Perinatal exposure to diesel exhaust origin secondary organic aerosol induces autism-like behavior in rats

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Research

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Abstract

**Background:** Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by impaired social communication, poor social interactions and repetitive behaviors. The exact cause and mechanism of autism remains unknown. Both genetic and environmental factors may involve in ASD. In this study, we used diesel exhaust (DE) origin secondary organic aerosol (DE-SOA) as environmental pollutants. DE-SOA was generated by oxidative reaction of mixing DE with ozone. The aim of present study is to examine autism-like behaviors and related gene expressions in rats exposed to DE-SOA perinatally. Sprague-Dawley pregnant rats were exposed to clean air (control), DE and DE-SOA in the exposure chamber for 5 h per day (from 10:00 pm to 3:00 am), 5 days a week excluding weekends from gestational day 14 to postnatal day 21 with their pups. At postnatal day 21, the male and female offspring rats were allocated into three different groups as follows: 1) rats exposed to clean filtered air; 2) rats exposed to DE; 3) rats exposed to DE-SOA. Social behaviors were investigated at 10~13-weeks-old rats using a 3-chambered social behavior test, social dominance tube test and marble burying test. Prefrontal cortex was collected to examine neurological and immunological markers, and glutamate concentration, using real-time RT-PCR and ELISA methods.

**Results:** DE-SOA-exposed male and female rats showed poor sociability and social novelty preference, socially dominant behavior and increased repetitive behavior compared with the control rats. The mRNA expression levels of serotonin receptor (5-HT(5B)) and brain-derived neurotrophic factor (BDNF) were down-regulated whereas interleukin 1 b (IL-b), and heme oxygenase 1 (HO-1) were upregulated in the prefrontal cortex of male and female rats exposed to DE-SOA compared to the control rats. Glutamate concentration was increased significantly in the prefrontal cortex of both male and female rats exposed to DE-SOA.

**Conclusion:** Our results indicate that perinatal exposure to DE-SOA may induce autism-like behavior in rats by modulating neurological and immunological markers in the prefrontal cortex.

**Background**

Exposure to air pollutants may trigger neurodevelopmental and neurodegenerative disorders like autism spectrum disorder (ASD) and Alzheimer's disease [1-4]. Epidemiological and experimental studies have indicated that associations exist between exposure to air pollution during brain developmental period and occurrence of ASD. In human studies, it was reported that maternal exposure to air pollution particulate matter (PM$_{2.5}$) during pregnancy was associated with a greater risk of the ASD in children [5, 6]. Furthermore, case-control study conducted in Southwestern Pennsylvania indicated that PM$_{2.5}$ exposure throughout pregnancy to two years old age was associated with increased risk of ASD [7]. In animal studies, the male mice exposed to ambient ultrafine particles during early postnatal period that is equivalent to human third trimester appeared features of ASD including learning and memory deficit, repetitive impulsive-like behavior, neuroinflammation, microglial activation, ventriculomegaly and excitatory/inhibitory imbalance [8, 9].
ASD is a neuro-developmental disorder and is characterized by impaired social interaction, difficulties in language or communication and repetitive behaviors. According to the Centers for Disease Control and Prevention (CDC) USA, recent ASD prevalence in the US is 1 in 59 children and it represents a public health issue and a large burden for education, social service and economy [10]. Both genetic and environmental factors are considerable for ASD [11] (Goines and Ashwood, 2013). Currently, many researchers have paid attention on association of exposure to air pollutants with elevated risk for ASD. However, the precise etiology and pathophysiology of autism remains unclear. In this study, using diesel exhaust (DE) originated secondary organic aerosol (SOA) as a model of air pollutant, we aimed to examine effects of the early life exposure to DE-SOA on autism-like behavior and neuroimmune responses including neurological and immunological biomarkers, mast cell and microglia activation in rats.

PM$_{2.5}$ consists of diesel exhaust particles which are major precursor of SOA formation. Previously, we have shown the effects of nanoparticle-rich diesel exhaust exposure on brain functions and behavior in adult mice [12-18]. Oxidation of volatile organic compounds in the atmosphere caused SOA formation [19]. Household and office appliances such as laser printer and copiers can also produce SOA [20, 21]. However, there are limited reports for the effects of exposure to SOA on neurodevelopmental changes. This prompted us to study the effects of inhalation exposure to DE-SOA during brain developmental period on ASD-like behavior.

