Alpha2B-Adrenergic Receptor Overexpression in the Brain Potentiate Air Pollution-induced Behavior and Blood Pressure Changes

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ABSTRACT

Fine ambient particulate matter ($PM_{2.5}$) is able to induce sympathetic activation and inflammation in the brain. However, direct evidence demonstrating an essential role of sympathetic activation in $PM_{2.5}$-associated disease progression is lacking. We assess the contribution of $\alpha 2B$-adrenergic receptor (Adra2b) in air pollution-associated hypertension and behavioral changes in this study. Wild-type mice and Adra2b-transgenic mice overexpressing Adra2b in the brain (Adra2b[Tg]) were exposed to concentrated $PM_{2.5}$ or filtered air for 3 months via a versatile aerosol concentrator exposure system. Mice were fed with a high salt diet (4.0% NaCl) for 1 week at week 11 of exposure to induce blood pressure elevation. Intra-arterial blood pressure was monitored by radio-telemetry and behavior changes were assessed by open field, light-dark, and prepulse inhibition tests. $PM_{2.5}$ exposure increased Adra2b in the brain of wild-type mice. Adra2b overexpression enhanced the anxiety-like behavior and high salt diet-induced blood pressure elevation in response to air pollution but not filtered air exposure. Adra2b overexpression induced upregulation of inflammatory genes such as TLR2, TLR4, and IL-6 in the brain exposed to $PM_{2.5}$. In addition, there were increased frequencies of activated effector T cells and increased expression of oxidative stress-related genes, such as SOD1, NQO1, Nrf2, and Gclm in Adra2b[Tg] mice compared with wild-type mice. Our results provide new evidence of distinct behavioral changes consistent with anxiety and blood pressure elevation in response to high salt intake and air pollution exposure, highlighting the importance of centrally expressed Adra2b in the vulnerability to air pollution exposure.

Key words: adrenergic receptor; behavior; air pollution; particulate matter; blood pressure.

Noncommunicable diseases account for over 70% of air pollution deaths. Air pollution is responsible for 19% of all cardiovascular deaths worldwide and indeed has been increasingly implicated as an important, and as yet poorly characterized risk factor for neurodevelopmental disorders in children and neurodegenerative disorders in elderly humans (Kioumourtzoglou et al., 2016; Wu et al., 2015). An intermediate factor that may potentially help explain the association between air pollution and susceptibility to cardiovascular events is increase in blood pressure. Although dietary and environmental factors are widely recognized as facilitators of hypertension, the precise mechanisms continue to be studied and debated. Studies by our group have indicated that exposure to $PM_{2.5}$, a ubiquitous environmental risk, is akin to chronic stress and is associated with neuroinflammation and sympathetic activation (Liu et al., 2014). Chronic exposure to $PM_{2.5}$ elevated blood pressure in wild-type (WT) mice and humans, which are paralleled by increases in surrogate markers of sympathetic activation (Liu et al., 2014; Munzel et al., 2017). Conversely, central nervous system (CNS) inhibition of inhibitor kappa-B kinase (IKK)/nuclear factor-kappaB (NF-κB) in air pollution-exposed mice, reversed CNS inflammation and restored peripheral energy homeostasis and insulin sensitivity (Liu et al., 2014). In both rodents and humans, stress is known to engage fear and threat circuitry in the brain resulting in a stereotypical biological and behavioral response, typified by include elevation of blood pressure, anxiety, and depression (Munzel et al., 2017). Studies examining the neurocircuitry of stress in rodents indicate that multiple stress responsive areas are activated, including the prefrontal cortex, hypothalamus, amygdala, and the hippocampus. Activation of this circuitry results in sympathetic nervous system (SNS) activation which in turn results in peripheral immune alterations.

To investigate the role of the sympathetic system in air pollution "stress"-induced adverse health effects, we examined the blood pressure and behavior changes in WT mice and brain α2B-adrenergic receptor (Adra2b)-overexpressing mice (Kintsurashvili et al., 2009). Activation of α2A and α2C in the CNS inhibits the SNS and lowers blood pressure, whereas α2B activation in the CNS activates SNS and increases blood pressure (Kanagy, 2005). We hypothesized that air pollution will potentiate hypertension, induce adverse, behavioral changes, and enhance inflammation in peripheral organs by activating sympathetic adra2b signaling. Air pollution exposure is a life-long unavoidable risk factor to most people living in polluted areas. As such, identifying the genes/conditions that predispose the subjects to air pollution may help to develop treatments for air pollution-associated adverse health effect. FVB/N mice have been shown to be less vulnerable to exposure of air pollutants such as ozone (Savov et al., 2004). In this study, we used brain-specific Adra2b-overexpressing FVB/N mice (Adra2b^{Tg}) to examine the role of sympathetic activation in regulating air pollution sensitivity. We found that adra2b overexpression in the brain enhanced air pollution-induced anxiety-like behavior and high salt diet-induced blood pressure elevation. Adra2b overexpression induced an upregulation of inflammatory genes such as TLR2, TLR4, and IL-6 in the brain exposed to $PM_{2.5}$. In addition, there were increased frequencies of activated effector T cells and increased expression of oxidative stress-related genes, such as SOD1, Nrf2, and Gclm in Adra2b^{Tg} mice compared with WT mice.

METHODS

Animals and animal care. Adra2b transgenic (Adra2b^{Tg}) mice (Kintsurashvili et al., 2009) on FVB/N background were purchased from Jackson Laboratories (Bar Harbor, Maine) and bred in the animal facilities at the University of Maryland School of Medicine and Case Western Reserve University. Eight weeks old male Adra2b transgenic positive FVB/N mice and negative FVB/N (WT) were used in this study. Animals were maintained in the animal facility at 21°C on a 12-h light/12-h dark cycle with free access to water and food (normal chow diet). High salt intake is an important acute hypertensive stressor that induces blood pressure elevation in susceptible individuals (Tobian, 1991). Mice were randomly assigned to normal salt diet (0.75% NaCl) or high salt diet (4.0% NaCl, Teklad diet) treatment for 1 week after 11 weeks of exposure when the effects of air pollution have been established. Blood pressure was measured by radio-telemetry (Data Sciences International, St. Paul, Minnesota) as detailed below. The protocols and the use of animals were approved by and in accordance with the Institutional Animal Care and Use Committee at the University of Maryland School of Medicine and Case Western Reserve University.

Whole-body inhalational exposure to concentrated ambient fine particulate matter. Mice were exposed by inhalation to either filtered air (FA) or concentrated ambient $PM_{2.5}$ at University of Maryland, Baltimore for 6 h/day, 5 days/week, for a total of 12 weeks, in a Versatile Aerosol Concentrator and Enrichment System (VACES) (Rao et al., 2014). This whole-body small animal air pollution exposure system allows 8- to 10-fold concentration of ambient $PM_{2.5}$ particles. All mice, including both FA and $PM_{2.5}$ exposure groups, were exposed at exactly the same time. FA-exposed mice received an identical protocol with the addition of a high-efficiency particulate air filter (Pall Life Sciences, East Hills, New York) positioned in the inlet valve to remove $PM_{2.5}$ particles in the FA stream, as described previously (Liu et al., 2014). The monitoring of the exposure environment and ambient aerosol was described previously in our publications (Rao et al., 2014; Sun et al., 2009).

$PM_{2.5}$ concentration and composition in the exposure chamber. Teflon filters (PTFE, 37 mm, 2 μm pore; PALL Life Sciences, Ann Arbor, Michigan) used in the VACES were collected and weighed before and after sampling. The filters were weighted after temperature and humidity acclimation on a Mettler Toledo Excellence Plus XP microbalance in a temperature- and humidity-controlled weighing room. The filter membranes were changed every week and concentration of each membrane was measured by gravimetric analysis. Weight gains were used to calculate exposure mass concentrations of $PM_{2.5}$ in the exposure chambers. Elemental composition of the $PM_{2.5}$ particles was measured by high-resolution inductively coupled plasma-mass spectrometry (ICSP-MS) (ELEMENT2, Thermo Finnigan, San Jose, California), as previously described by us (Xu et al., 2011).

