

Activation of the Stress Axis and Neurochemical Alterations in Specific Brain Areas by Concentrated Ambient Particle Exposure with Concomitant Allergic Airway Disease

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OBJECTIVE: Exposure to ambient particulate matter (PM) has been linked to respiratory diseases in people living in urban communities. The mechanism by which PM produces these diseases is not clear. We hypothesized that PM could act on the brain directly to stimulate the stress axis and predispose individuals to these diseases. The purpose of this study was to test if exposure to PM can affect brain areas involved in the regulation of neuroendocrine functions, especially the stress axis, and to study whether the presence of preexisting allergic airway disease aggravates the stress response.

DESIGN: Adult male rats ($n = 8/\text{group}$) with or without ovalbumin (OVA)-induced allergic airway disease were exposed to concentrated air particles containing PM with an aerodynamic diameter $\leq 2.5 \mu\text{m}$ ($\text{PM}_{2.5}$) for 8 hr, generated from ambient air in an urban Grand Rapids, Michigan, community using a mobile air research laboratory (AirCARE 1). Control animals were exposed to normal air and were treated with saline.

MEASUREMENTS: A day after $\text{PM}_{2.5}$ exposure, animals were sacrificed and the brains were removed, frozen, and sectioned. The paraventricular nucleus (PVN) and other brain nuclei were microdissected, and the concentrations of aminergic neurotransmitters and their metabolites were measured using high-performance liquid chromatography with electrochemical detection. Serum corticosterone levels were measured using radioimmunoassay.

RESULTS: A significant increase in the concentration (mean \pm SE, $\text{pg}/\mu\text{g}$ protein) of norepinephrine in the PVN was produced by exposure to concentrated ambient particles (CAPs) or OVA alone (12.45 ± 2.7 and 15.84 ± 2.8 , respectively) or after sensitization with OVA (19.06 ± 3.8) compared with controls (7.98 ± 1.3 ; $p < 0.05$). Serum corticosterone (mean \pm SE, ng/mL) was significantly elevated in the OVA + CAPs group (242.786 ± 33.315) and in the OVA-presensitized group (242.786 ± 33.315) compared with CAP exposure alone (114.55 ± 20.9). Exposure to CAPs (alone or in combination with OVA pretreatment) can activate the stress axis, and this could probably play a role in aggravating allergic airway disease.

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In the past decade, numerous studies have indicated a causal association between ambient particulate matter (PM) in major metropolitan areas and potential health risks (Pope 2004). Daily death rate and hospital admissions due to pulmonary and cardiovascular problems have been shown consistently to parallel elevated PM air pollution in several epidemiologic reports (Brook 2005; Pope 2004; Samet et al. 2000; Schwartz 1999; von Klot et al. 2005).

Exposure to PM with an aerodynamic diameter $\leq 2.5 \mu\text{m}$ ($\text{PM}_{2.5}$) has been associated with the incidence of respiratory conditions (Johnson and Graham 2005; Peel et al. 2005). Studies in various parts of the world have linked PM exposure to allergic airway diseases such as asthma (Barnett et al. 2005; D'Amato et al. 2005; Kappos et al. 2004; Trasande and Thurston 2005). PM exposure is known to cause acute respiratory events such as acute asthma exacerbations that are reflected as an increase in the symptom score as well as an increase in hospitalization and use of medication (Penttinen et al. 2001; von Klot et al. 2005). These responses are especially prevalent in populations with preexisting pulmonary disorders such as chronic bronchitis and

chronic obstructive pulmonary diseases (Clarke et al. 1999). Although coarse PM ($\leq 10 \mu\text{m}$; PM_{10}) can penetrate the human larynx and is deposited in the trachea and larger airways, finer $\text{PM}_{2.5}$ is believed to be more toxic because of its large surface area and ease of accumulation in the alveoli of the lungs (Lippmann et al. 2005). The composition of PM is also believed to play a role in inflammatory reactions. Trace elements of anthropogenic origin such as lanthanum, vanadium, manganese, and sulfur (Morishita et al. 2004), nitric oxide (Barnett et al. 2005), tobacco smoke (Tatum and Shapiro 2005), and elemental or organic carbon from vehicle emissions (Lippmann et al. 2005; Peel et al. 2005) are all known to promote various respiratory conditions.

