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Differential effects of inhalation exposure to $PM_{2.5}$ on hypothalamic monoamines and corticotrophin releasing hormone in lean and obese rats

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ABSTRACT

Acute exposure to airborne pollutants, especially particulate matter ($PM_{2.5}$) is known to increase hospital admissions for cardiovascular conditions, increase cardiovascular related mortality and predispose the elderly and obese individuals to cardiovascular conditions. The mechanisms by which $PM_{2.5}$ exposure affects the cardiovascular system is not clear. Since the autonomic system plays an important role in cardiovascular regulation, we hypothesized that $PM_{2.5}$ exposure most likely activates the paraventricular nucleus (PVN) of the hypothalamus to cause an increase in sympathetic nervous system and/or stress axis activity. We also hypothesized that these changes may be sustained in obese rats predisposing them to higher cardiovascular risk. To test this, adult male Brown Norway (BN) rats were subjected to one day or three days of inhalation exposures to filtered air (FA) or concentrated air particulate (CAP) derived from ambient $PM_{2.5}$. Corpulent JCR-LA rats were exposed to FA or CAP for four days. Animals were sacrificed 24 h after the last inhalation exposure. Their brains were removed, frozen and sectioned. The PVN and median eminence (ME) were microdissected. PVN was analyzed for norepinephrine (NE), dopamine (DA) and 5-hydroxy-indole acetic acid (5-HIAA) levels using HPLC-EC. ME was analyzed for corticotrophin releasing hormone (CRH) levels by ELISA. One day exposure to CAP increased NE levels in the PVN and CRH levels in the ME of BN rats. Repeated exposures to CAP did not affect NE levels in the PVN of BN rats, but increased NE levels in JCR/LA rats. A similar pattern was observed with 5-HIAA levels. DA levels on the other hand, were unaffected in both BN and JCR/LA strains. These data suggest that repeated exposures to $PM_{2.5}$ continue to stimulate the PVN in obese animals but not lean rats.

1. Introduction

Exposure to particulate matter (PM) is known to increase the risk for cardiovascular diseases worldwide (Anenberg et al., 2010). Exposure to fine PM such as $PM_{2.5}$ affects heart rate variability in experimental rodents (Kamal et al., 2011; Rohr et al., 2011), the elderly (Creason et al., 2001; Liao et al., 1999) and also in young healthy males (Magari et al., 2002) suggesting that it affects cardiac autonomic control. The mechanism by which $PM_{2.5}$ exposure affects cardiovascular function is unclear. There has been some evidence to suggest that inflammatory changes in the lungs that results in the release of chemical mediators could contribute to changes in the autonomic control of cardiac rhythm (Godleski et al., 2000). Besides affecting heart rate variability, $PM_{2.5}$ exposure is also known to increase blood pressure in normotensive healthy adults (Urch et al., 2005) and in patients with preexisting cardiovascular disease (CVD) (Zanobetti et al., 2004). These observations suggest that the association between $PM_{2.5}$ exposure and CVD risk may be due to decreased vagal or increased sympathetic tone (Magari et al., 2002).

One of the important central sites for sympathetic nervous system (SNS) regulation is the paraventricular nucleus (PVN) of the hypothalamus. The PVN has efferent connections to the intermediolateral cell column of the thoracic and lumbar spinal segments that in turn connects to various parts of the body through pre and post ganglionic neurons (Drake et al., 2005). The SNS is responsible for increasing heart rate and blood pressure during stressful situations or in response to external stimuli and is an essential part of the homeostatic mechanism (Brodal, 2004). Activation of the PVN could therefore be a mechanism by which PM exposure increases SNS activity to affect cardiovascular functions.