Open-field test. For open-field test, Adra2b^{Tg} mice and WT mice were placed in 50 cm × 50 cm × 38 cm arenas under 30–35 lux illumination with an overhead video-tracking system (San Diego Instruments) after 12 weeks of exposure to FA or $PM_{2.5}$. Locomotion of animals (distance traveled) was then continuously monitored by the experimenter throughout the 60-min habituation period. Travel distance and duration in the center were analyzed by TopScan v2.0 (CleverSys, Inc.).

Prepulse inhibition. The prepulse inhibition was performed, as previously described (Zanos et al., 2016). Following air pollution exposure for 12 weeks, the mice were placed in the startle chambers (SR-LAB, San Diego Instruments, San Diego, California) for a habituation period of 30 min. Each chamber consists of a Plexiglas

cylinder (8.9 cm length × 2.8 cm diameter on a 20.4 × 12.7 cm plastic base) located within a ventilated enclosure (internal dimensions: 35 × 38.6 × 32 cm). After the habituation period, the mice were exposed to a 5-min adaptation period with a constant background noise (67 dB), followed by 5 initial startle stimuli (120 dB, 40 ms duration each). Subsequently, the mice were stimulated with 5 different trial types: pulse alone trials (120 dB, 40 ms duration), 3 prepulse trials of 76, 81, and 86 dB of white noise bursts (20 ms duration) preceding a 120 dB pulse by 100 ms, and background (67 dB) no-stimuli trials. Each of these trials was randomly presented 5 times. Startle response was measured by recording the rapid force change of body reaction in the startle chambers using the SR-LAB software. The percentage prepulse inhibition (% PPI) was calculated as follows: [(magnitude on pulse alone trial − magnitude on prepulse + pulse trial)/magnitude on pulse alone trial] × 100.

Light-dark box test. For the light-dark box (LDB) test, the mice were placed in an arena (35 × 35 × 35 cm) that is divided into two by a partition with a door. The larger chamber (23 × 35 × 35 cm) was illuminated brightly (400 lux) and the adjacent smaller chamber (12 × 35 × 35 cm) was darker (<5 lux). The mice were allowed to move freely between the 2 chambers through the door connecting the 2 chambers. The mice were monitored and videotaped for 10 min after placing them into the dark chamber. The transition numbers through the door, the time stayed in the light side, and the latency to enter the light chamber were recorded and analyzed using Anno Star software (CleverSys Inc., Reston, Virginia).

Radiotelemetry measurement of mouse blood pressure. Mouse radiotelemetry (Data Sciences International) was used for blood pressure and heart rate measurements, as we previously described (Liu et al., 2014). Briefly, the right common carotid artery was cannulated with the catheter of the transmitter under anesthesia. The transmitter body was placed in a subcutaneous pocket in the flank. The surgery was carried out in a dedicated surgery area which was previously disinfected with UV light and 70% alcohol. After surgery, the mice were housed individually with access to food/water ad libitum and allowed to recover for 1 week. After 1 week of recovery, the blood pressure, heart rate, and physical activities data were collected and stored. Dataquest A.R.T. software (Data Sciences) was used to analyze the data.

α2B-adrenergic antagonist imiloxan (1 mg/kg) was injected intraperitoneally into FA- or PM2.5-exposed WT and Adra2b^{Tg} mice to block α2B signaling and evaluate if PM-induced blood pressure elevation in Adra2b^{Tg} mice is dependent on α2B. Continuous beat-by-beat blood pressure values were recorded for 30 min before and 2 h after administration of imiloxan. The values from the 45th to the 60th minutes after drug injection were used to characterize the response and to avoid measuring stress-induced blood pressure changes.

Urine collection and norepinephrine analysis. Urine samples were collected during the week before implantation of telemetry transmitters. The mice were placed into the urine collection cages immediately after daily exposures and the urine was then collected the next morning. The urine was stored at −80°C until analysis. The concentrations of norepinephrine (NE) and vanillylmandelic acid (VMA) in the urine were analyzed using ELISA kit (Abnova, Taipei City, Taiwan), according to the manufacturer's instructions.

Quantitative real time-PCR. Total RNA was extracted from whole-brain tissues using Trizol (Invitrogen, Carlsbad, California) and

cDNA was reversely transcribed using a High Capacity cDNA Transcription kit (Applied Biosystems, Carlsbad, California), according to the manufacturers' protocols. Quantitative real time polymerase chain reaction (qPCR) was performed in triplicate on a Lightcycler 480 system (Roche, Indianapolis, Indiana). The expression of target genes was calculated using the ΔΔCt method relative to housekeeping gene β-actin (Livak and Schmittgen, 2001).

Flow cytometry. The cells were stained with anti-CD3, anti-CD44, anti-CD62L, anti-Ly6C, and F4/80 at 4°C for 30 min, followed by 3 washes with 1× PBS and subsequently resuspended in flow buffer (1× PBS containing 2% FBS). The cells were then run on an Aminis Flowsight imaging flow cytometer (Millipore Sigma, Billerica, Massachusetts) and data were analyzed using Amnis IDEAS software (Millipore Sigma). All antibodies were purchased from Biolegend, eBioscience, or BD Bioscience.

Data analysis. Data are expressed as means ± standard error of the mean (SEM) unless otherwise specified. Unpaired 2-tailed *t*-tests were used for the comparison of adrenergic receptor expression or PM2.5 concentration between FA and PM2.5 using Graphpad Prism software (Version 7). Two-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was used to compare the means among WT (FA), WT (PM), Adra2b^{Tg} (FA), and Adra2b^{Tg} (PM) in behavior study, detections of inflammatory marker and oxidative stress, using Graphpad Prism software (Version 7). Repeated measure 2-way ANOVA with Bonferroni's post hoc test was used for the analysis of blood pressure. A *p* value of <.05 was considered statistically significant.

RESULTS

Characterization of Concentrated Air Pollution Exposures
First, we examined the gene and protein expression of Adrenergic receptors in the brain of FA or PM2.5 exposed mice (Figure 1). PM2.5 exposure increased the gene expression of Adra2b more than 2-fold in the brain of WT mice by real-time PCR (Figure 1B). By contrast, there were no significant differences in the expressions of α2A and α2C adrenergic receptors between FA and PM group (Figs. 1A and 1C). We also examined the gene expression of β adrenergic receptors in the brain tissues. After PM2.5 exposure, gene expression of β1 adrenergic receptor increased (Figure 1D), whereas β2 adrenergic receptor decreased in the brain tissue of WT mice (Figure 1E). There was no change in β3 adrenergic receptor level after PM2.5 exposure (Figure 1F). These results suggest that a2B, β1, and β2 adrenergic receptors may participate in central sympathetic remodeling in response to PM2.5 (Figs. 1B, 1D, and 1E). Because α2B is the major adrenergic receptor in the CNS to activate SNS and increase blood pressure(Kanagy, 2005), we further examined the protein level of Adra2b in the brain after PM2.5 exposure. Adra2b protein expression in the brain increased ~1.5-fold (representative bands in Figure 1G and bar graph in Figure 1H) in PM2.5 exposed mice compared with FA group.