Although the substances associated with PM can cause inflammation on their own, the presence of $\text{PM}_{2.5}$ in respiratory tissue can incite inflammatory reactions as well. Inhalation of $\text{PM}_{2.5}$ results in its accumulation in the deeper portions of the lungs where it may stimulate macrophages and epithelial cells or cause an influx of neutrophils and eosinophils and promote cytokine and chemokine release (Tao et al. 2003). This may increase the permeability

of the airways, and PM may enter the interstitial space where it could elicit an allergen-induced response (D'Amato et al. 2005). This would result in an increase in T-helper 2 cell ($\text{T}_\text{H}2$) cytokines such as interleukin (IL)-4 and IL-13 that play an important role in the pathophysiology of allergic asthma (Marshall 2004).

Stress could be another important factor that promotes asthma. High levels of psychosocial stress are known to predict the onset of asthma and are correlated with high asthma morbidity (Sandberg et al. 2000). The elevation of stress hormones such as glucocorticoids is known not only to suppress the immune system but also to induce a shift in the $\text{T}_\text{H}1/\text{T}_\text{H}2$ cytokine balance toward a $\text{T}_\text{H}2$ cytokine response, which favors the onset of asthma and allergic diseases (Marshall and Agarwal 2000). However, the effect of PM on the stress axis has not been investigated before.

We hypothesized that exposure to $\text{PM}_{2.5}$ can cause an activation of the stress axis and that this effect would be more pronounced in animals that have existing allergic airway disease. This could be mediated through neurotransmitters such as norepinephrine (NE) and dopamine (DA) that are crucial for the stimulation of the stress axis. To test this hypothesis, we used an ovalbumin (OVA)-induced asthma model and exposed these animals to concentrated air particles (CAPs) containing $\text{PM}_{2.5}$. The activation of the stress axis in these animals was measured by examining neurotransmitter levels in different areas of the brain related to neuroendocrine functions and by correlating them with serum corticosterone.

Materials and Methods

Adult male Brown Norway (BN) rats 12–13 weeks old were obtained from Harlan Sprague-Dawley (Indianapolis, IN) and

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divided into two groups ($n = 16$). We sensitized one group to OVA by intranasally instilling a 0.5% solution of OVA in saline (150 μL /nasal passage) for 3 consecutive days. Control animals received saline intranasally. The airway sensitization of these animals was conducted in J.R.H.'s laboratory at Michigan State University. Fourteen days after the last intranasal instillation, all the rats were moved to the mobile air research laboratory (AirCARE 1; Sioutas et al. 1997) located at Calvin College (Grand Rapids, Michigan). Approximately 1 hr before the start of the inhalation exposure, rats were intranasally instilled with a 1.0% solution of OVA in saline (150 μL /nasal passage for antigen challenge) or with saline alone (controls; no antigen challenge). Rats were further divided into two groups ($n = 8$), placed in whole-body exposure chambers, and exposed to fine CAPs drawn from the local urban atmosphere or to filtered air drawn from room air (filtered air controls). We used a Harvard/U.S. Environmental Protection Agency ambient fine particle concentrator to generate the CAP exposures. The concentration of $\text{PM}_{2.5}$ in the CAP mixture was 500 $\mu\text{g}/\text{m}^3$. Animals were exposed to CAPs for 8 hr. Twenty-four hours after exposure, they were sacrificed by excess pentobarbital administration. The protocols were approved by the Institutional Animal Care and Use Committee at Michigan State University, and experiments were performed according to National Institutes of Health guidelines (National Institutes of Health 2002).

Brain microdissection. At the time of sacrifice, the brain along with the olfactory bulb was quickly removed and frozen on dry ice. Serial coronal sections (300 μm thick) of the brain were obtained using a cryostat (Slee Mainz, London, UK) maintained at -10°C . The sections were transferred to precleaned microscopic slides placed on a cold stage at -10°C . Various nuclei of the hypothalamus including the paraventricular nucleus (PVN), medial preoptic area (MPA), arcuate nucleus (AN), median eminence, supra-chiasmatic nucleus (SCN), and the substantia nigra (SN) were microdissected by the Palkovits's micropunch technique (MohanKumar et al. 1998; Palkovits 1973) using a 500- μm -diameter punch with the rat brain stereotaxic atlas (Paxinos and Watson 1987) as a reference. Tissue samples were obtained bilaterally, and all the subdivisions of the nuclei were included. They were stored at -70°C until analysis for neurotransmitter concentrations using high-performance liquid chromatography with electrochemical detection (HPLC-EC).