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Besides playing an important role in SNS regulation, the PVN is also a key player in regulating stress axis activity. The PVN receives rich noradrenergic, dopaminergic and serotonergic innervations from the hindbrain. These neurotransmitters are important for activation of corticotrophin releasing hormone (CRH) neurons (Muller and Nistico, 1989) that are located in the PVN that results in CRH release from terminals in the median eminence (ME). We have previously observed that acute exposure to $PM_{2.5}$ can increase NE levels in the PVN which could have implications in both SNS and stress axis activity (Sirivelu et al., 2006). However, it is not clear if $PM_{2.5}$ will continue to increase NE levels with repeated exposures of 3 days or more. Since the stress axis is capable of adapting to stressful stimuli (Ostrander et al., 2006), we hypothesized that repeated exposure to $PM_{2.5}$ would result in subdued changes in NE levels in the PVN. We wanted to verify downstream changes in stress axis activity by measuring CRH levels in the ME. Besides producing serious health effects in the elderly, harmful effects of $PM_{2.5}$ exposure are more pronounced in obese individuals (Dubowsky et al., 2006). This could be due to elevated stress axis or SNS activity. To study this, we used spontaneously obese JCR/LA-cp rats that are corpulent, have hyperlipidemia and are insulin resistant (Russell et al., 1989). We hypothesized that PM exposure would produce marked increases in NE levels in the PVN of these rats. To test these hypotheses, we exposed Brown Norway (BN) rats to $PM_{2.5}$ for 1 and 3 days and JCR/LA rats for 4 days. We measured NE and other monoamines in the PVN and CRH levels in the ME.

2. Materials and methods

2.1. Animals and treatment

Adult male BN rats were obtained from Charles River Laboratories (Portage, MI). Four-month-old JCR/LA rats were obtained from Charles River laboratories as well. A group of 8-month-old JCR/LA rats were kindly donated by Dr. J.C. Russell, University of Alberta. Animals were exposed to $PM_{2.5}$ as described earlier (Keeler et al., 2007; Sirivelu et al., 2006) using a mobile laboratory (AirCARE1) (Dvonch et al., 2004) located either at Calvin College in Grand Rapids, Michigan (BN rats), or at Maybury Elementary School in Detroit, Michigan (JCR rats). The inhalation lab contains a Harvard/U.S. Environmental Protection Agency ambient fine particle concentrator and 2 stainless steel Hinners-type whole body chambers capable of holding 16 rats. The chambers could hold a volume of 0.32 m^3 of air. $PM_{2.5}$ was generated using the EPA concentrator with air drawn from the local urban atmosphere. One of the chambers was used for $PM_{2.5}$ exposure, while the other was used for exposing animals to HEPA-filtered clean air at the same flow rate (control) (Keeler et al., 2007). In the first experiment, BN rats were exposed to concentrated air particulate (CAP) for 1 or 3 days in Grand Rapids, MI and sacrificed 24 h after exposure by pentobarbital administration. JCR/LA rats were exposed to CAP for 4 days in Detroit in another experiment. At the time of sacrifice, body weight was measured, blood was collected and the brains were removed, frozen and sectioned as described before (Sirivelu et al., 2006). All the protocols used in this experiment were approved by the Institutional Animal Care and Use Committee at Michigan State University.

2.2. Characteristics of $PM_{2.5}$

Continuous ambient $PM_{2.5}$ concentration data were collected using a tapered Element Oscillating Microbalance (TEOM) monitor (Rupprecht and Patashnick, Model 1400AB). Major components of organic and elemental carbon, sulfates, nitrates, ammonia and crustal/urban dust were determined as previously described (Keeler et al., 2007).

2.3. Brain microdissection

Serial sections (300 μm thickness) of the brains were obtained using a cryostat (Slee Mainz, London, UK) on clear glass slides. The sections were then placed on a cold stage (−10 °C) and the hypothalamic nuclei of interest, namely the PVN and ME, were microdissected using the Palkovit's microdissection technique with a 500 μm diameter punch. We used the Rat Brain Stereotaxic Atlas as a reference (Paxinos and Watson, 1987). The co-ordinates for microdissecting the PVN and ME were −1.8 to −2.12 mm and −2.12 to −3.3 mm posterior to the bregma, respectively.