To test the role of sympathetic activation in air pollution-associated adverse health effect, a mouse model with brain-selective overexpression of α2B-adrenergic receptor (Adra2b) (Kintsurashvili et al., 2009) was used. The Adra2b transgenic mice used a human platelet-derived growth factor-β promoter to target neurons in the cortex, hippocampus, hypothalamus, and cerebellum, and has shown a 1.8-fold increase of protein expression in the whole brain tissue compared with WT mice
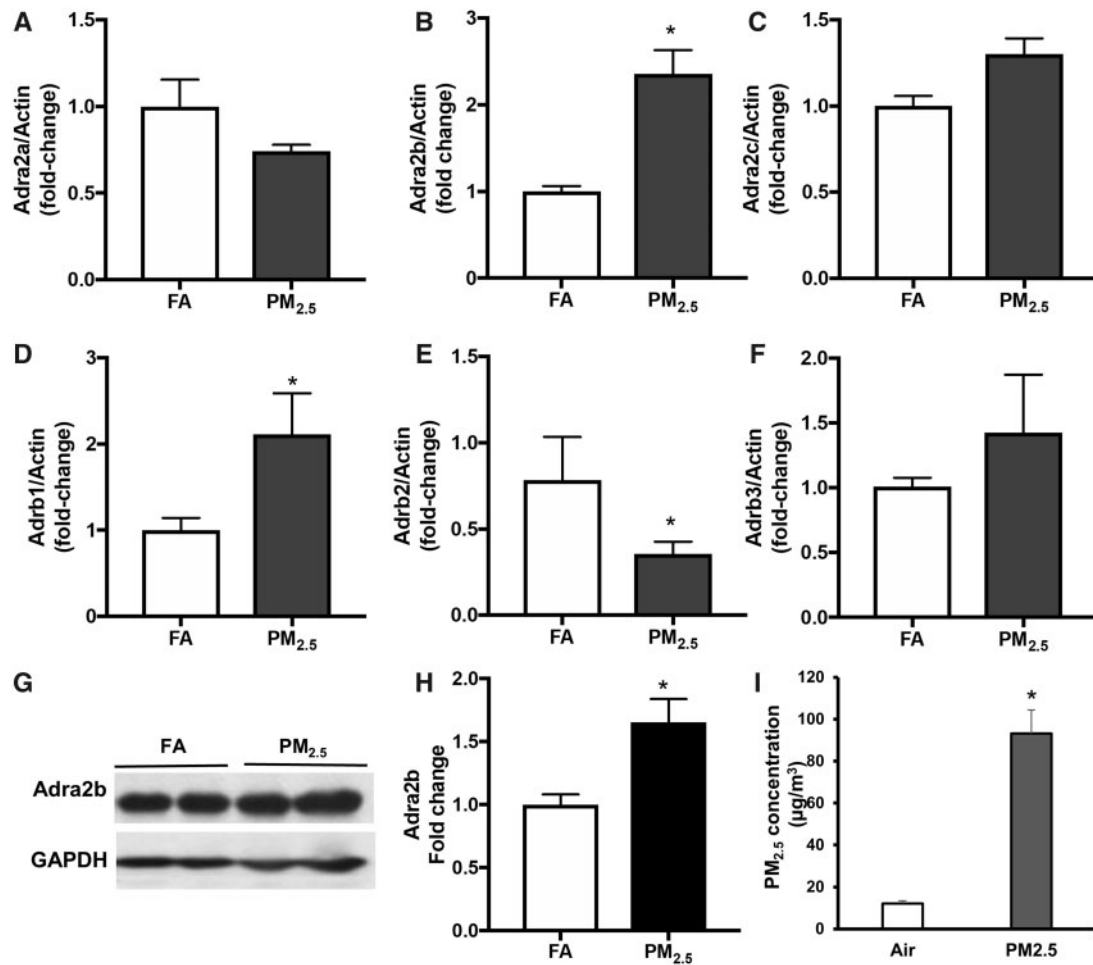
Figure 1. Effect of $PM_{2.5}$ exposure on the gene expression of adrenergic receptors: Adra2a (A), Adra2b (B), Adra2c (C) mRNA levels in the brain from FA- or $PM_{2.5}$-exposed WT mice. Adrb1 (D), Adrb2 (E), and Adrb3 (F) mRNA levels in the brain from FA- or $PM_{2.5}$-exposed WT mice. Representative image (G) and statistic bar graph (H) showing Adra2b protein levels in the brain from FA- or $PM_{2.5}$-exposed mice. I, $PM_{2.5}$ concentration in ambient air and PM2.5 exposure chamber. Data are presented as means ± standard error (SE). $N = 5$–7 for each group. $^*p < .05$.

(Kintsurashvili *et al.*, 2009). We had previously shown that administration of α2A-adrenergic agonist, guanfacine attenuated air pollution-induced increases in blood pressure (Liu *et al.*, 2014), and α2A in the CNS inhibits the sympathetic nervous activity. We postulated that if air pollution-mediated increase in blood pressure involves central sympathetic activation, then transgenic overexpression of α2B receptors in the brain will augment blood pressure responses to $PM_{2.5}$ exposure (Kanagy, 2005). Adra2b transgenic mice (Adra2b^{Tg}) and WT controls were exposed to FA or concentrated $PM_{2.5}$ (PM), 6 h/day, 5 days/week for a total of 12 weeks. The average concentrations of $PM_{2.5}$ (mean ± SD) in the exposure chamber and ambient air were 93.1 ± 37.1 and 12.2 ± 4.4 µg/m^3 respectively (Figure 1I). We have previously demonstrated that these levels of exposure represent relevant doses of exposure routinely experienced in many cities in Asia (Rajagopalan and Brook, 2015). The XRF elemental composition analysis of the particles collected from filter membranes provides detailed chemical component characterization of the particulate matter during the exposure period (Table 1).

Effects of Adra2b on the Sensitivity to Air Pollution-induced Blood Pressure Elevation

Implantable radio-telemetry devices allow continuous reliable measurement in freely moving animals while tail-cuff method

has restraint stress. Therefore, we examined the blood pressure change using state-of-the-art radiotelemetry. After 11 weeks of air pollution exposure, blood pressure slightly increased in Adra2b^{Tg} mice compared with WT mice under normal chow diet condition, although not statistically different (Figs. 2A and 2B). Heart rate was reduced in Adra2b^{Tg} mice exposed to $PM_{2.5}$ (Figure 2C). This may due to the baroreflex response to compensate for blood pressure change. Because FVB/N mice are more resistant to blood pressure changes (Hein *et al.*, 1995) and we did not observe a difference in blood pressure under normal chow diet condition, we then fed all the mice with 4% high salt diet with concomitant exposure to corresponding FA or $PM_{2.5}$ for 1 week (week 12). High salt intake is an acute risk factor for hypertension and can synergistically interact with other risk factors to increase blood pressure (Chen *et al.*, 2018). It is able to desensitize baroreflexes in salt-sensitive animals (Andresen, 1989; Victor *et al.*, 1986). Therefore, we used high salt diet as a stressor to examine if the combination of high salt diet and $PM_{2.5}$ could induce blood pressure elevation. Both systolic (Figure 2D) and diastolic (Figure 2E) blood pressures were increased in the Adra2b^{Tg} mice exposed to $PM_{2.5}$ and high salt diet, compared with WT mice or FA-exposed Adra2b^{Tg} mice on high salt diet. No significant difference in heart rate was seen among the 4 groups after combined treatment of high salt diet and FA/$PM_{2.5}$ (Figure 2F).

Table 1. Elemental Concentrations (ng/m^3) of Ambient Air or Concentrated Particles During the Exposure

	Ambient Air		Exposure to PM$_{2.5}$	
	Mean	SD	Mean	SD
S	512.7	154.9	4658.8	1655.3
Na	149.9	27.1	990.0	227.4
Fe	79.3	25.8	932.3	404.0
Si	31.6	45.8	755.6	745.1
Ca	56.8	21.4	683.0	220.8
Mg	36.7	5.6	280.6	88.6
K	25.2	8.4	245.5	92.6
Al	8.9	16.4	220.7	339.3
Zn	25.8	11.0	173.2	78.9
U	12.0	18.6	110.1	150.2
Br	10.0	3.4	84.6	27.2
Ba	5.0	3.7	62.3	32.7
Ti	5.3	2.4	53.1	31.1
Cu	7.8	16.2	42.5	13.5
Lu	4.0	3.7	30.7	13.1
Mn	2.5	1.1	27.2	16.8
P	1.6	1.0	26.2	12.9
Er	2.0	0.9	22.7	10.4
Te	4.0	7.1	21.2	30.0
Sb	3.0	4.4	18.5	22.9
Ni	2.9	7.5	15.6	11.2
Sr	2.0	0.2	15.4	2.2
Pb	2.0	0.9	13.8	6.6
W	2.0	1.1	13.3	5.4
Sn	3.0	3.4	9.8	10.8
Cr	0.7	0.4	8.5	2.6
V	0.7	1.5	6.6	18.7
In	1.0	2.0	6.3	7.7
As	1.0	0.4	5.0	3.8

Adra2b Enhances Anxiety in Open-field Test

We next examined the behavior changes in Adra2b^{Tg} and WT mice exposed to air pollution. As shown in Figure 3A, there were no significant differences in distance traveled between FA- and PM$_{2.5}$-exposed animals of either WT or Adra2b^{Tg} genotype under normal chow diet condition. However, overexpression of Adra2b in the brain reduced the distance traveled in the center and the duration in the center in both FA- and PM$_{2.5}$-exposed Adra2b^{Tg} mice, compared with the WT mice (Figs. 3B and 3C), suggesting that Adra2b^{Tg} had an anxiety like behavior regardless the exposure of FA or PM$_{2.5}$. We also observed a greater increase in heart rate (Figure 3D), but not blood pressure (Supplementary Figure 1), in Adra2b^{Tg} mice exposed to PM$_{2.5}$ compared with FA, in open field, measured by the mouse radiotelemetry.