Neurotransmitter analysis. At the time of analysis, tissue samples were homogenized in 150 μL of 0.1 M HClO_4 , and an aliquot of 10 μL was saved for protein analysis. The remaining sample was centrifuged briefly at

10,000 $\times g$, and 75 μL of the supernatant with 25 μL of internal standard (0.05 M dihydroxy benzyl amine; Sigma Chemical Co., St. Louis, MO) was injected into the HPLC system. The concentrations of NE, DA, the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC), and the serotonin metabolite 5-hydroxyindole acetic acid (5-HIAA) were measured as described previously (MohanKumar et al. 1994, 1998). Briefly, the HPLC-EC apparatus consisted of an LC-10 AT/VP pump (Shimadzu, Columbia, MD), a phase II 5- μm ODS reverse-phase C_{18} column (Phenomenex, Torrance, CA), a glassy carbon electrode (Bioanalytical Systems, West Lafayette, IN), a model CTO-10 AT/VP column oven (Shimadzu, Columbia, MD) maintained at 37°C , and an LC-4C amperometric detector (Bioanalytical Systems, West Lafayette, IN). The data were integrated using a computer with the Class-VP chromatography Laboratory Automated Software system (version 4.2; Shimadzu).

The mobile phase was made with pyrogen-free water and contained monochloroacetic acid (14.14 g/L), sodium hydroxide (4.675 g/L), octanesulfonic acid disodium salt (0.3 g/L), EDTA (0.25 g/L), and acetonitrile (3.5%), to which tetrahydrofuran (1.4%) was added. The mobile phase was then filtered and degassed through a Milli-Q purification system (Millipore Co., Bedford, MA) and pumped at a flow rate of 1.8 mL/min. The range of the detector was 1 nA full scale, and the potential of the working electrode was 0.65 V. The sensitivity of the system was < 1 pg.

Protein determination. Protein concentrations in the homogenates were determined using the micro-bicinchoninic acid assay (micro BCA; Pierce, Rockford, IL) and neurotransmitter concentrations were expressed as picograms per microgram protein.

Radioimmunoassay. Double-antibody radioimmunoassay was used to measure corticosterone levels in the serum as described previously (Francis et al. 2000). Corticosterone standards and the ^{125}I -labeled corticosterone were obtained from Diagnostic Products Inc. (Los Angeles, CA). The primary and secondary antibodies were raised in our laboratory and were used at dilutions of 1:17,500 and 1:11,000 respectively. The sensitivity of the corticosterone assay was 0.2 ng/mL.

Statistical analysis. The differences in serum corticosterone levels, neurotransmitters, and their metabolites among the various treatment groups were analyzed by one-way analysis of variance followed by post hoc Fisher's least significant difference test.

Results

Paraventricular nucleus. Exposure to CAPs alone or after sensitization with OVA produced significant changes in NE concentrations in the

PVN (Figure 1). Exposure to CAPs alone increased NE concentrations (mean \pm SE, pg/ μg protein) by more than 75% (12.45 \pm 2.7) compared with animals exposed to air + saline (7.98 \pm 1.3; $p < 0.05$). Pretreatment with OVA increased NE levels to 15.84 \pm 2.8. Exposure to CAPs after sensitization with OVA increased NE concentrations even further (19.06 \pm 3.8) and produced a greater than 2-fold increase in NE levels compared with the group treated with air + saline. A trend for a decrease in DA concentrations was observed after exposure to CAPs, although it was not statistically significant. There was no significant change in the concentrations of 5-HIAA and DOPAC after exposure to CAPs.

Medial preoptic area. In contrast to the PVN, CAP exposure resulted in an increase in the concentrations of DA in the MPA, whereas the concentrations of NE, 5-HIAA, and DOPAC remained unchanged (Figure 2). The concentration of DA in animals exposed to CAPs (0.68 \pm 0.2) or OVA alone (4.51 \pm 1.4) was not different from that of the controls treated with air + saline (0.93 \pm 0.1). However, sensitization with OVA before CAP exposure increased DA levels significantly (6.58 \pm 2.8) compared with those of the control and the CAPs + saline group ($p < 0.05$).

Arcuate nucleus. Unlike the PVN and the MPA, NE levels (mean \pm SE, pg/ μg protein) increased in the AN after OVA sensitization

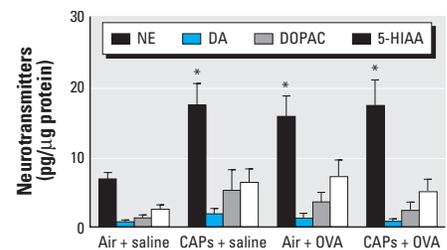


Figure 1. Neurotransmitter concentrations in the PVN after exposure to CAPs with or without pretreatment with OVA. Animals treated with either OVA or saline ($n = 8$ per group) were sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air. * $p < 0.05$ compared with the air + saline group (control).