2.4. Neurotransmitter analysis using HPLC

NE and other monoamines in the PVN were measured using a Shimadzu Prominence UFLC system (Shimadzu, Columbia, MD). Briefly, it consisted of a LC-20 AD Prominence Pump, a DGu-20A3 degasser, a SIL 20AC autosampler, and a CTO 20AC column oven maintained at 37 °C. We used a ODS reverse phase C-18 column (Phenomenex, Torrance, CA) for the separation of neurotransmitters. The mobile phase contained 14.5 g of chloroacetic acid, 0.3 g of octane sulfonic acid, 0.25 g of ultrapure EDTA, 4.675 g of sodium hydroxide, 17.5 ml of acetonitrile and 14 ml tetrahydrofuran per liter with pH adjusted to 3.1. The flow rate of the mobile phase was set at 1.8 ml/min. The PVN punches were homogenized in 60 μl 0.1 M perchloric acid and 5 μl of the homogenate was used for protein estimation using bicinchoninic acid assay (Pierce, Rockford, IL) as described earlier. The remaining homogenate was centrifuged at 13 000 rpm for 10 min and 15 μl of the supernatant was injected into the HPLC system using an autoinjector. 15 μl of dihydroxybenzylamine (0.05 M) was added to the supernatants as an internal standard. The chromatograms were analyzed using the Class VP software version 7.4 SP3 (Shimadzu, Columbia, MD). NE and other monoamine values were expressed as pg/μg of protein.

2.5. ELISA

CRH levels in the ME were measured using a competitive ELISA kit (Phoenix Pharmaceuticals, Burlingame, CA). Samples were assayed in duplicates as per the manufacturer's instructions. CRH levels were expressed as pg/μg of protein.

2.6. Statistical analysis

Differences in body weight between BN and JCR rats were compared using ANOVA followed by Fisher's LSD post hoc analysis. Differences in neurotransmitter levels and CRH levels between different exposures in BN rats were compared by ANOVA followed by Fisher's LSD test. Neurotransmitter values in 4-month-old and 8-month-old JCR/LA rats were pooled to get a $n = 8$ per group because there were no significant differences between them. Differences in neurotransmitter concentrations and CRH levels in JCR/LA rats were then compared by Student's t test.

3. Results

3.1. Body weight

Fig. 1 shows the effects of $PM_{2.5}$ exposure on body weight in BN and JCR/LA rats. Both acute and chronic $PM_{2.5}$ exposure did not affect body weights in BN rats. A similar effect was observed in JCR/LA rats exposed to 4 days of CAPs. JCR/LA rats were significantly

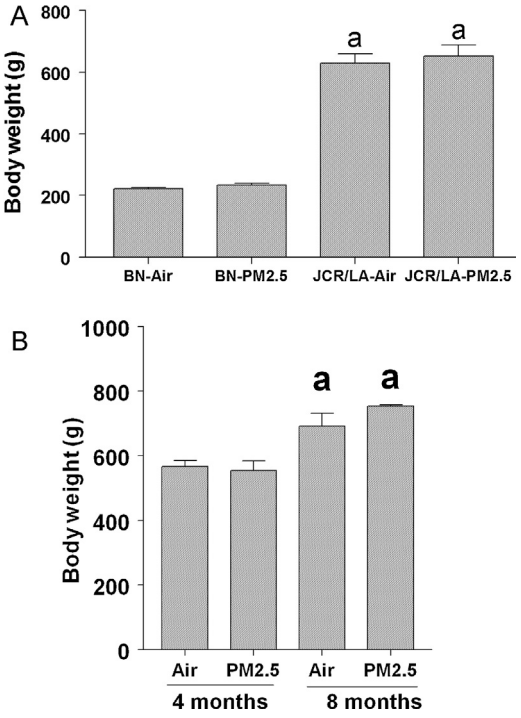

Fig. 1. Effect of $PM_{2.5}$ exposure on body weights in BN and JCR/LA rats. (A) Body weights in BN rats compared to JCR/LA rats. There were no treatment differences, but only strain differences. (B) Body weight differences between the 2 age groups of JCR/LA rats. Older rats were heavier than the younger rats. There were no treatment differences. 'a' indicates $p < 0.05$ compared to BN rats or younger JCR/LA rats.