Effects of Adra2b on the Vulnerability to Air Pollution-induced Behavior Changes in Light-dark Box and Prepulse Inhibition Tests

To further examine anxiety-related behavior changes after air pollution exposure, exposed mice were further subjected to the LDB and prepulse inhibition tests. Exposure to PM$_{2.5}$ significantly reduced time spent in light area in Adra2b^{Tg} mice but not WT mice (Figure 3E) consistent with anxiogenic effects, although bouts in the light area were not affected (Figure 3F). These results suggest that air pollution may enhance anxiety in susceptible individuals and Adra2b signaling is an important risk factor for air pollution-induced anxiety change. PM$_{2.5}$ exposure reduced percentage of prepulse inhibition response

following a 76 dB prepulse stimulation in Adra2b^{Tg} mice but not in WT mice. Similar trends were observed in 81 or 86 dB prepulse stimulation (Figure 3G). There was no significant difference in startle response among the 4 groups, although startle responses in the WT PM, Adra2b^{Tg} FA, and Adra2b^{Tg} PM groups were all slightly reduced compared with that in the WT FA group (Figure 3H).

Adra2b Activation in Air Pollution Exposure

To confirm a pathophysiologic role of Adra2b in PM$_{2.5}$-induced blood pressure elevation, blood pressure was monitored on FA/PM$_{2.5}$-exposed WT and Adra2b^{Tg} mice on high salt diet after the intraperitoneal injection of the Adra2b-specific antagonist imiloxan. The blood pressure reductions were greater in PM$_{2.5}$-exposed mice in both WT and Adra2b^{Tg} groups (Figure 4A), suggesting that Adra2b activation is involved in PM$_{2.5}$-induced blood pressure elevation. The imiloxan-induced heart rate decrease was similar between WT FA and WT PM$_{2.5}$, although PM$_{2.5}$-exposed Adra2b^{Tg} mice had a smaller reduction of heart rate (Supplementary Figure 2). In addition, urine levels of NE and VMA, a metabolite of NE and epinephrine, were significantly increased in PM$_{2.5}$-exposed mice in both WT and Adra2b^{Tg} groups (Figs. 4B and 4C).

Effects of Adra2b Activation and Air Pollution Exposure on Inflammation

Inflammation has been confirmed as an important mechanism underlying air pollution-induced adverse health effects. Indeed, activation of the SNS has been shown to profoundly influence peripheral immune function (Chavan et al., 2017). To examine the involvement of Adra2b in PM$_{2.5}$-induced inflammation, we detected the mRNA expression of inflammatory markers in the brain of PM$_{2.5}$-exposed mice fed on a normal chow diet. As depicted in Figure 5, inflammation-associated genes, nitric oxide synthase 2 (NOS2), interferon gamma (IFNγ), and interleukin-12 (IL-12) increased in PM$_{2.5}$-exposed WT mice. Overexpression of Adra2b also enhanced the gene expressions of IFNγ and IL-12, although PM$_{2.5}$ exposure did not further enhance their expression in Adra2b^{Tg} mice (Figs. 5A–C). In addition, air pollution exposure increased the mRNA expression of Toll-like receptor 4 (TLR4), Toll-like receptor 2 (TLR2), and interleukin-6 (IL-6) in the brain of Adra2b^{Tg} but not WT mice, indicating that Adra2b overexpression enhanced the sensitivity to air pollution-induced inflammation (Figs. 5D–F). No significant changes in anti-inflammatory genes interleukin-4 (IL-4) and interleukin-10 (IL-10) were detected after PM$_{2.5}$ exposure in both WT and Adra2b^{Tg} mice (Figs. 5G and 5H). Consistent with these results, flow cytometry also showed that the frequency of activated effector T cells (CD3$^+$CD44$^-$CD62L$^-$), but not activated memory T cells (CD3$^+$CD44$^+$CD62L$^-$), increased in both lung and spleen from PM$_{2.5}$-exposed WT mice (Figs. 6A–D). No significant change in the amount of inflammatory macrophage (Ly6C$^+$F4/80$^+$) after PM$_{2.5}$ exposure was observed in either lung or spleen from WT and Adra2b^{Tg} mice, although Ly6C$^+$F4/80$^+$ inflammatory macrophage increased in the spleen of Adra2b^{Tg} mice compared with WT mice (Figs. 6E and 6F).

Effects of Adra2b Activation and Air Pollution Exposure on CNS Oxidative Stress

We and others have shown previously that air pollution exposure is associated with enhanced oxidative stress (Rao et al., 2018; Sorensen et al., 2003; Xu et al., 2011). To investigate if Adra2b activation is associated with oxidative stress following air pollution exposure, we examined the expression of oxidative
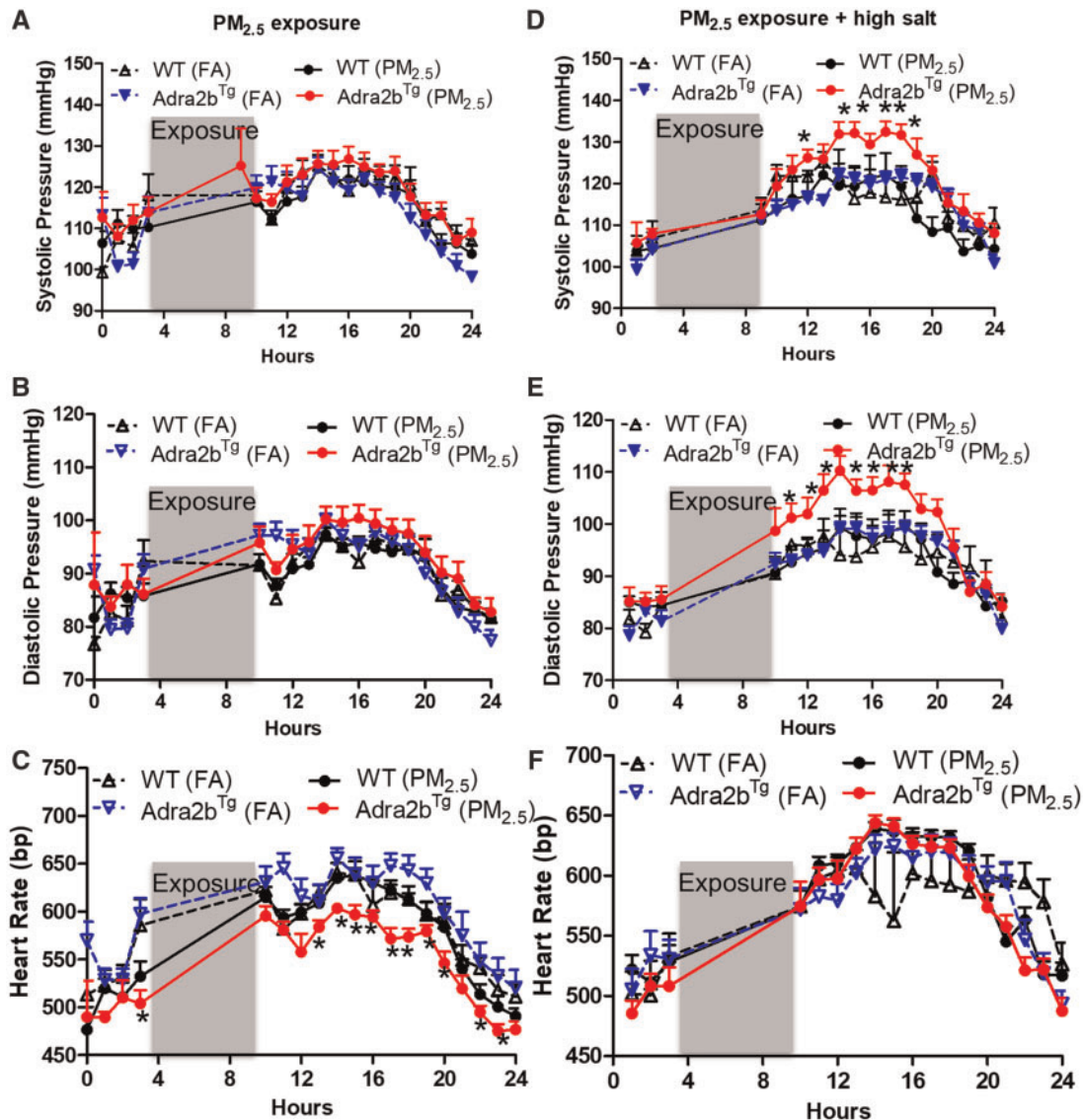
Figure 2. Effects of Adra2b on the sensitivity to air pollution-induced blood pressure elevation: A–F, WT and Adra2b^{Tg} mice were exposed to FA or PM$_{2.5}$ for 11 weeks, then fed with 4% high salt diet simultaneously with exposure for 1 week (week 12). Systolic (A and D), diastolic (B and E) blood pressure, and heart rate (C and F) were examined before (A–C) and after (D–F) high salt diet feeding. Data are presented as means ± SE. N = 3–4 for each group. *$p < .05$ compared with FA within the same genotype.

