb, V, Zn, including platinum group elements Pt, Pd, and Rh. Amount of Cobalt (Co), Nickel (Ni), Zinc (Zn) and Arsenic (As) were found to be elevated in the brains of rats exposed to polluted air compared to those of rats exposed to purified air from the same location. Heavy metal uptake was found to be highest ($p<0.05$) during the first 3 months of exposure (30%) Co, 400% (Ni), 75% (As), and 75% (Zn), which corresponds to 0.005 µg/g, 0.05 µg/g, 0.3 µg/g, and 19 µg/g respectively of metal/g of brain tissue (Figure 2). The contents in the brains did not reveal size preference excluding particle effects due to size.

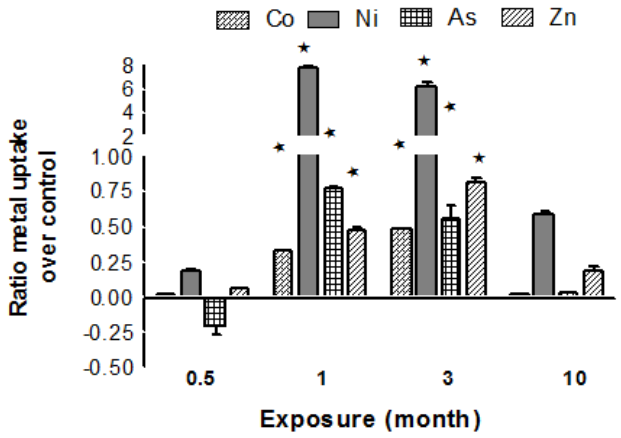


Figure 2. Relative increase of metal accumulation after exposure to different size nanoparticles compared to free air as a control. Bars: standard deviations; *: $p < 0.05$.

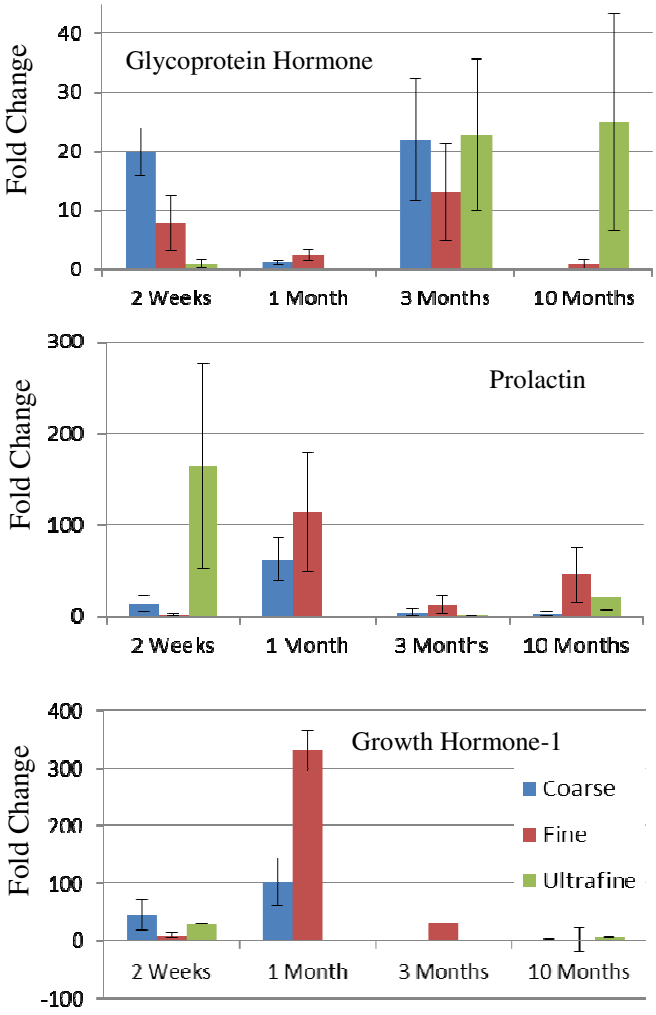


Figure 3. Quantitative PCR for pituitary hormones expressed as fold change as compared to clean air.

Gene expression studies by Affymetrix GeneChip Rat Genome 230 2.0 microarray chips harboring probes for 28,000 genes revealed changes in genes related to immediate responses by short and long-time exposure, which then were validated by quantitative PCR. Based on the microarray data, we performed qPCR for genes encoding pituitary hormones including alpha subunit of Glycoprotein hormones (cell to cell interaction), Prolactin (role in cell growth, differentiation, apoptosis and angiogensis), Growth Hormone-1 (role in growth, cell reproduction and regeneration), and the beta subunit of Thyroid Stimulating Hormone (role in regulation of thyroid hormone level and thyroid adenomas). We found that exposure to air nanopollutants had a significant effect on these hormones at short exposure times compared with clean air (Figure 3), except Thyroid Stimulating Hormone which was not affected (data not shown).

## 4 DISCUSSION

The bioaccumulation of the four heavy metals reported in Figure 2 was significant for 1 and 3 months exposure times, suggesting that 2 weeks is not enough time for the delivery and accumulation of heavy metals in the brain. Regarding the differences observed between particles sizes, a growing literature indicates that fine and ultrafine particles exert adverse health effects of greater magnitude than the particles with bigger sizes. It appears that the smallest particles that exist in the urban environment can cross the blood brain barrier and are by far the most abundant particles by number in urban environments such as Los Angeles [22]. Because these particles are emitted mainly by vehicular emissions and other combustion sources, they contain a high content of redox-cycling organic chemicals that could be released deep into the lungs [23].

Although heavy metals have been related to tissue malignancies [24], heavy metals content of airborne particles [25] has not yet been considered in the etiology of central nervous system diseases. Upon short exposure, we found that heavy metals from air nanoparticles accumulated in the brain tissues which associated with altered expression level of pituitary hormones. Further investigations to study the impact of these pituitary hormones due to air nano pollution are ongoing. In addition, changes in proinflammatory genes in brain tissues are being investigated.

## 5 ACKNOWLEDGEMENT

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