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Full Length Article

Role of TLR4 in olfactory-based spatial learning activity of neonatal mice after developmental exposure to diesel exhaust origin secondary organic aerosol

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ABSTRACT

Exposure to ambient air pollutants has been reported to have various adverse health impacts. Ambient particulate matter comprises primary particles released directly via engine exhaust and secondary organic aerosols (SOAs) formed from oxidative reactions of the ultrafine particle fraction of diesel exhaust (DE). Toll-like receptor 4 (TLR4) is well known to initiate the inflammatory cascade in the central nervous system. However, whether and how DE and DE-SOA exposure influences TLR4 signaling in the immature brain remains unclear. We attempted to evaluate the roles of TLR-4, inflammatory mediators and microglial markers in the impaired spatial learning ability of neonatal mice exposed to DE and DE-SOAs. Pregnant C3H/HeN (TLR4-intact) and C3H/HeJ (TLR4- mutated) mice were exposed to clean air, DE or DE-SOA from gestational day 14 to postnatal day (PND) 10 (5 h/day for 5 days) in exposure chambers. PND11 neonatal mice were examined for their performance in the olfactory-based spatial learning test. After the spatial learning test, the hippocampi of the mice were removed and real-time RT-PCR analysis was performed to examine the neurological and immunological markers. Both male and female C3H/HeN and C3H/HeJ neonatal mice exposed to DE and DE-SOAs showed poor performance in the test phase of spatial learning as compared to the mice exposed to clean air. However, this spatial learning deficit was prominent in C3H/HeJ neonatal mice. In the neonatal C3H/HeN male mice exposed to DE and DE-SOAs, the mRNA expression levels of the NMDA receptor subunits (NR1, NR2B), proinflammatory cytokines, tumor necrosis factor-α and cyclooxygenase-2, oxidative stress marker, heme oxygenase-1, and microglial marker, Iba1, in the hippocampus were significantly increased, but these changes were not observed in female mice. Our findings indicate that activation of the neuroimmune system and TLR4 signaling may possibly be involved in environmental pollutant-induced spatial learning impairment in neonatal mice.

1. Introduction

Epidemiological studies have indicated that chronic exposure to ambient particulate matter has various deleterious health effects, including increased risk of heart diseases, neonatal asthma, COPD and stroke (Hong et al., 2002; Pollock et al., 2017; Pope et al., 2002). Particulate air pollutants, in general, are composed of primary particles directly released via the vehicle engine exhaust and the ultrafine component of diesel exhaust (DE) in the atmosphere are oxidized to form secondary organic aerosol (SOA) (Virtanen et al., 2010). In urban environments, SOAs represent the major pollutants, not only in the atmosphere, but also in indoor residential settings (Virtanen et al., 2010; Wang et al., 2012; Youssefi and Waring, 2012).

Numerous studies have been conducted to examine the association between exposure to DE and development of neurological diseases. In one study, high dose DE exposure was found to lead to elevation in the levels of brain proteins associated with neurodegenerative diseases, and it was suggested that neuroinflammation might precede the emergence of preclinical markers of neurodegenerative disease (Levesque et al., 2011). Another study examined the effect of diesel exhaust on different areas of the rat brain and suggested that different brain regions may show unique responses to changes in proinflammatory cytokine expressions induced by prolonged exposure to DE (Gerlofs-Nijland et al., 2010). Another study pointed out the

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association of traffic-related air pollutants and PM2.5 with pediatric asthma (Pollock et al., 2017). While it is established beyond doubt that air pollution has deleterious effects on human health, susceptibility to the effects of air pollution may be more pronounced during certain periods of life, such as the neonatal period, during which the central nervous system is still developing.

In the atmosphere, volatile organic compounds (VOCs) react with ozone to form SOAs, which are components of PM 2.5 (Castro et al., 1999; Wang et al., 2012). In present times, SOA formation takes place not only in the atmosphere, but also indoors, by ozonolysis of terpenoids in residential and office spaces (Castro et al., 1999; Youssefi and Waring, 2012). It has been reported that printing processes can also result in SOA formation, because VOCs from laser printers can react with ozone to generate SOAs (Wang et al., 2012). The daily PM 2.5 environmental standard (particles less than 2.5 μm in aerodynamic diameter and the respirable fraction of particulate matter) in Japan is less than 35 $\mu g/m^3$. Most previous studies have used exposure to whole diesel exhaust, such as exhaust gas from diesel engines. However, a numerically large fraction of the particulates in diesel exhaust is ultrafine in size, and the particulate fraction may be important for toxicity (Jackson et al., 2011).