stress-related genes in the brain tissue. Superoxide dismutase 1 (SOD1) and NAD(P)H quinone dehydrogenase 1 (NQO1) (Figs. 7A and 7B) but not nuclear factor erythroid 2-related factor 2 (Nrf2) and glutamate-cysteine ligase modifier subunit (Gclm) (Figs. 7C and 7D) were increased slightly in the brain of WT mice after PM$_{2.5}$ exposure. The mRNA expressions of SOD1, Nrf2, and Gclm were increased in the brain of Adra2b^{Tg}, relative to WT mice (Figs. 7A–D). Furthermore, PM$_{2.5}$ exposure increased the expression of NQO1, but not SOD1, Nrf2, and Gclm in the brain of Adra2b^{Tg} mice compared with FA-exposed Adra2b^{Tg} mice (Figure 7B).

DISCUSSION

Several previous studies, including our own, have indicated that central sympathetic activation is associated with air pollution-induced adverse health effects (de Hartog et al., 2009; Liu et al.,

2014). Using a genetic approach of adrenergic receptor α2B over-expression in the brain as a model of central sympathetic activation, we provide direct evidence that sympathetic activation in response to air pollution results in behavioral and blood pressure changes to salt loading.

Ambient air pollution exposure has been associated with a number of human diseases such as hypertension, atherosclerosis, diabetes, stroke, etc. However, individuals may not be equally vulnerable to air pollution-induced adverse health effect. Age, behaviors, and underlying susceptibility are regarded as factors that can influence health effects of air pollution (Gent and Bell, 2010; Makri and Stilianakis, 2008). To identify if sympathetic activation could predispose individuals to air pollution, we used a mouse model that overexpresses Adra2b selectively in the brain. The Adra2b receptor plays a critical role in regulating noradrenaline signals and receptor responsiveness (Small et al., 2001). In the current study, we observed a selective elevation of Adra2b expression in the brain of PM$_{2.5}$-exposed mice.
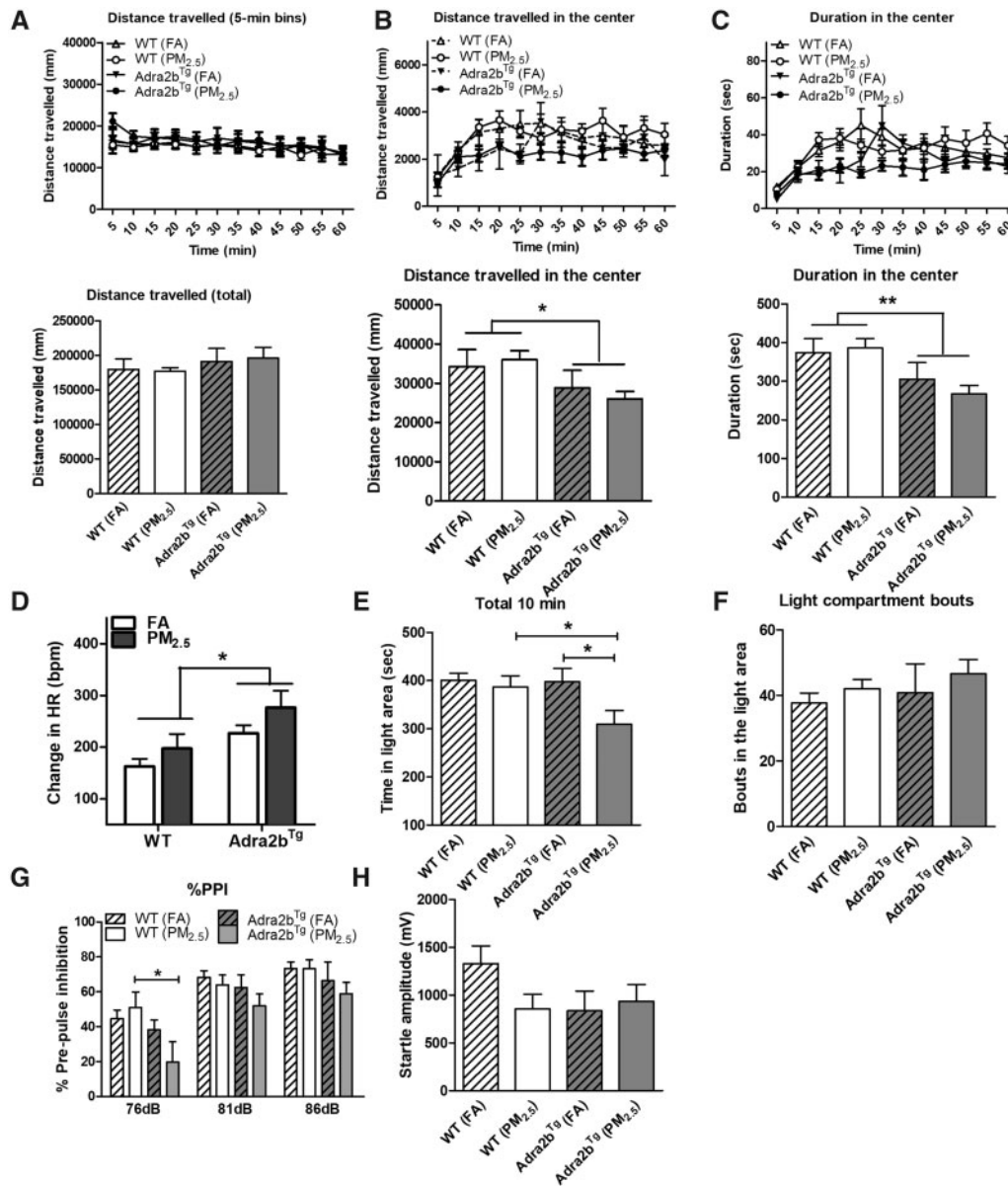
Figure 3. Effects of Adra2b on anxiety-like behavior changes: A–C, FA- or PM$_{2.5}$-exposed WT and Adra2b^{Tg} mice on a normal chow diet were subjected to open-field test. Total travel distance (A; upper panel, traveling speed [distance/min]; lower panel, cumulative distance traveled during the 60 min of detection period), travel distance in the center (B; upper panel, traveling speed [distance/min] in the center; lower panel, cumulative distance traveled in the center during the 60 min of detection period), and duration in the center (C; upper panel, the duration/minute stayed in the center; lower panel, cumulative duration stayed in the center during the 60 min of detection period) were measured. D, Heart rate changes in open-field test. Time in light area (E) and light compartment bouts (F) in the LDB test. % prepulse inhibition (G) and startle response (H) in the prepulse inhibition test. Data are presented as means ± SE. $N = 5$–7 for each group. $^*p < .05$; $^{**}p < .01$.