We have generated diesel exhaust originated secondary organic aerosol (DE-SOA) by adding ozone to diesel exhaust and established the SOA inhalation chambers in our Research Institute. We have been studying the effect of early exposure to air pollutant DE-SOA on neurotoxicity in later life using animal models. We have shown that novel object recognition ability was impaired and accompanied with abnormally increased N-methyl-D-aspartate (NMDA) receptor expression in the hippocampus in adult male mice exposed to DE-SOA for 3 months [22]. Furthermore, one-month DE-SOA exposure in female mice caused tendency to decrease maternal performance accompanied with decreased expression levels of estrogen receptor (ER)-α, and oxytocin receptor in [22]. In addition, neonatal mouse model was established in our laboratory to examine early detection of learning disability by DE-SOA and found that olfactory-based spatial learning activity was impaired in neonatal mice [23]. Furthermore, we have showed that exposure to DE-SOA during brain developmental period impaired social behavior and altered social behavior related gene expression in the hypothalamus of adult male mice [24]. Recently, we have developed valproic acid (VPA)-induced autism model rats and showed that reduction of gamma amino butyric acid (GABA) synthetic enzyme glutamic acid decarboxylase (GAD) 67 protein in both male and female VPA-exposed rats. Thus, we suggest that imbalance of glutamate/GABA was the possible mechanism for ASD [25].

Some animal studies have shown that inhaled 13C particles from graphite rods in rats and intranasally instilled manganese oxide particles in monkeys translocate to central nervous system via olfactory nerve [26, 27]. In addition, our previous data showed that increased proinflammatory cytokines mRNAs expressions were observed in olfactory bulb of mice intranasally instilled with carbon black nanoparticles
(Win-Shwe et al., 2006). Therefore, we hypothesized that the toxic constituents from DE-SOA may translocate to brain via olfactory nerve pathway or systemic circulation and induce neuroinflammation and affect social behavior. The purpose of this study was to detect the effects of perinatal (gestational and neonatal) exposure to DE-SOA on autism-like behavior and neuroimmune response in rats.

**Materials And Methods**

**Animals**

Forty-eight timed pregnant Sprague-Dawley rats (gestational day; GD 8) were purchased from Oriental Yeast Co., Ltd. (Tokyo Japan) and exposed to clean air (control, n = 16), DE (n = 16) and DE-SOA (n = 16) from GD 14 to postnatal day (PND) 21 in the whole body exposure chambers. Food and water were given *ad libitum*. Date of birth was recorded as PND 0 and the offspring were housed in cages with dam under controlled environmental condition (temperature, 22 ± 0.5°C; humidity, 50 ± 5%; lights on 07:00–19:00 h). The pregnant mice were exposed in the exposure chamber for 5 h per day (from 10:00 pm to 3:00 am), 5 days a week excluding weekends from gestational day 14 to postnatal day 21 with their pups.

The number of pups born were 128 (60 male and 68 female) in the control, 130 (68 male and 62 female) in the DE and 135 (63 male and 72 female) in the DE-SOA groups. We used 3 male and 3 female pups from each dam (total 48 male and 48 female pups). Among them, 16 male and 16 female rats were used for 3 chamber social behavior test and marble burying test, among them 14 were used for neurochemical analyses and 2 were used for immunohistochemical analyses. Another 16 male and 16 female rats were used for test animals for social dominance test and other 16 male and 16 female rats were used for pair match. Furthermore, 4 male and 4 female rats from each group were used for aged match stranger 1 and stranger 2.

The pups were weaned at PND 21 and 3~4 pups of same sex were housed in a plastic cage. Experimental design was depicted in the Fig. 1. Social behavioral tests were performed at 10~13-week-old. Behavioral testing was performed between 09:00 and 13:00 h. Before performing each test, the apparatus to be used was cleaned with 70% ethanol. After completing social behavioral test, the rats were sacrificed under deep pentobarbital anesthesia and the left and right prefrontal cortex were collected from each group and frozen quickly in liquid nitrogen, then stored at −80°C until the extraction of the total RNA. The experimental protocols were approved by the Ethics Committee of the Animal Care and Experimentation Council of the National Institute for Environmental Studies (NIES), Japan (AE-19-36, AE-20-05). All efforts were made to minimize the number of animals used and their suffering.