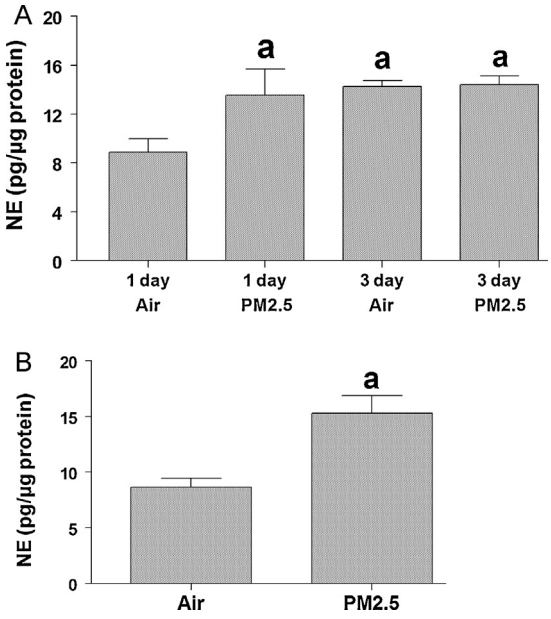

Fig. 2. Effect of $PM_{2.5}$ exposure on norepinephrine levels in the PVN. (A) NE levels in BN rats after single and multiple day $PM_{2.5}$ exposure. There were significant increases after 1 day exposure between treatments, but not after 3 day exposure. 'a' indicates significant difference from acute-air treated group, $p < 0.05$. (B) NE levels in the PVN of JCR/LA rats. There were significant differences between the treatment and control group, 'a' indicates $p < 0.05$.

heavier than BN rats (Fig. 1A) and 8-month-old JCR/LA rats were heavier than the 4-month-old rats (Fig. 1B).

3.2. Comparisons of CAP characteristics between Grand Rapids and Detroit

Table 1 describes differences in $PM_{2.5}$ mass and major components in CAPs collected in Detroit and Grand Rapids. Average mass concentrations of concentrated $PM_{2.5}$ ($\mu g/m^3$) in Detroit (291) were almost half of what was generated during exposures in Grand Rapids (519 and 595 for 1 and 3 day exposures, respectively). Major components in Detroit $PM_{2.5}$ were fairly consistent throughout the exposure period with little day-to-day variation in organic carbon (OC) and sulfates. By comparison, the last day of repeated exposures in Grand Rapids was marked by high sulfates and total mass. Also, organic carbon in $PM_{2.5}$ was consistently higher in Grand Rapids compared to Detroit, suggesting more input from traffic sources.

3.3. NE levels in the PVN

In BN rats, acute exposure to $PM_{2.5}$ produced a moderate but significant increase in NE levels (mean ± SE, pg/µg protein) in the PVN (13.5 ± 2.14) when compared to rats exposed to air (8.9 ± 1.1, $p < 0.05$). While NE levels after repeated exposure to air and $PM_{2.5}$ did not differ from each other (14.3 ± 0.5 and 14.4 ± 0.7 in air and $PM_{2.5}$ treated groups, respectively), they were significantly different from the group acutely exposed to air. In JCR/LA rats, repeated exposure to $PM_{2.5}$ exposure produced a significant increase in NE levels in the PVN (15.3 ± 1.6) compared to animals exposed to air (8.7 ± 0.8, $p < 0.05$) (Fig. 2A and B).

3.4. Other neurotransmitters in PVN

$PM_{2.5}$ exposure did not affect DA levels in BN and JCR/LA rats (Table 2). In contrast, 5-HIAA levels (mean ± SE; pg/µg protein) increased 2–3 fold with both acute (7.6 ± 2.5) and repeated

Table 1
Comparison of various components of CAPs in the different exposures in Grand Rapids and in Detroit. OC represents organic carbon and EC represents elemental carbon.