Overexpression of Adra2b in the brain sensitized the blood pressure response of FVB/N mice to air pollution under high salt loading conditions. High salt intake and salt sensitivity have been associated with sympathetic activation (Fujita, 2014). We did not observe hypertensive response in Adra2b-expressing FVB/N mice after PM$_{2.5}$ exposure under normal salt loading conditions. This may be consistent with previously noted findings that FVB/N strain may be more resistant to blood pressure changes (Hein et al., 1995). This may relate to the compensatory mechanisms to maintain blood pressure balance in vivo. For example, the hypertensive response of PM$_{2.5}$ in Adra2b^{Tg} mice may reduce heart rate through baroreflex response to maintain blood pressure balance. High salt intake, conversely, may desensitize baroreflexes in salt-sensitive animals (Andresen, 1989;

Victor et al., 1986). Therefore, Adra2b^{Tg} mice under high salt diet condition may have a disrupted baroreflex response to compensate for salt-induced pressor effects, resulting in a blood pressure elevation. Interestingly, it has also been shown that inhalation of PM$_{2.5}$ may also affect cardiac vagal influence and baroreflex sensitivity when combined with modified diets (Carll et al., 2017), suggesting a more complicated relationship between high salt intake and air pollution exposure. In consistency with this, the difference in heart rate between Adra2b^{Tg} (PM$_{2.5}$) group and other groups disappeared after treatment of a high salt diet, indicating that there might be a synergistic effect between high salt intake and air pollution exposure which disrupted the baroreflex compensatory mechanism(s), resulting in blood pressure elevation. In our study, imiloxan-induced heart
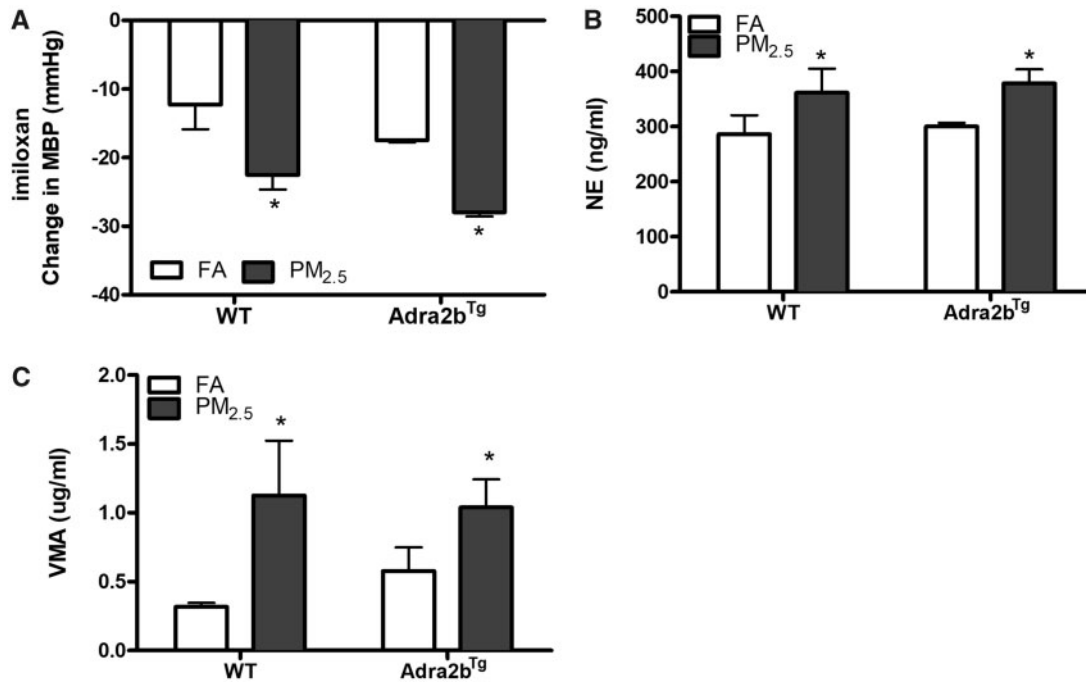
Figure 4. Sympathetic activation in PM$_{2.5}$ exposure: A, FA- or PM$_{2.5}$-exposed WT and Adra2b^{Tg} mice on a high salt diet were injected with the Adra2b-specific antagonist imiloxan and mean blood pressure (MBP) was monitor by telemetry. Changes in MBP were shown. N = 3–4 for each group. Norepinephrine (NE, B) and vanillylamandelic acid (VMA, C) levels were tested in the urine of exposed WT or Adra2b^{Tg} mice. Data are presented as means ± SE. *$p < .05$.

rate reduction was smaller in Adra2b^{Tg} (PM$_{2.5}$) group compared with Adra2b^{Tg} (FA) group, suggesting that there might be a higher level of baroreflex response and associated sympathetic inhibition although it may not be sufficient to maintain normal blood pressure. In contrast, Adra2b blockade by imiloxan had greater effects on the changes in blood pressure, NE and VMA in PM$_{2.5}$-exposed animals. In combination with the findings that PM$_{2.5}$ exposure enhanced Adra2b expression, these results suggest that Adra2b-associated sympathetic activation plays an important role in air pollution-induced elevations in blood pressure. Activation of the SNS relays stress interpretation from the brain to the immune system. This is pertinent as SNS activation centrally results in direct release of catecholamines and stimulation of adrenergic receptors in peripheral lymphoid cells resulting in alteration of their inflammatory potential and migration capacity (Chavan et al., 2017). Indeed, air pollution exposure resulted in robust evidence of sympathetic excess, together with increase in the expression of inflammation-related genes, an increase of T cell activation in both lung and spleen and CNS expression of TLR2, TLR4, and IL-6. Sympathetic activation could either directly regulate inflammatory response through adrenergic receptors, or indirectly via blood flow, immune cell distribution, and proinflammatory peptides (Pongratz and Straub, 2014). Immune cells express a variety of adrenergic receptors which have been directly associated with the regulation of immune activation (Bacou et al., 2017; Dasu et al., 2014; Scanzano and Cosentino, 2015). However, the exact role of Adra2b in direct regulation of immune function was not well understood (Scanzano and Cosentino, 2015). Similarly, Adra2b activation also enhanced the expression of antioxidant genes such as SOD1, NQO1, Nrf2, and Gclm in the brain. Recent studies have demonstrated an important homeostatic role of Nrf2 in the mitigation of blood pressure in response to hypertensinogenic stimuli (Gao et al., 2017). Ablation of Nrf2 selectively in areas of the brain resulted in potentiation of blood pressure in

response to angiotensin II (Gao et al., 2017). Although we cannot determine if redox stress directly result in sympathetic activation in our experiments, prior observations have shown that air pollution exposure increases redox stress via nicotinamide adenine dinucleotide phosphate oxidase pathways (Rao et al., 2018).