**Preparation of exposure chambers for clean air, DE or DE-SOA**

The whole-body inhalation exposure chambers for clean air, DE or DE-SOA were generated at the National Institute for Environmental Studies, Japan as described previously [22, 28] (Fig. 2). Briefly, an 81-diesel engine (J08C; Hino Motors Ltd., Hino, Japan) was used to generate diesel exhaust. The engine was operated under a steady-state condition for 5 h a day. Our driving condition of diesel engine was not
simulated to any special condition as in the real world. The engine operating condition (2000 rpm engine speed and 0 Nm engine torque) promotes the generation of high concentrations of nano-size particles. There are three chambers: a control chamber receiving clean air filtered through a HEPA filter and a charcoal filter (referred to as “clean air”), the diluted exhaust (DE which was without mixing O$_3$), DE-SOA which was generated by mixing DE with ozone at 0.6 ppm after secondary dilution. Secondary dilution ratio in DE and DE-SOA chambers were the same which resulted in the same particle and gaseous concentrations when O$_3$ was not mixed. Actually, the concentrations of particles in DE-SOA was higher when O$_3$ was mixed and concentrations of DE and DE-SOA were 101 ± 9 µg/m$^3$ and 118 ± 23µg/m$^3$, respectively. The increased mass concentration was due to the generation of secondary particles. The temperature and relative humidity inside each chamber were adjusted to approximately 22 ± 0.5°C and 50 ± 5%, respectively. The particle characteristics inside the exposure chamber were shown in Table 1. In detail, sample air was taken from the inhalation chamber (2.25 m$^3$) using stainless steel tubing. The gas concentrations (CO, CO$_2$, NO, NO$_2$, and SO$_2$) were monitored using a gas analyzer (Horiba, Kyoto, Japan). CO and NOx concentrations in both chambers were similar, but NO and NO$_2$ are different each other because NO was oxidized to NO$_2$ by reacted with O$_3$. The particle size distributions were measured using a scanning mobility particle sizer (SMPS 3034; TSI, MN). The modal sizes of the particles used in the present study were 22.69 ± 1.47 nm for DE and 24.45 ± 1.21 nm for DE-SOA. The particles were collected using a Teflon filter (FP-500; Sumitomo Electric, Osaka, Japan) and a Quartz fiber filter (2500 QAT-UP; Pall, Pine Bush, NY, USA), and the particle mass concentrations were measured using a Teflon filter. The particle weights were measured using an electrical microbalance (UMX 2, Mettler-Toledo, Columbus; OH, USA; readability 0.1 µg) in an air-conditioned chamber (CHAM-1000; Horiba) under constant temperature and relative humidity conditions (21.5°C, 35%). For the Quartz fiber filter, the quantities of elemental carbon (EC) and organic carbon (OC) were determined using a carbon analyzer (Desert Research Institute, NV, USA). EC to TC ratio in the present study were 0.15 ± 0.06 for the control chamber, 0.36 ± 0.03 for DE chamber and 0.38 ± 0.03 for DE-SOA exposure chamber.

### Table 1. Particles and gaseous compounds in the exposure chambers

|                  | Size (nm) | Particle number (cm$^{-3}$) | Concentration (µg/m$^3$) | EC/OC | WSOC/OC | Temperature (°C) | Relative humidity (%) |
|------------------|-----------|-----------------------------|--------------------------|-------|---------|------------------|-----------------------|
| **Diesel exhaust particles** |           |                             |                          |       |         |                  |                       |
| Clean air        | –         | 2.36 × 10$^2$ ± 1.31 × 10$^1$ | 5.8 ± 8.00               | 0.00 ± 0.00 | 0.24 ± 0.10 | 23.15 ± 0.22 | 50.47 ± 1.20          |
| DE-SOA           | 24.45 ± 1.21 | 2.50 × 10$^4$ ± 7.51 × 10$^4$ | 118.15 ± 22.52           | 0.00 ± 0.00 | 0.07 ± 0.01 | 23.12 ± 0.15 | 50.71 ± 1.48          |
| DE               | 22.69 ± 1.47 | 2.25 × 10$^5$ ± 8.61 × 10$^4$ | 101.28 ± 8.73            | 0.00 ± 0.00 | 0.06 ± 0.01 | 22.74 ± 0.13 | 51.49 ± 2.05          |