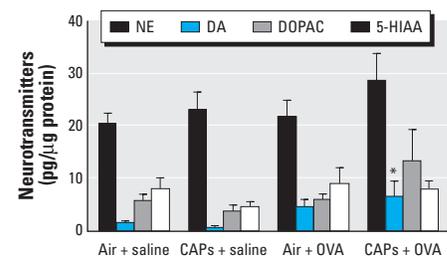


Figure 2. Neurotransmitter concentrations in the MPA after exposure to CAPs with or without pretreatment with OVA. Animals treated with either OVA or saline ($n = 8$ per group) were sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air. * $p < 0.05$ compared with control animals exposed to filtered air.

(31.68 ± 4.5) compared with the air + saline controls (14.75 ± 2.4; *p* < 0.05) but not after exposure to CAPs alone (19.77 ± 2.9). Although there was a trend for an increase in the concentrations of DA, 5-HIAA, and DOPAC after CAP exposure, these changes were not statistically significant (Figure 3).

Olfactory bulb. Exposure to CAPs with or without sensitization to OVA and OVA alone were all capable of increasing NE levels in the olfactory bulb (Figure 4). NE concentrations in control rats treated with air + saline were 8.8 ± 0.8 and increased significantly after exposure to CAPs (14.2 ± 0.8; *p* < 0.05). Sensitization with OVA kept NE levels elevated at 16.2 ± 1.8, and the combination of CAPs and OVA maintained it at the same level (16.1 ± 1.6). There were no significant changes in the other neurotransmitters after CAP exposure in the olfactory bulb.

Monoamines in other areas. There were no differences in the levels of monoamines in other areas of the hypothalamus: the SCN, median eminence, ventromedial hypothalamus, or dorsomedial hypothalamus. Similarly, the SN and the cortex did not show any significant differences in neurotransmitter concentrations (Table 1).

Serum corticosterone levels. Levels of corticosterone in the serum (mean ± SE, ng/mL; Figure 5) paralleled the levels of NE in the

PVN. Exposure to CAPs alone (114.55 ± 20.9) or after sensitization with OVA (242.786 ± 33.315) or exposure to OVA by itself (294.04 ± 26.228) produced significant increases in serum corticosterone compared with those in the control group exposed to filtered air (56.096 ± 10.2; *p* < 0.05). Moreover, exposure to CAPs after OVA sensitization, and presensitization with OVA increased serum corticosterone levels significantly compared with exposure to CAPs alone (*p* < 0.05).

Discussion

The results of the present study indicate for the first time that exposure to CAPs can affect the concentrations of neurotransmitters in specific areas of the brain. Changes in neurotransmitters were observed in the olfactory bulb and discrete areas of the hypothalamus, such as the PVN, AN, and MPA. Neurotransmitter concentrations in other areas of the hypothalamus and the brain remained unchanged, indicating that the effects of CAPs are highly specific. Exposure to CAPs also increased circulating levels of corticosterone, paralleling the increase in NE levels in the PVN, indicating an activation of the hypothalamo-pituitary-adrenal axis or stress axis. Challenge with OVA alone produced a marked increase in stress axis activity that indicated this may be a homeostatic response to suppress inflammation. However, when OVA-sensitized animals were exposed to CAPs, stress axis activity remained elevated but no additive effect was observed. This could indicate that both OVA and CAPs act through common pathways to stimulate the stress axis.

In the present study, exposure to CAPs or OVA sensitization did not produce any change in the whole hypothalamus (data not shown). However, marked changes were observed in specific nuclei of the hypothalamus that regulate various neuroendocrine functions such as feeding, reproduction, stress, and circadian activity. Among these, the activation of the stress axis that was observed in this study is the most striking. This response could be potentially mediated through the hypothalamus. The PVN of the hypothalamus accounts for the highest concentration of corticotrophin-releasing hormone (CRH) perikarya and can effect adrenocorticotrophic hormone (ACTH) release from the pituitary upon stimulation (Szafarczyk et al. 1987). A number of neurotransmitters are known to influence CRH secretion (Muller and Nistico 1988). Among these, NE is believed to play a prominent role. The PVN receives rich noradrenergic innervation from the brainstem, and neurotoxic blockade of its noradrenergic input decreases ACTH secretion (Szafarczyk et al. 1987, 1988). Moreover, direct administration of NE into the PVN also results in an activation of the stress axis (Itoi et al. 1994). On the contrary, noradrenergic antagonists have blocked stress axis activation, emphasizing the importance of this neurotransmitter in stimulating CRH neurons (Muller and Nistico 1988). In this study, exposure to CAPs alone resulted in a robust increase in NE levels in the PVN. A similar response was observed in OVA-sensitized animals, indicating that OVA by itself can activate the stress axis. This effect was accompanied by elevated corticosterone levels in animals