In consistency with our previous report (Liu et al., 2014), chronic PM$_{2.5}$ exposure resulted in sympathetic activation and peripheral inflammation. We have shown that chronic exposure to PM$_{2.5}$ increased low-frequency blood pressure variability and urinary NE in C57BL/6 mice, whereas central inhibition of IKK/NF-κB reversed central inflammation and restored peripheral energy homeostasis and insulin sensitivity in PM$_{2.5}$-exposed mice (Liu et al., 2014). In humans, exposure to PM$_{2.5}$ and ozone has also been associated reduced heart rate variability, index of sympathetic activation (Park et al., 2005; Park et al., 2008). In addition, β blockers were able to reduce the low frequency heart rate variability in response to pollutants in human subjects (Park et al., 2005). It seems that older populations may be more sensitive to air pollution-induced alteration in sympathetic pathways (Creason et al., 2001; Holguin et al., 2003; Liao et al., 1999). Sympathetic pathways have also been associated with depression and anxiety disorders (Roth et al., 2008; Veith et al., 1994). We and others have indicated that chronic air pollution exposure may result in anxiety and depressive like behaviors in both humans and mice (Fonken et al., 2011; Hogan et al., 2015; Vert et al., 2017). However, whether the sympathetic signaling plays a role in air pollution-associated behavior changes is not clear. Several clinical and preclinical studies indicate that stress promotes the development of anxiety and depressive like behaviors (Gilman et al., 2013; Kendler et al., 1999; McLaughlin et al., 2010). In models of repetitive social defeat (RSD), anxiety like behavior in the open field and light-dark preference tests develop after RSD (3–6 cycles) and persists for days (Wohleb et al., 2014, 2013). Commonly used mouse models of anxiety include open field test, LDB test, and elevated plus/T maze. Each
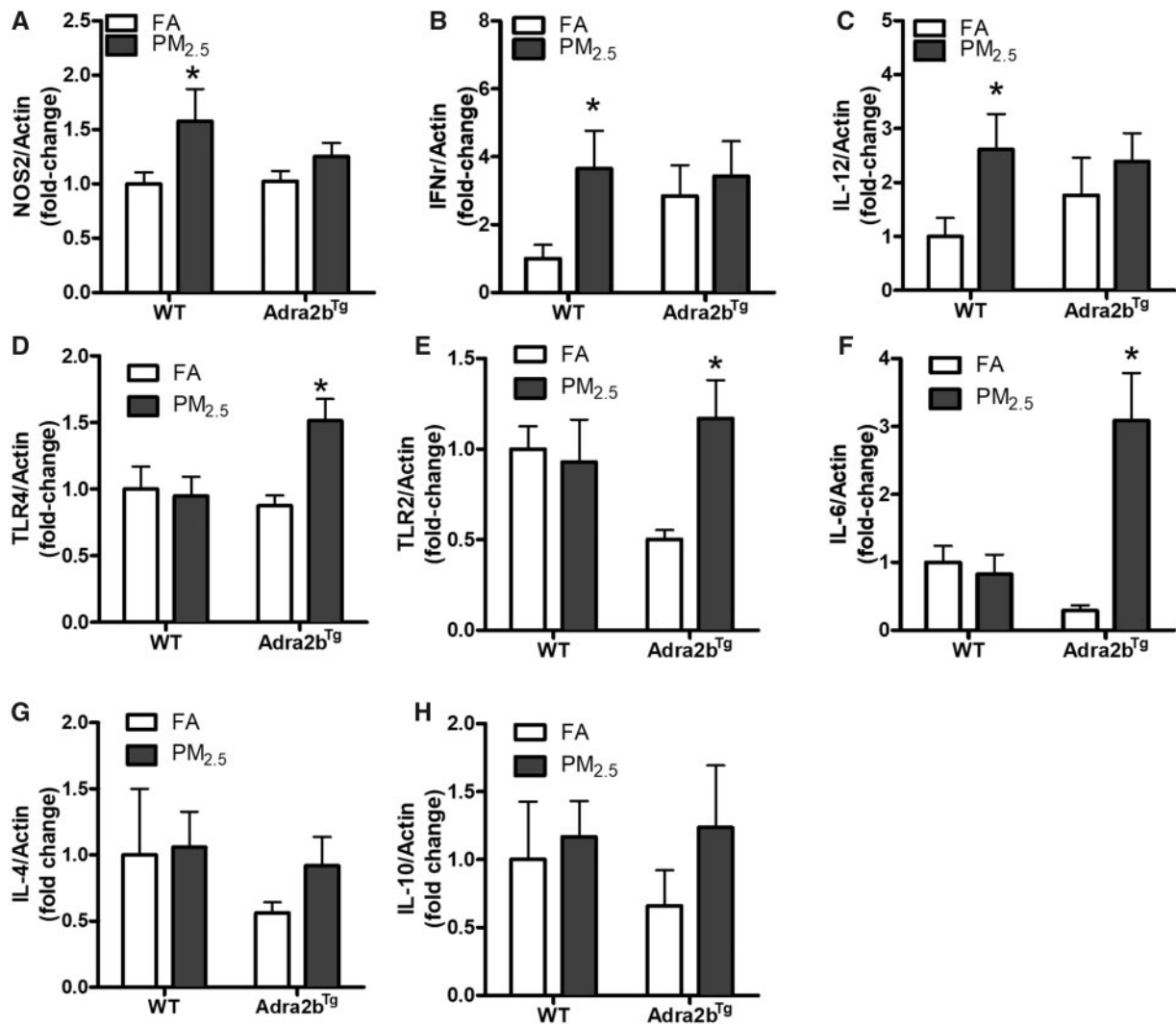
Figure 5. Adra2b activation in $PM_{2.5}$-induced inflammatory gene expression: NOS2 (A), INFr (B), IL-12 (C), TLR4 (D), TLR2 (E), IL-6 (F), IL-4 (G), and IL-10 (H) expressions in the brain from FA- or $PM_{2.5}$-exposed WT and $Adra2b^{Tg}$ mice on a normal chow diet. Data are presented as means ± SE. N = 5–7 for each group. *$p < .05$ compared with FA.

test may differ according to the feature of aversive signal, response, and sensitivity to different stresses (Bourin et al., 2007; Steimer, 2011). Open field test is sensitive to the anxiolytic-like effects of benzodiazepine receptor- and 5-HT_{1A} receptor agonists but not to the effects of other compounds such as selective serotonin reuptake inhibitors and tricyclics (Prut and Belzung, 2003). Many models including open field test were developed and validated based on benzodiazepines, their sensibility on drugs acting on other system remains questionable (Bourin et al., 2007). LDB test, developed in 1980s, evaluates the innate aversion of rodents to brightly illuminated areas and their spontaneous exploratory behavior (Bourin et al., 2007). Prepulse inhibition test measures the inhibitory effect of a weak acoustic auditory stimulus on a subsequent startle response (Longenecker et al., 2016). Prepulse inhibition deficits are associated with psychosis and schizophrenia. In the open field test, we found that central overexpression of Adra2b significantly reduced distance traveled and time in the center, suggesting that SNS is involved in the anxiety response. In support of our findings, the deletion variant (Del301-303) of Adra2b that results in reduced agonist-promoted receptor desensitization and

persistent receptor activation (Small et al., 2001) has been shown to be associated with vulnerability to emotional disorders such as anxiety and abnormal emotional response (de Quervain et al., 2007; Naudts et al., 2012). In the LDB test, $PM_{2.5}$ exposure suppressed the willingness of transgenic mice to explore in a risky environment, suggesting there was a synergistic effect between air pollution and Adra2b overexpression on anxiety-like behavior. Prepulse inhibition deficits were identified in transgenic mice exposed to $PM_{2.5}$. These results further support the conclusion that sympathetic activity controls the susceptibility of mice to air pollution-induced behavioral changes.

In contrast to our previous observation that air pollution exposure increases blood pressure in C57BL/6 mice (Liu et al., 2014), we did not observe a significant effect of air pollution exposure on baseline blood pressure in both WT and $Adra2b^{Tg}$ mice after 12 weeks of $PM_{2.5}$ exposure. This may be due to the differences in exposure duration and/or strain of mice. Indeed, FVB/N mice are salt-resistant and may be less vulnerable to air pollution exposure and environmental challenges (Hein et al., 1995; Masuzaki et al., 2003; Savov et al., 2004). However, the relative resistance to blood pressure changes in FVB/N mice also