### GASEOUS COMPOUNDS

|                  | CO (ppm) | SO$_2$ (ppm) | NO$_2$ (ppm) | NO (ppm) | O$_3$ (ppm) | CO$_2$ (%) |
|------------------|----------|--------------|--------------|----------|-------------|------------|
| Clean air        | 0.15 ± 0.02 | 0.00 ± 0.00 | 0.00 ± 0.00 | 0.00 ± 0.00 | –           | 0.06 ± 0.00 |
| DE-SOA           | 2.79 ± 0.04 | 0.00 ± 0.00 | 1.48 ± 0.03  | 1.14 ± 0.02 | 0.34 ± 0.03 | 0.06 ± 0.00 |
| DE               | 2.94 ± 0.05 | 0.01 ± 0.00 | 1.64 ± 0.03  | 0.58 ± 0.01 | 1.05 ± 0.03 | –          |

Data were expressed as mean ± SD.

**Behavioral Assessment**
Sixteen male and female rats were used for social behavior analyses.

**Sociability and social novelty preference**

Sociability and preference for social novelty test were performed as reported previously [22]. The apparatus used is a rectangular, three-chambered Plexiglas box (100 cm x 100 cm x 35 cm), with equal sizes of the three chambers. The dividing partitions are also made of clear Plexiglas, with small doorways on each (10 cm x 10 cm) that allow free access of the animals among the chambers. Wired cups (diameter 15 cm; height, 30 cm) are placed in each of the side chambers to house unfamiliar animals. For habituation, the subject rats from three different groups (DE or DE-SOA-exposed and the control rats) are placed in the middle chamber and allowed to explore for 5 min. During the habituation phase, the wired cup in each of the side chambers was empty (E). Following habituation, for the sociability test, an unfamiliar rat (stranger 1 (S1), age-matched rat) is placed in the wired cup in one of the side chambers; the subject rats are allowed to explore for 10 min. The location of stranger 1 in the left or right-side chamber is systematically alternated between trials. The social novelty preference test is performed immediately after the sociability test. For this test, another unfamiliar rat (stranger 2 (S2), age-matched rat) is placed in the wired cup on the other side that had been empty during the first 10-minute session, and the subject rat is allowed to explore the two strangers for 10 min. The time spent in exploring the wired cups on either side will be measured. The time that the subject rat spent exploring the wired cup is measured as the time spent with its head facing the cup from a distance of within 1 cm.

**Social dominance behavior (Tube test)**

Social dominance was tested in a transparent Plexiglas tube measuring 45 cm in length and 4 cm in (inside) diameter, a size just sufficient to permit one rat to pass through without reversing direction [29]. For training, each rat was released at alternating ends of the tube and allows to run through the tube. Each animal was given five training trials on each of two successive days. For the social dominance test, animals were placed at opposite ends of the tube and released simultaneously. An animal was declared the “winner” when its opponent backed out of the tube. The maximum test time was set to 2 min. The tube was cleaned with 70% ethanol before each trial.

**Marble burying test**

Marble burying test is a useful model of anxiety-like behavior and repetitive behavior. Each rat was placed for 20 min into a clean rat cage (40 cm X 24 cm X 15 cm) with 5 cm deep bedding and 20 glass marbles placed in a regular pattern and evenly spaced. The number of marbles that were buried at least 2/3 of the area by the rat was measured.

**Quantification of mRNA expression levels**

After completion of behavioral tests, 13-week-old male and female rats (n = 14 from each group) were sacrificed under deep pentobarbital anesthesia and the left prefrontal cortex was collected from each group for mRNA analyses. Briefly, the total RNA was extracted from the prefrontal cortex samples using
the BioRobot EZ-1 and EZ-1 RNA tissue mini kits (Qiagen GmbH, Hilden, Germany). Then, the purity of the total RNA was examined, and the quantity was estimated using the ND-1000 NanoDrop RNA Assay protocol (NanoDrop, Wilmington, DE, USA), as described previously [13, 30]. Next, we performed first-strand cDNA synthesis from the total RNA using SuperScript RNase H-Reverse Transcriptase II (Invitrogen, Carlsbad, CA, USA), according to the Manufacturer's protocol. We examined the mRNA expression levels using real-time RT-PCR (Light Cycler 96, Roche, Germany). The tissue 18S rRNA level was used as an internal control. The primer sequences used in the present study are shown below. Some primers (5-hydroxytryptamine (serotonin) receptor 5B (5-HT5B), NM_024395; brain-derived neurotrophic factor (BDNF), NM_012513; interleukin (IL)-1β, NM_008361; cyclooxygenase (COX)2, NM_011198; HO-1, NM_010442) and neuroligin3 (Nlgn3), NM_134336 were purchased from Qiagen, Sample and Assay Technologies. Other primer was designed in our laboratory as follows: 18S (forward 5'-TACCACATCCAAAAGGCAG-3', reverse 5'-TGCCCTCCAATGGATCCTC-3'), tumor necrosis factor (TNF) α (forward 5'-GGTTCCTTTGTGGCACTTG-3', reverse 5'-TTCTCTTGGTGACCGGGAG-3'). Data were analyzed using the comparative threshold cycle method. Then, the relative mRNA expression levels were expressed as mRNA signals per unit of 18S rRNA expression.