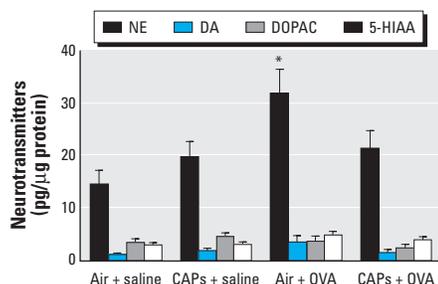


Figure 3. Neurotransmitter concentrations in the AN after exposure to CAPs with or without pretreatment with OVA. Animals treated with either OVA or saline (*n* = 8 per group) were sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air. **p* < 0.05 compared with the air + saline group (control).

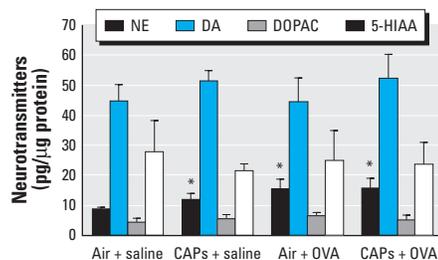


Figure 4. Neurotransmitter concentrations in the olfactory bulb after exposure to CAPs with or without pretreatment with OVA. Animals treated with either OVA or saline (*n* = 8 per group) were sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air. **p* < 0.05 compared with respective controls.

Table 1. Neurotransmitter concentrations (mean ± SE, pg/ug protein) in discrete brain nuclei after exposure to CAPs.

Area	Treatment	NE	DA	DOPAC	5-HIAA
Median eminence	Air + saline	36.04 ± 8.1	44.79 ± 13.8	17.49 ± 5.0	3.75 ± 0.5
	CAPs + saline	34.43 ± 6.4	63.09 ± 27.6	18.76 ± 6.1	3.05 ± 0.7
	Air + OVA	35.40 ± 6.9	23.76 ± 2.4	6.08 ± 2.2	5.41 ± 0.8
	CAPs + OVA	24.23 ± 5.5	60.99 ± 16.5	4.15 ± 1.1	3.82 ± 0.9
SCN	Air + saline	12.42 ± 2.2	1.0 ± 0.3	3.1 ± 0.8	3.27 ± 0.5
	CAPs + saline	15.92 ± 3.1	1.2 ± 0.5	4.62 ± 0.6	5.82 ± 0.6
	Air + OVA	15.18 ± 1.2	1.3 ± 0.4	4.26 ± 0.7	6.04 ± 1.3
	CAPs + OVA	19.89 ± 3.5	2.5 ± 0.9	3.56 ± 0.7	4.29 ± 0.6
Ventromedial hypothalamus	Air + saline	11.86 ± 2.8	1.63 ± 0.8	2.11 ± 0.4	4.53 ± 0.6
	CAPs + saline	21.89 ± 2.3	1.64 ± 0.7	2.45 ± 0.4	4.83 ± 0.2
	Air + OVA	25.86 ± 4.5	1.02 ± 0.1	3.27 ± 0.6	4.80 ± 0.4
	CAPs + OVA	20.99 ± 3.0	2.02 ± 0.4	2.89 ± 0.3	5.19 ± 1.1
Dorsomedial hypothalamus	Air + saline	13.89 ± 2.4	1.35 ± 0.5	2.26 ± 0.6	5.32 ± 0.8
	CAPs + saline	13.38 ± 2.7	1.66 ± 0.7	2.16 ± 0.4	5.31 ± 0.4
	Air + OVA	13.12 ± 2.7	1.16 ± 0.2	2.39 ± 0.4	6.00 ± 0.5
	CAPs + OVA	15.47 ± 3.5	1.84 ± 0.4	2.03 ± 0.3	6.10 ± 0.6
SN	Air + saline	3.08 ± 0.5	2.24 ± 0.7	1.42 ± 0.1	5.02 ± 0.5
	CAPs + saline	4.99 ± 0.7	2.24 ± 1.0	2.30 ± 0.4	5.21 ± 0.8
	Air + OVA	5.15 ± 0.8	2.35 ± 0.3	2.70 ± 0.4	6.18 ± 0.5
	CAPs + OVA	4.71 ± 0.7	2.73 ± 0.7	2.47 ± 0.4	5.85 ± 0.5
Cortex	Air + saline	2.32 ± 0.3	0.27 ± 0.1	0.99 ± 0.1	3.02 ± 0.3
	CAPs + saline	1.86 ± 0.3	0.39 ± 0.2	1.36 ± 0.6	2.91 ± 0.4
	Air + OVA	2.06 ± 0.2	0.47 ± 0.3	1.60 ± 0.7	3.14 ± 0.3
	CAPs + OVA	1.89 ± 0.2	0.84 ± 0.4	2.23 ± 0.8	2.83 ± 0.2