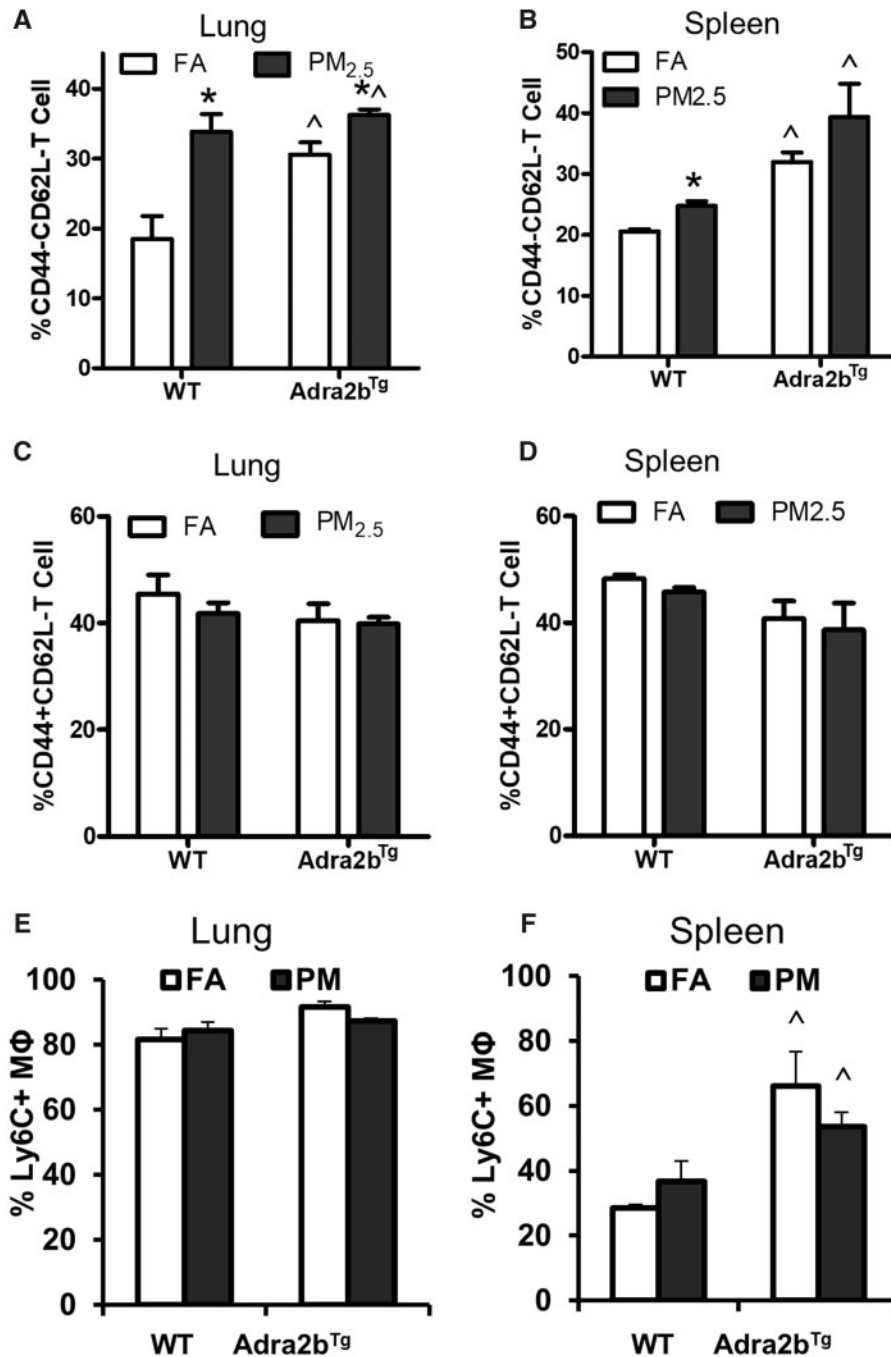**Figure 6.** Adra2b activation in $PM_{2.5}$-induced T cell activation: activated effector T cell ($CD44^-CD62L^-$, A and B) and activated memory T cell ($CD44^+CD62L^-$, C and D) in the lung and spleen from FA- or $PM_{2.5}$-exposed WT and Adra2b^{Tg} mice on a normal chow diet. E and F, Percentage of inflammatory macrophage (Ly6C+ MΦ) in the lung and spleen from FA- or $PM_{2.5}$-exposed WT and Adra2b^{Tg} mice. Data are presented as means ± SE. N = 5–7 for each group. *$p < .05$ compared with FA within the same genotype;^$p < .05$ compared with WT exposed to the same aerosol.

make it a good model to study exposure sensitivity to air pollution. This may also be the reason that $PM_{2.5}$ exposure alone in WT FVB/N mice did not cause any behavioral changes in open field, LDB, and prepulse inhibition tests. We have shown that air pollution exposure alone provoked anxiety-like and depressive-like responses in C57BL/6 mice (Fonken et al., 2011) and C3H/HeNHsd mice (Hogan et al., 2015), although no significant behavioral changes were observed in FVB/N mice after exposure in this study. This study also shows that increasing the salt intake in $PM_{2.5}$-exposed mice with genetic

predisposition, ie, increased brain Adra2b expression, can lead to hypertension. Air pollution exposure has been recently linked to criminal activities (Kristiansson et al., 2015; Lu et al., 2018), suicide mortality (Lin et al., 2016), and depressive/anxiety symptoms (Pun et al., 2017) in epidemiologic studies. This study may provide a better understanding of the mechanisms underlying the link between air pollution exposure and psychologic/behavior changes. It may also contribute to the identification of vulnerable populations to air pollution exposure.
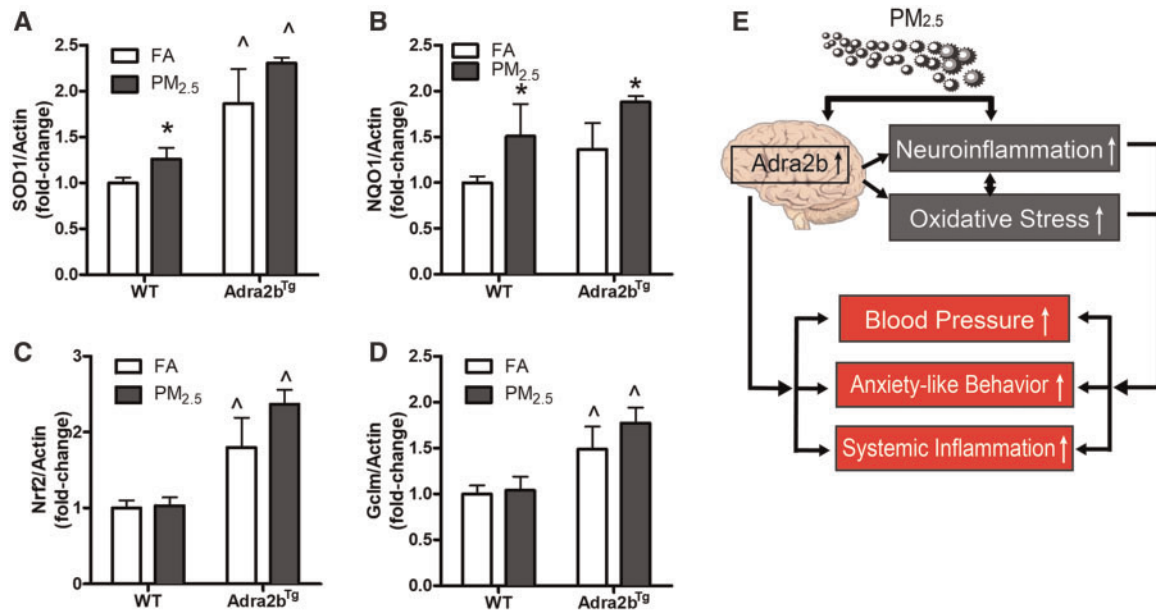
Figure 7. Adra2b activation in $PM_{2.5}$-induced oxidative stress: Gene expression of SOD1 (A), NQO1 (B), Nrf2 (C), and Gclm (D) in the brain from FA- or $PM_{2.5}$-exposed WT and $Adra2b^{Tg}$ mice on a normal chow diet. E, Hypothetical mechanism. Data are presented as means $\pm$ SE. $N = 5–7$ for each group. $*p < 0.05$ compared with FA within the same genotype; $\hat{}p < .05$ compared with WT exposed to the same aerosol.

It should be noted that there are several limitations within this study. First, our study only focused on the Adra2b signaling and there might be other pathways regulating the SNS activation in air pollution exposure which requires further investigations. Second, the urinary levels of NE and VMA examined in our study were not normalized by creatinine and therefore could not exclude the possibility that $PM_{2.5}$ exposure may affect urinary NE/VMA levels via altering kidney function. Third, this study used self-control for high salt diet treatment which results in small difference in $PM_{2.5}$ exposure duration (11 weeks of $PM_{2.5}$ exposure for normal chow diet vs. 12 weeks of $PM_{2.5}$ exposure for high salt diet). Given the fact that the $PM_{2.5}$ concentrations throughout the 12 weeks of exposure were consistent (Supplementary Figure 3), one more week of $PM_{2.5}$ exposure would presumably have minimal added effect beyond 11 weeks.

In conclusion, our results provide support for an important and direct role for central sympathetic activation in behavioral (anxiety) and peripheral cardioimmune alterations (Figure 7E).

SUPPLEMENTARY DATA

Supplementary data are available at *Toxicological Sciences* online.

DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

AUTHORS' CONTRIBUTIONS

X.R. contributed to the experimental design, performed experiments, collected samples, analyzed date, and drafted the manuscript. L.D.A. performed the radio-telemetry surgery. P.Z. performed the behavior tests. R.S.G., C.X., L.D., Y.M.C., and P.R. helped with the tissue collection and exposure. P.A.J., T.D.G., R.N., S.B., J.Z. provided critical comments on study design and manuscript. L.C.C. helped with element characterization of air particulate matters. S.R. conceived of and lead the study, drafted the manuscript.

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