Measurement of glutamate concentration

After completing social behavioral test, the rats (n = 14 from each group) were sacrificed under deep pentobarbital anesthesia and the right prefrontal cortex was collected from six male and female rats of each group and frozen quickly in liquid nitrogen, then stored at –80°C until protein analysis. The right prefrontal cortex from 6 rats of each male and female groups were homogenized in a falcon tube containing 10 ml of cool sterile saline and centrifuged at 3000 rpm/min for 5 min at 4°C. The supernatant was used for subsequent glutamate detection by glutamate research ELISA assay kit (Ref: BA E-2300, Neuroscience, Inc., Tokyo, Japan) according to the manufacturer's instructions.

2.1 Statistical analysis

All the data were expressed as the mean ± standard error (S.E.). The statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, Version 5.0 (Nankodo Inc., Tokyo, Japan). The data were analyzed using a one-way analysis of variance with a post-hoc analysis using the Bonferroni/Dunn method. Differences were considered significant at \( P < 0.05. \)

Results

General toxicity assessment
To determine the general toxicity of DE or DE-SOA, body and brain weights of the rats were measured at the time of sampling. Although body weight and brain weight of DE-exposed male and female rats were increased compared to the control and DE-SOA exposed group, no statistically significant difference was observed among the groups (Table 2).

**Table 2. Body weight and brain weight of male and female offspring rats**

|                | Male rat          |          | Female rat         |          |
|----------------|-------------------|----------|--------------------|----------|
|                | Weight (g)        | Weight (mg) | Weight (g)        | Weight (mg) |
| Control        | 298.0 ± 7.0       | 1917.4 ± 22.6 | 192.4 ± 9.0       | 1844.3 ± 27.0 |
| DE             | 315.4 ± 3.7       | 2059.1 ± 37.6 | 219.0 ± 7.7       | 1900.6 ± 51.5 |
| DE-SOA         | 296.6 ± 5.4       | 1936.3 ± 31.5 | 203.4 ± 6.0       | 1762.0 ± 22.4 |

**DE-SOA exposure impaired sociability and social novelty preference**

Sociability test: The control rats approached more time to S1 than E cup ($P < 0.05$). DE-exposed rats equally approached to E and S1 cups. DE-SOA exposed rats approached more time to E than S1 cup ($P < 0.05$). Both male and female rats showed similar pattern (Fig. 3A).

Social novelty preference test: The control rats approached more time to S2 than S1 cup ($P < 0.05$). DE-exposed rats equally approached to S1 and S2 cups. DE-SOA exposed rats approached more time to S1 than S2 cup ($P < 0.05$). Both male and female rats showed similar pattern (Fig. 3B).

It was suggested that the control rats recognized novel or new one and DE or DE-SOA exposed rats had a poor ability to discriminate between familial and novel ones.

**DE-SOA exposure induced social dominance behavior**

In social dominance tube test, male rats exposed to DE-SOA showed increased win% compared to the control and DE exposed rats ($P < 0.01$, Fig. 4A) and female rats exposed to DE-SOA showed increased win% compared to the control rats ($P < 0.01$, Fig. 4B).

It was suggested that male and female rats exposed to DE-SOA had social dominance or aggressive behavior.

**DE-SOA exposure induced repetitive behavior**

In the marble burying test, male rats exposed to DE or DE-SOA showed significantly increased percentage of buried marble compared to the control rats ($P < 0.05$, Fig. 5A) and female rats exposed to DE-SOA...
showed significantly increased percentage of buried marble compare to the control rats ($P < 0.01$, Fig. 5B).