Toll-like receptor 4 (TLR4) is expressed on a variety of brain cells, such as the microglia (Glezer et al., 2007; Olson and Miller, 2004), astrocytes (Bsibsi et al., 2006; Farina et al., 2005), oligodendrocytes (Bsibsi et al., 2002) and neurons (Lafon et al., 2006). The binding of TLR4 by lipopolysaccharide (LPS) triggers the NF-κB transduction pathway and induces the release of critical proinflammatory cytokines that are necessary to activate potent immune responses (Akira et al., 2001; Beutler, 2002). TLRs might not only serve to sense microbial pathogen-associated molecular patterns (PAMPs), but also to sense certain endogenous factors that act as damage-associated molecular pattern (DAMP) molecules (Rubartelli and Lotze, 2007). Molecules identified as potential DAMP molecules include the heat shock proteins, fibrinogen, fibronectin, heparan sulfate, soluble hyaluronan, oxidized LDL, gangliosides, fatty acids and other cues of dying cells, and these molecules can stimulate TLR4 and TLR2 signaling (Marshak-Rothstein, 2006; Beg, 2002; McMahon et al., 2005). Activation of TLR4 and NF-κB may be neuroprotective, by either increasing the cell resistance or removing toxic molecules via increase in the phagocytotic capacity of activated microglia (Glezer et al., 2006). However, little is known about the role of TLR4 in learning and memory function and neuroimmune crosstalk following exposure to environmental pollutants.

Our research group has been engaged in systematic investigation of the effects of DE and DE-SOAs on the central nervous system, and has found, based on the findings of impaired gene expressions in the hippocampus and hypothalamus, that exposure to DE-SOAs is potentially associated with decreased memory function and maternal performance in mice (Win-Shwe et al., 2014). In another related work, we established an olfactory-based spatial learning test to assess the behaviors of neonatal mice; in the same study, we demonstrated changes in the expressions of the N-methyl D-aspartate (NMDA) receptor subunits and of the signaling pathway gene Ca^{2+}/calmodulin-dependent protein kinase II in the hippocampi of mice exposed to DE-SOA (Win-Shwe et al., 2015). We noticed that exposure to ambient pollutants such as DE and DE-SOAs in utero or early neonatal period might have a significant impact on the learning ability, role in learning, memory and behavioral development in the offspring/neonates. This knowledge prompted us to further investigate the effects of in utero and neonatal exposure to DE and DE-SOA on the brain in neonatal mice.

In this study, using TLR4-intact and TLR4-mutated mice, we attempted to evaluate the role of TLR4 in the spatial learning ability of mice and the changes in the expression levels of the receptor subunits of the memory function-related gene NMDA, inflammatory mediators, oxidative stress markers and microglial markers in DE-/DE-SOA-exposed neonatal mice.

2. Materials and method

2.1. Animals

Pregnant C3H/HeN (TLR4-intact) (n = 24) and C3H/HeJ (TLR4-mutated) (n = 24) mice were purchased from Japan SLC Co. (Tokyo, Japan). Postnatal day (PND) 11 mice were used for this experiment. One male and one female pulps from same dam (8 male and 8 female pups from 8 dams each of C3H/HeN and C3H/HeJ) were used for assessment by the olfactory-based spatial learning test. The cages used for the pregnant mice were special cages (thick saw dust flooring to avoid stress), differing from the usual wire cages that we usually use for pregnant mice at our Institute. During the exposure period, the animals were kept in their respective inhalation chambers. Food (a commercial CE-2 diet, CLEA Japan, Inc., Tokyo, Japan) and water were given *ad libitum*. The pups were housed in the wire cages with their own dam under controlled environmental conditions (temperature, 22 ± 0.5 °C; humidity, 50 ± 5%; lights on 0700–1900 h). This study was conducted with the approval of the Ethics Committee of the Animal Care and Experimentation Council of the National Institute for Environmental Studies (NIES), Japan.

2.2. Generation of SOAs

SOAs were generated at the National Institute for Environmental Studies, Japan. An

81-model diesel engine (J08C; Hino Motors Ltd., Hino, Japan) was used to generate the diesel exhaust. The details of the exposure system have been described previously (Fujitani et al.,

Table 1
Characteristics of diesel exhaust particles and gaseous compounds in exposure chambers.