Animals were treated with either OVA or saline (*n* = 8 per group) and sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air.

exposed to CAPs alone or a combination of CAPs and OVA treatment. Both NE levels in the PVN and serum corticosterone were high even in the OVA-sensitized animals upon exposure to filtered air. This observation is supported by another study in which sensitization with OVA alone activates the PVN and the central nucleus of the amygdala, another important nucleus in the stress circuitry (Costa-Pinto et al. 2005). These findings suggest that the stress axis is activated by immune challenge/allergic airway disease and that exposure to CAPs also contributes to this effect.

Our present observations indicate that NE levels are elevated in the AN as well. Noradrenergic innervation to the AN may also be involved in the stress axis. We have previously shown that NE levels in the AN are elevated after an immune stressor such as (MohanKumar et al. 1998). AN has also been implicated in autonomic functions such as IL-1 respiratory processing mediated by carotid body receptor (Banks and Harris 1988), suggesting that apart from the PVN, the AN also may be involved in stress-induced autonomic alterations.

Our previous studies involving chronic activation of the stress axis with a T_H1 cytokine such as IL- β for a period of 5 days indicate that NE levels in the PVN stabilize at the end of the treatment period and are not significantly different from the controls (MohanKumar et al. 2003). However, in the present study, although the animals were repeatedly exposed to OVA for 3 days, we still observed an increase in stress axis activity. This could be because OVA induces a predominantly eosinophilic response that produces a T_H2 -type immune response with increases in IL-4, IL-5, IL-10, and IL-13 (Erin et al. 2005; Kalomenidis et al. 2005). Unlike T_H1 cytokines that directly affect the stress axis, T_H2 cytokines, especially IL-4 and IL-13, are involved in the isotype switching from IgM to IgE, which is the antibody responsible for the generation of classical allergic reactions (Marshall 2004). However, in this study we observed an increase in stress axis activity with

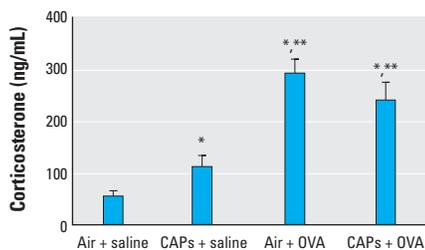


Figure 5. Serum corticosterone concentrations after exposure to CAPs with or without pretreatment with OVA. Animals treated with either OVA or saline ($n = 8$ per group) were sacrificed 24 hr after an 8-hr exposure to CAPs or filtered air.

* $p < 0.05$ compared with the air + saline group. *** $p < 0.05$ compared with the CAPs + saline group.

OVA alone. This may indicate a role for T_H2 cytokines in stress axis stimulation, a possibility that warrants further investigation. Therefore, the nature, duration, and intensity of a specific immune response and its effect on the stress axis may depend on a delicate balance between these two classes of cytokines (Marshall 2004).

The activation of the stress axis after CAP exposure or OVA sensitization could be a homeostatic mechanism to counter the inflammatory response elicited by these paradigms. One of the mechanisms by which homeostasis is achieved could be by facilitating the elimination of tissue eosinophils through the airway lumen (Uller et al. 2006). On the other hand, corticosterone could cause a shift in the T_H1/T_H2 response, increasing the production of IL-4 and IL-13 that could increase the secretions in airways promoting allergic airway diseases (Erin et al. 2005; Marshall 2004).

CAP exposure has produced a marked elevation in DA concentrations in the MPA. The present study was conducted using male rats, and in males, DA in the MPA is involved in sexual motivation and copulatory behavior (Bitran et al. 1988; Pehek et al. 1988). Moreover, the MPA receives chemosensory stimuli from the olfactory bulb (Murphy and Schneider 1970), and these inputs have been implied to be obligatory for the increase in DA in the MPA during copulation (Triemstra et al. 2005). The increase in DA observed in the MPA in this study could therefore be a result of olfactory stimulation.