These results indicate that male and female rats exposed to DE-SOA performed repetitive behavior.

**DE-SOA exposure altered the mRNA expressions of social behavior-related genes and neurotrophic factor in the prefrontal cortex**

We have investigated the effect DE or DE-SOA exposure on social behavior-related genes and neurotrophic factor mRNA expressions in the prefrontal cortex of rats. We found that the expression levels of serotonin receptor 5HT5B was significantly reduced in male and female rats exposed to DE-SOA compared with the corresponding control ($P < 0.05$, Fig. 5A, 5B). *Nlgn3* is a gene associated with ASD. We found *Nlgn3* mRNA expression was significantly decreased in male mice exposed to DE-SOA, but not in the female mice ($P < 0.05$, Fig. 5C, 5D). We have also observed that the expression levels of BDNF were significantly lower in male and female rats exposed to DE-SOA compared with the corresponding control ($P < 0.05$, Fig. 5E, 5F).

**DE-SOA exposure altered the mRNA expressions of inflammatory markers in the prefrontal cortex**

To detect the DE or DE-SOA-induced inflammation in the prefrontal cortex, we have also investigated the inflammatory markers such as IL-1 b, TNF-a and COX2 in the prefrontal cortex of rats. We found that the expression level of IL-1 b mRNA was remarkably increased in male and female rats exposed to DE-SOA compared with the corresponding control ($P < 0.01$; $P < 0.05$, Fig. 6A, 6B). No significant changes of TNF-a and COX2 were found. We also observed that the expression levels of HO-1 were significantly higher in male and female rats exposed to DE-SOA compared with the corresponding control ($P < 0.01$, $P < 0.05$, Fig. 6C, 6D).

**DE-SOA exposure increased the level of neuronal excitatory marker in the prefrontal cortex**

Excessive glutamate level is the indicator of neurotoxicity. We measured glutamate concentration in the prefrontal cortex and found that glutamate secretion was increased remarkably in both male and female rats exposed to DE-SOA compared with the control rats ($*P < 0.05$) (Fig. 7).

**Discussion**

In our previous study, we have shown that developmental exposure to DE-SOA impaired sociability and social novelty preference and hypothalamic expression of social behavior-related genes estrogen receptor alpha and oxytocin receptor in male mice [24]. Recently, we used VPA-induce autistic rat models to examine the role of excitatory and inhibitory neurotransmitters, especially GABA synthetic enzyme in the mechanism of ASD [25]. These studies prompted us to use rat models to examine the ASD related social behaviors and related gene expressions in ASD related brain region, prefrontal cortex. In 3 chamber social behavioral test, we found same pattern of sociability and social novelty preference were observed in male.
mice and rats. Our targets in previous mice study and the present rat study were different in animal strain, target brain area and target genes. The reason for using rat model in the present study were to examine the ASD related social behaviors other than 3 chamber test, sexual different effects and role of social behavior related genes such as serotonin, BDNF and inflammatory marker IL-1 b.

The major findings of the present study were that the perinatal exposure to DE-SOA induces 1) impairment of sociability and social novelty preference, increased social dominance behavior and increased repetitive behavior, 2) downregulation of social behavior related gene serotonin receptor 5HT5B, *Nlgn3* and neurotrophic factors BDNF, 3) upregulation of proinflamatory cytokine IL-1 b and oxidative stress marker HO-1, and 4) increased neurotoxic substance glutamate concentration in male and female rats. Our findings suggest that the perinatal exposure to DE-SOA induces autism-like behavior in rats by triggering neuroinflammation and neurodevelopmental disorder via neurological and immunological biomarkers in the brain.