Day	Mass	OC	EC	Nitrate	Sulfate	Ammonium	Crustal/Urban dust
BN rats 1 day exposure – Grand Rapids							
Day 1	519	280	7	23	58	51	36
BN rats 3 day exposure – Grand Rapids							
Day 1	388	140	3	4	106	50	14
Day 2	492	222	3	13	59	30	15
Day 3	904	198	8	35	417	81	20
Avg	595	187	4	18	194	54	16
JCR rats 4 day exposure – Detroit							
Day 1	289	128	7	20	59	28	29
Day 2	448	150	16	31	63	20	62
Day 3	251	121	7	36	64	31	35
Day 4	176	92	12	6	21	9	27
Avg	291	123	10	23	52	22	38

Table 2
Neurotransmitter levels in the PVN of $PM_{2.5}$ exposed BN and JCR/LA rats.

BN rats	Neurotransmitters	1 day exposure		3 day exposure	
		Air	$PM_{2.5}$	Air	$PM_{2.5}$
	Dopamine (pg/μg)	1.8 ± 1.2	2.0 ± 0.7	0.6 ± 0.1	0.6 ± 0.1
	5-HIAA (pg/μg)	2.6 ± 0.4	7.6 ± 2.5*	6.8 ± 0.9*	7.4 ± 0.2*

JCR/LA rats	Neurotransmitters	4 day exposure	
		Air	$PM_{2.5}$
	Dopamine (pg/μg)	0.5 ± 0.1	0.7 ± 0.1
	5-HIAA (pg/μg)	7.5 ± 1.1	12 ± 1.6*

Neurotransmitter levels in the PVN were measured by HPLC-EC after 1 day and 3 or 4 day $PM_{2.5}$ exposure in BN and JCR rats. There were no significant changes in DA levels, but 5-HIAA, a metabolite of serotonin increased significantly after repeated $PM_{2.5}$ exposures in both BN and JCR/LA rats.
* indicates $p < 0.05$.

(7.4 ± 0.2) $PM_{2.5}$ exposure compared to air exposure (2.6 ± 0.4) in BN rats. A similar increase in 5-HIAA levels was observed in JCR/LA rats exposed to $PM_{2.5}$ (12 ± 1.6) when compared to animals exposed to air (7.5 ± 1.1) ($p < 0.01$).

3.5. CRH levels in the ME

While acute $PM_{2.5}$ exposure produced a 3-fold increase in CRH levels in BN rats compared to air-treated rats, CRH levels after repeated exposure to $PM_{2.5}$ exposure were not significantly different compared to air-treated rats. CRH levels after both acute and repeated $PM_{2.5}$ exposure were comparable and were significantly different only from acute air exposed group in BN rats (Fig. 3A). In JCR/LA rats, $PM_{2.5}$ exposure produced a marked increase in CRH levels compared to animals exposed to air (Fig. 3B).

4. Discussion

Results from this study indicate that exposure to $PM_{2.5}$ produces a significant increase in NE and 5-HIAA levels in both BN and JCR/LA rats. However, this increase was apparent only after acute exposure in BN rats and was also noted after repeated exposure in JCR/LA rats suggesting that JCR/LA rats have sustained increases in neurotransmitter levels in the PVN. This may translate into chronic increases in sympathetic tone in JCR/LA but not BN rats. In BN rats, stress axis activation was observed after acute exposure as measured by increase in CRH levels in the ME. This response was lost after repeated exposure in these rats. There was a similar pattern in JCR/LA rats after repeated exposure. Taken together, these data suggest that there is an adaptive response in the stress axis after repetitive $PM_{2.5}$ exposures. However, this adaptation does not extend to noradrenergic activity in the PVN in JCR/LA rats suggesting that obesity may contribute to prolonged activation of the SNS after repeated CAPs exposure in these obese animals.