The olfactory bulb has been implicated in the neuroendocrine control of various autonomic activities. Stimulation of the olfactory bulb by several methods, including smoke exposure, has resulted in altered cardiovascular and respiratory parameters and increased sympathetic activity (Nakamura and Hayashida 1992). The olfactory bulb has connections to noradrenergic neurons of the brainstem (Cassell and Roberts 1991; Perez et al. 1987), especially those located in the nucleus of the solitary tract (NTS; A2 cell group) and the locus coeruleus (A6 cell group). Besides innervating the PVN, the NTS is also involved in the critical regulation of respiration (Haxhiu et al. 2005). In the NTS, a down-regulation of GABAergic inhibitory influences may lead to heightened airway responsiveness and sustained narrowing of the airways (Haxhiu et al. 2005). Because GABA (γ -aminobutyric acid) and NE are known to have a reciprocal relationship in most central functions (Muller and Nistico 1988), it is possible that the observed increase in noradrenergic activity in the olfactory bulb is related to a reduction in GABA levels, and this could translate into an altered respiratory response with the NTS as a possible relay center. Alternatively, neurons of the olfactory tract extend to the amygdala, which

sends fibers to the lateral hypothalamus and the NTS besides other areas (Price 2003). This could be another potential pathway by which the olfactory lobe can affect respiration. However, further studies are needed to investigate the interconnection between the olfactory bulb and brainstem noradrenergic neurons in precipitating respiratory effects as a result of CAP exposure.

The mechanism by which CAP exposure leads to changes in neurotransmitters in the brain is unclear. The constituents and composition of the PM may be one important factor to be considered in understanding potential mechanisms. Nitric oxide, a component of $PM_{2.5}$, can influence neurotransmitter systems (Rettori et al. 1992). Ultrafine particles in CAPs can translocate from the nasal cavity to the olfactory bulb, most likely through olfactory nerves (Oberdorster et al. 2004), where they may incite a foreign-body reaction, activating glial cells to release reactive oxygen species (Block et al. 2004; Veronesi et al. 2005). In fact, exposure to CAPs is known to increase the levels of nuclear factor κB in the brains of dogs indicating early inflammatory changes (Calderon-Garciduenas et al. 2003). It has been shown previously that lipopolysaccharide (LPS) is a constituent of CAPs as well as OVA, which was used for sensitization (Campbell et al. 2005). The same study demonstrated an increase in inflammatory cytokines, IL-1 α , and tumor necrosis factor- α (TNF- α) in the brain upon exposure to CAPs. However the concentrations of LPS found were too low to stimulate IL-1 production, and the ability of LPS at these levels to stimulate IL-1 production remains questionable. LPS is shown to mediate neuroendocrine changes in the hypothalamus through IL-1 (MohanKumar et al. 1999) as well as TNF- α (Turnbull and Rivier 1998). IL-1 has been shown to produce elevations of NE in the PVN and AN (MohanKumar and Quadri 1993; MohanKumar et al. 1998) as well as elevation of ACTH and corticosterone levels (Lacosta et al. 1998). Besides increasing brain IL-1, inhaled CAPs may affect the pulmonary epithelium to cause inflammation and an increase in circulating cytokines. An increase in peripheral cytokines may also have a direct effect on brain NE levels (MohanKumar and Quadri 1993). It is possible that the activation of the stress axis by CAPs may be mediated at least in part through elevated cytokine levels.

Thus, the present study demonstrates for the first time that exposure to CAPs containing $PM_{2.5}$ produces changes in neurotransmitter levels in specific areas of the brain and modulates neuroendocrine pathways involved in the stress axis and the autonomic control of respiratory functions. This could be one of the potential modes by which CAPs could affect the neuroendocrine and autonomic systems.

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Therefore, inhibition of endogenous NOC formation may account for some of the observed inverse associations between vegetable intake and many cancers and adverse reproductive outcomes.

To adequately evaluate the risk associated with consumption of nitrate in drinking water at the regulatory limit of 10 mg/L nitrate-N [background levels are typically < 1 mg/L (Nolan and Hitt 2003)], studies must account for the potentially different effects of dietary and water sources of nitrate. Well-designed studies include the assessment of exposure for individuals (e.g., case-control, cohort studies) in a time frame relevant to disease development, and the evaluation of factors affecting nitrosation. Estimating NOC formation via nitrate ingestion requires information on diet and drinking water nitrate, inhibitors of nitrosation (e.g., vitamin C, polyphenols), nitrosation precursors (e.g., red meat, nitrosatable drugs), and medical conditions that may increase nitrosation (e.g., inflammatory bowel disease).