ASD is a neurodevelopmental disorder and is characterized by impaired social interaction, difficulties in language or communication, and repetitive behaviors. In the present study, impairment of social interaction, increased social dominance and increased repetitive behavior were observed in DE-SOA exposed male and female rats. The exact causes of ASD has not been known and both genetic and environmental factors are suggested to contribute to ASD. Fragile X syndrome, Rett syndrome or cytogenetic abnormalities are reported to be associated with ASD [32, 33]. *NLGN3, NLGN4X* and *SHANK3* are synaptic genes and mutation of these genes are involved in idiopathic autism [32-36]. We have found that poor sociability and social novelty preference in fragile X mental retardation (*Fmr1*) and *Nlgn3* knockout male and female rats with sex specific manner (unpublished data). Regarding environmental factors, maternal infection in first trimester [37], poor pregnancy outcome such as low birth weight, preterm, small for age maternal hemorrhage, socioeconomic status, drugs and environmental toxic substance exposure are risk factors for ASD [37-39]. Maternal education level with poor socioeconomic status may influence the risk for ASD. Exposure to valproate, an epileptic drug, during first trimester of pregnancy is a risk factor for ASD. It was reported that gestational exposure to valproate have 8-fold increased risk to have ASD in children [40, 41]. Previously, we have demonstrated that developmental exposure to valproic acid induced poor social behavior in adult rats by modulating the expression levels of social behavior-related genes and inflammatory mediators accompanied with changes in GABA enzyme glutamic acid decarboxylase 67 (GAD67) in the hippocampus [25]. In that study we have showed that GAD67 deficiency may be associated with glutamate/GABA imbalance in autistic brain. We have also reported that early life exposure of BALB/c mice to DE-SOA may affect their late-onset hypothalamic expression of social behavior related genes (ERa and oxytocin receptor) and induced impaired social behavior [24]. Taken together, environmental factors such as maternal infection, nutrition, antenatal care, education, drug usage and exposure to chemicals or pollutants during pregnancy are risk factors for ASD.

Recently we have reported that developmental exposure to VPA and arsenic impaired social behavior by modulating serotonin receptors and decreasing BDNF [25, 42]. In the present study, we found downregulation of mRNA expression levels of social behavior related gene serotonin receptor 5HT5B and
neurotrophic factors BDNF in the prefrontal cortex of male and female rats exposed to DE-SOA. Among serotonin receptors, 5HT5B was selected because this receptor is involved in social behavior [43]. BDNF involved in synaptic plasticity which is critical for learning and memory functions. Downregulation of BDNF may impair synaptic plasticity and may also affect development and function of serotonin neurons [44].

Immune dysfunction such as abnormalities in T cells, B cells and NK cells, production of autoantibodies, increasing proinflammatory cytokines were reported in autism [45-47]. Patients with ASD showed that an increase of proinflammatory and regulatory cytokines in the cerebrospinal fluid [48]. In the present study, proinflammatory cytokine IL-1β and oxidative stress marker HO-1 mRNAs were increased significantly in prefrontal cortex of male and female rats exposed to DE-SOA. Regarding brain immune cells, it has been reported that microglial activation was observed in brain especially in prefrontal cortex, cerebellum and cerebral white matter of patients with ASD [49, 50]. These results are consistent with our present findings showing activation of microglia in the prefrontal cortex of male rats exposed to DE-SOA. In addition, we found that mast cell expression was increased in the prefrontal cortex of male rats exposed to DE-SOA. We found sex-specific effects of DE-SOA on microglia and mast cell activation.

Glutamate is excitatory amino acid neurotransmitter and excessive glutamate release caused excitotoxicity and induced the neuronal death [50]. We found that glutamate concentration was increased significantly in the prefrontal cortex of male and female rats exposed to DE-SOA perinatally. Increased glutamate level in DE-SOA exposed rats may Blockage of re-uptake by glutamate transporter in the presynaptic neurons and decreased GABA synthetic enzymes are possible causes of increased glutamate concentration. Although we did not examine GABA, an inhibitory amino acid neurotransmitter, or its synthetic enzyme GAD level, excitatory-inhibitory imbalance may trigger autism-like behavior in DE-SOA exposed rats.

Human studies have indicated that pregnant mothers who live or work near highway and busy roads were prone to expose air pollutants including diesel exhaust particles and were increased the risk of ASD [51-54]. Some studies have indicated that critical window for prevalence of childhood ASD was third trimester of pregnancy [9, 55, 56] because cortical synaptogenesis reaches peak at that period [57]. In addition, infection-induced maternal immune activation triggered neuroinflammation in the placenta and fetal brain which causing ASD-like behavior in childhood [58]. Taken together, location of residential area, exposure dose, gestational period of exposure, maternal risk factors, socioeconomic status of parents are predisposing factors for air pollution-induced increased risk of ASD.