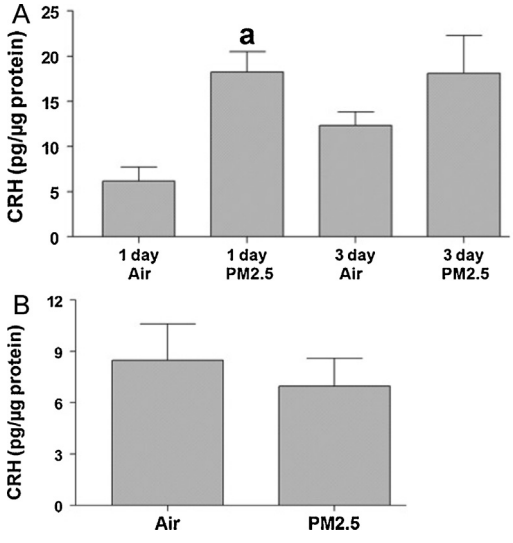

Fig. 3. Effect of multiple day $PM_{2.5}$ exposure on CRH levels in the ME. (A) CRH levels in BN rats after 1 and 3 day $PM_{2.5}$ exposure. There were significant changes in CRH levels only after $PM_{2.5}$ exposure for 1 day. 'a' indicates significant difference from acute-air exposed group; $p < 0.05$. No differences were observed in CRH levels after repeated exposures. (B) CRH levels in the ME of JCR/LA rats. There were no treatment effects on CRH levels in these rats.

The PVN receives rich noradrenergic innervations from A1 and A2 brainstem NE neurons (Sawchenko and Swanson, 1983a). It has efferent connections to the brainstem and the intermediolateral cell column (IML) of the spinal cord (Swanson and Kuypers, 1980) and has reciprocal connections with brain areas that are involved in cardiovascular functions (Sawchenko and Swanson, 1983b; Swanson and Kuypers, 1980). Increases in NE levels in the PVN contribute to sympathoexcitation in rats (Zhang and Felder, 2008). A number of studies have shown that exposure to $PM_{2.5}$ is strongly associated with cardiovascular mortality (Anenberg et al., 2010) and is thought to promote atherosclerosis (Kunzli et al., 2010) and cause vascular stiffening (Lenters et al., 2010) leading to increase in blood pressure. Small increases in heart rate and endothelial dysfunction after PM exposure are believed to reflect PM-induced autonomic imbalance (Brook et al., 2011). PM-induced effects on autonomic functions are reflected as changes in heart rate variability (Creason et al., 2001), arrhythmias (Liao et al., 2011), premature ventricular contractions (He et al., 2011) and elevated blood pressure (Urch et al., 2005). The increases in blood pressure were apparent within a short period of few hours (Urch et al., 2005). These observations suggest that the SNS may be involved in this phenomenon.

Since the PVN is capable of activating the SNS in response to environmental and physiological influences, it was logical to measure NE levels in the PVN both after single and repetitive exposures to $PM_{2.5}$ in BN rats. The increase in NE levels that were observed after acute exposure agrees with our previously published study (Sirivelu et al., 2006). The interesting observation in the present study was that this difference between $PM_{2.5}$ exposure and the air-treated group was not apparent after chronic exposure. Since NE levels in this group was comparable to those observed after acute $PM_{2.5}$ exposure, and did not increase further compared to the control group, it is possible that there is an adaptation response to $PM_{2.5}$ exposure. The stress axis is capable of adapting to repetitive stressful episodes (MohanKumar et al., 2003). Therefore, it is likely that the lack of further increases in NE levels after multiple exposures to $PM_{2.5}$ is part of an adaptive response. In contrast to BN rats, there was almost a 2-fold increase in NE levels after $PM_{2.5}$ exposure compared to air exposure in JCR/LA rats. The reason for increase in NE levels after $PM_{2.5}$ exposure is not clear. It is very likely that PM exposure can incite an

inflammatory process leading to an oxidative stress response within the lungs (Brook and Rajagopalan, 2010). The resultant release of intermediary molecules such as free radicals (Factor, 2011) and reactive oxygen species can affect central sites specifically the rostroventrolateral medulla (RVLM) to influence blood pressure and cardiovascular functions, since scavenging of superoxide in the RVLM can prevent increase in blood pressure in response to peripheral chemoreflex stimulation with potassium cyanide (Nunes et al., 2010).