Only a few such studies evaluated risk among potentially susceptible groups (reviewed by Ward et al. 2005), and two

studies found significantly elevated risks associated with water nitrate levels below the regulatory limit (Brender et al. 2004; De Roos et al. 2003). Higher nitrate levels in drinking water were associated with an increased risk of colon cancer among individuals with high red meat or low vitamin C intakes (De Roos et al. 2003). Higher water nitrate ingestion was linked with neural tube defects in the offspring of women who used nitrosatable drugs during the peri-conceptual period (Brender et al. 2004).

We agree with L'hironde et al. that diarrhea, in addition to high water nitrate exposure, can cause methemoglobinemia in infants; in our article (Ward et al. 2005) we stressed the need for further studies to clarify the role of drinking water nitrate exposure. Nevertheless, it is important to note that the regulatory limit does not include a safety factor; rather, it is based on available data supporting no observed adverse effect for methemoglobinemia in infants (the most sensitive subpopulation) [U.S. Environmental Protection Agency (EPA) 1991]. Therefore, we do not agree that the regulatory limit is overprotective as suggested by L'hironde et al.

Until more well-designed studies are conducted and evaluated, we reject the conclusions by L'hironde et al. that enough evidence has been gathered to safely raise the drinking water limit for nitrate. Raising the regulatory limit, and thereby allowing the increased intake of drinking water nitrate, would likely result in increased exposure to endogenously formed potentially carcinogenic and neurotoxic *N*-nitroso compounds and possibly result in new cases of methemoglobinemia.

The authors declare they have no competing financial interests.

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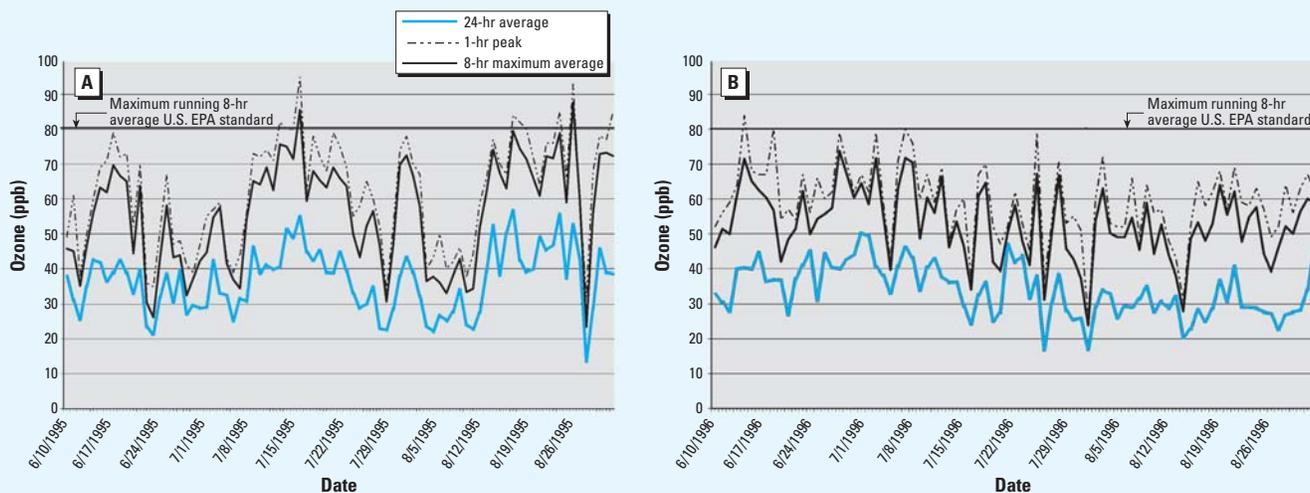
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ERRATA

In Figure 1 of the article by Triche et al. [Environ Health Perspect 114:911–916 (2006)], the 24-hr average and the 8-hr maximum average were labeled incorrectly. The corrected figure appears below:



On page 873 of the article by Sirivelu et al. [Environ Health Perspect 114:870–874 (2006)], two sentences were incorrect: “IL-1” was omitted from the first sentence and placed incorrectly in the second. The corrected sentences are as follows:

We have previously shown that NE levels in the AN are elevated after an immune stressor such as IL-1 (MohanKumar et al. 1998). AN has also been implicated in autonomic functions such as respiratory processing mediated by carotid body receptor (Banks and Harris 1988), suggesting that apart from the PVN, the AN also may be involved in stress-induced autonomic alterations.

EHP regrets the errors.