We did not know the no effect of DE on social novelty preference performance and social behavior-related genes such as 5HT5B, BDNF, IL-1β, HO1 and glutamate in the present study. The difference between DE and DE-SOA was constituents and there were 1000 constituents from DE and DE-SOA and we could not say exactly the which constituents were influence the behavior and related biomarkers. Recently, we are investigating the candidate chemicals which have adverse effects in DE and DE-SOA in inhalation chambers.
Sex-specific effects were observed in ASD and boys are 5 times greater risk for ASD than girls. In our previous study, impairment of social behavior and related gene expression changes were prominent in male rats of VPA-induced autism model [25]. In the present study, no remarkable statistical differences of ASD related behaviors were observed between male and female rats. However, in 3 chamber social behavioral test, during sociability performance, total exploration time were 210 sec (Control), 110 sec (DE) and 125 sec (DE-SOA) in male rats and 180 sec (Control), 120 sec (DE) and 113 sec (DE-SOA) in female rats. Pattern was similar but less pronounce differences were observed in female rats. During social novelty preference performance, DE and DE-SOA-exposed male and female rats did not prefer stranger 2 rats and that novelty preference was lower in male rats compared to female rats. Regarding ASD related gene, we found Nlgn3 mRNA expression was significantly decreased in DE-SOA exposed male rats, but not in female rats in the present study. Different hormonal milieu, receptor type and function, maturation of neural network may contribute the sex-specific effects in ASD. Moreover, compensatory effects of deficient ASD related genes may protect female from ASD [59].

**Conclusion**

Developmental exposure to DE-SOA induced autism-like behaviors such as poor social interaction, social dominance and repetitive behavior in male and female rats. These poor social behaviors are accompanied with changes of neurological and immunological biomarkers in the prefrontal cortex. It is possible that the toxic substances from DE-SOA may translocate to the fetal brain via the olfactory nerve route or systemic circulation and induce neuroinflammation. Neuroimmune interaction, synaptic dysfunction, immune dysregulation and E-I imbalance are major contributing factors for development ASD in response to environmental pollutant exposure. Further studies are needed to elucidate the role of other social behavior related neurological and immunological biomarkers changes in environmental pollutant induced ASD-like behavior.

**Declarations**

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**Authors’ contributions**

TTWS designed the experiment, YF performed exposure management, CKTT participated in behavioral tests and histology and molecular analysis, TTWS wrote manuscript. ST and SH critically revised manuscript.

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**Availability of data and materials**

The data available from the corresponding author on reasonable request.

**Ethical approval and consent to participate**

Not applicable.

**Consent for publication**

All authors agreed to this publication.

**Competing interest**

The authors declare that they have no competing interest.

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Figures
Figure 1

Experimental protocol including generation of DE-SOA and exposure chambers. Forty-eight timed pregnant Sprague-Dawley rats were exposed to clean air (control, n = 48), DE (n = 48) or DE-SOA (n = 48) from GD 14 to PND 21 in the whole-body exposure chambers. Sixteen male and female offspring from the control, DE and DE-SOA groups were used for social behavioral tests at 10~13-week-old. After completing social behavioral test, brain samples were collected for molecular and histochemical analyses.

Figure 2
Assessment of social behavior using 3 chamber social behavior test. (A) sociability test, (B) social novelty preference test in the control, DE and SOA-exposed male and female rats (n = 16, *P < 0.05). NS: not significant.

Figure 3

Assessment of social dominance behavior using tube test. Social dominance behavior in (A) male and (B) female rats of the control, DE and SOA-exposed rats (n = 16, **P < 0.01 vs corresponding control or DE-exposed rats).
Figure 4

Assessment of repetitive behavior using marble burying test. Repetitive behavior in (A) male and (B) female rats of the control, DE and SOA-exposed rats (n = 16, **P < 0.01, *P < 0.05 vs corresponding control).

Figure 5
mRNA expression levels of neurological biomarkers. (A) Serotonin receptor 5HT5B, (B) Nlgn3 and (C) BDNF in the prefrontal cortex of the control, DE and SOA-exposed male and female rats. (n = 14, Control; *P < 0.05 vs. Control).

Figure 6

mRNA expression levels of immunological biomarkers. (A, B) IL1-β and (C, D) HO-1 in the prefrontal cortex of the control, DE and SOA-exposed male and female rats. (n = 14, **P < 0.01 vs. Control; *P < 0.05 vs. Control).
Figure 7

Assessment of neurotoxic substance in the brain. Glutamate concentration in the prefrontal cortex of the control, DE and SOA-exposed male and female rats (n = 6, *P < 0.05).