CRH is also known to play an important role in cardiovascular and autonomic responses (Brown and Fisher, 1985). CRH-induced cardiovascular excitation is thought to be mediated through sympathetic and vagal efferents (Fisher, 1993). Although most CRH neurons located in the PVN are involved in ACTH secretion and stress axis activity (Fisher, 1993), some CRH neurons project to the brainstem especially the RVLM (Milner et al., 1993) that and may be important for cardiovascular regulation. Some CRH neurons also project to the intermediolateral cell column of the spinal cord (Sawchenko, 1987) and these CRH neurons are considered to be preautonomic. Under physiological conditions, very few CRH type 1 receptors that mediate SNS activity are detectable. However they undergo rapid up regulation in the event of stress or increased CRH secretion (Luo et al., 1994; Rivest et al., 1995). In BN rats, CRH levels increased after acute exposure to $PM_{2.5}$. In contrast, CRH levels after repeated $PM_{2.5}$ exposure were not significantly different from the air exposed group. In JCR/LA rats, we observed a similar trend in CRH levels after repetitive $PM_{2.5}$ exposure.

This could be an adaptive response to $PM_{2.5}$ exposure as well. This can also be linked to the obese phenotype of these animals. We have previously observed that NE levels do increase in the PVN without producing any change in CRH levels in the ME of obese rats when they are placed on a high fat diet for long periods of time (Shin et al., 2010). The lack of a CRH response in the face of elevated NE levels has been attributed to a reduction in $\alpha 2$ adrenergic receptor levels in the PVN in obese rats (Levin, 1996). A similar phenomenon may be in operation in JCR/LA rats after multiple, repetitive exposure to $PM_{2.5}$ and needs further investigation. The increase in NE levels in obese animals after repetitive $PM_{2.5}$ exposures could also be due to the persistent increase in serum lipids in these animals. Elevations in free fatty acids can stimulate NE release in the hypothalamus (our unpublished observation). Since JCR/LA rats have a hyperlipidemic background, the combination of $PM_{2.5}$ exposure-induced stress with higher serum lipid levels could be a cause for the persistence in NE elevations in these animals. The lack of increase in CRH but elevated NE levels in the PVN could favor an increase in SNS activity in these animals predisposing these rats to unexpected cardiovascular outcomes. This difference in responsiveness to $PM_{2.5}$ exposure could place obese individuals at higher risk for autonomic dysregulation.

Besides affecting SNS activity, the PVN is also the central site for integrating stress responses. Emotional, immunological, and physical stress result in an increase in glucocorticoid secretion. This is brought about by a cascade of events starting with neurotransmitter stimulation of CRH neurons in the PVN, that releases CRH from the ME. CRH enters the portal circulation to stimulate adrenocorticotropin (ACTH) secretion from the pituitary. ACTH acts directly on the adrenal gland to stimulate glucocorticoid secretion (Muller and Nistico, 1989). Since CRH levels were suppressed after repeated exposures to $PM_{2.5}$ in both BN and JCR/LA rats, it is possible to conclude that the animals generate an adaptive response to the stress of $PM_{2.5}$ exposure.

The differences in stress axis responses could also be due to variations in the composition of $PM_{2.5}$ between the two sites. Comparison of $PM_{2.5}$ from Detroit and Grand Rapids showed modest differences in both total mass and major constituents. While both exposure sites are located in urban Midwestern cities near major roadways, higher organic carbon in $PM_{2.5}$ from Grand Rapids suggests that this site had greater impact from traffic sources on days of exposure. Furthermore one of the days during exposures in Grand Rapids was marked by a high sulfate content of ambient $PM_{2.5}$. How these qualitative differences in CAPs might affect the responses in brain centers that we observed is open to speculation. Since our previous report in BN rats (Sirivelu et al., 2006), there has been little new information with regard to stress-axis activation in response to airborne particulate matter. Using similar systems, we and others have reported $PM_{2.5}$-related changes in heart rate variability that is related to metal content(Chen et al., 2010; Kamal et al., 2011; Rohr et al., 2011). We did not analyze metals in the present study and as such their contribution to the stress responses we describe are unknown. It is plausible that, in addition to underlying metabolic differences in JCR/LA versus BN rats, exaggerated stress responses to $PM_{2.5}$ are related in part to specific components. Further studies are needed to confirm the neuroendocrine responses to various components of $PM_{2.5}$, and elucidate the underlying processes of the adaptive stress response after repeated exposures.

Conflict of interest statement

The authors declare that there is no conflict of interest.

Acknowledgments

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