	Diesel exhaust particles			Temperature	Relative humidity		
	Size (nm)	Particle number (cm^3)	Concentration ($\mu g/m^3$)	°C	(%)	EC/TC	WSOC/OC
Clean air	–	2.98 ± 0.72	2.15 ± 1.15	23.03 ± 0.34	49.39 ± 0.83	0.18 ± 0.07	0.45 ± 0.41
DE-SOA	25.44 ± 1.32	$3.54 \times 10^6 \pm 8.73 \times 10^4$	106.84 ± 12.70	22.81 ± 0.21	50.56 ± 1.04	0.41 ± 0.03	0.20 ± 0.07
DE	24.90 ± 1.42	$3.06 \times 10^6 \pm 7.24 \times 10^4$	85.57 ± 9.17	22.31 ± 0.18	51.40 ± 1.33	0.40 ± 0.03	0.25 ± 0.09

	Gaseous compounds						
	CO (ppm)	SO_2 (ppm)	NO_x (ppm)	NO_2 (ppm)	NO (ppm)	O_3 (ppm)	CO_2 (ppm)
Clean air	0.27 ± 0.04	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	–	0.05 ± 0.00
DE-SOA	2.96 ± 0.05	0.00 ± 0.00	1.28 ± 0.02	1.00 ± 0.03	0.28 ± 0.02	0.07 ± 0.00	0.07 ± 0.00
DE	2.82 ± 0.05	0.01 ± 0.00	1.20 ± 0.02	0.41 ± 0.01	0.79 ± 0.02	–	0.07 ± 0.00

Data were expressed as mean ± SD.

2009; Win-Shwe et al., 2014). The engine was operated under a steady-state condition for 5 h per day. In the present study, our driving conditions for the diesel engine did not simulate any special condition in the real world. The engine operating conditions (2000 rpm engine speed and 0 Nm (Newton meter) engine torque) in this study permitted a high concentration of nano-sized particles to be released. There were three chambers in the system: a control chamber receiving clean air filtered through a charcoal filter, a chemical filter, a high-efficiency particulate air (HEPA) filter, and an ultra-low-penetration air (ULPA) particulate filter (collection efficiency 99.999% at particle sizes of 0.1–0.2 μm) (referred to as the "clean air" chamber), a chamber containing diluted exhaust (DE chamber, prior to reaction with O_3), and a chamber containing DE-SOAs (DE-SOA chamber) generated by mixing DE with 0.6 ppm of O_3 after secondary dilution. The exposure air is distributed from the primary dilution tunnel and immediately diluted with more clean air (secondary dilution). The characteristics of the diesel exhaust particles and gaseous compounds in the exposure chambers are described in Table 1. The temperature and relative humidity inside each chamber were adjusted to approximately 22 ± 0.5 °C and $50\% \pm 5\%$, respectively. The particle characteristics were evaluated from the air samples taken from inside the exposure chambers. Air samples were obtained from the breeding space of 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). The CO and NO_x concentrations in chambers were similar, however, those of NO and NO_2 differed, because mixing with O_3 resulted in oxidation of NO to NO_2. The particle size distributions were measured using a scanning mobility particle sizer (SMPS 3034; TSI, Shoreview, MN, USA). The sizes of the particles used in the present study were 25.56 ± 1.75 nm for DE and 25.46 ± 1.51 nm for DE-SOA. The particles were collected using a Teflon filter (FP-500; Sumitomo Electric, Osaka, Japan) and 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) maintained under constant temperature and relative humidity conditions (21.5 °C, 35%). For the Quartz fiber filter, the quantities of elemental carbon and organic carbon were determined using a carbon analyzer (Desert Research Institute, Reno, NV, USA). An analysis of the particle composition (DE and DE-SOA) showed that the percentage of OC relative to the total carbon in the diluted exhaust was about 60%, and that DE and the DE-SOAs showed nearly the same carbon composition.

2.3. Experimental schedule

Pregnant C3H/HeN (TLR4-intact) (n = 24) and C3H/HeJ (TLR4-mutated) (n = 24) mice were allocated to three different groups ($n = 8$ per group), as follows: (1) mice exposed to clean filtered air; (2) mice exposed to DE, and (3) mice exposed to DE-SOAs, from gestational day 14 to PND 10 (5 h/day for 5 days) in the exposure chambers (Fig. 1). We picked up one male and female pups from each mother and we used 8 male and 8 female pups from each group for spatial learning test. On PND11, the performance of the neonatal male and female mice ($n = 8$ per group from three groups of C3H/HeN or C3H/HeJ mice) in the olfactory-based spatial learning test was examined. The time to reach the target location, the location of the mother and littermates was recorded for each neonatal mouse using a video-assisted computer system (Any-maze software, Muromachi Kikai Co. Ltd., Tokyo, Japan). After the spatial learning test was completed, the mice were sacrificed and the hippocampi were removed from the mice and examined by RT-PCR analyses for neurological and immunological markers.

2.4. Olfactory- based spatial learning test

A rounded cage, 40 cm in diameter, with clean wood chip bedding was used as the test cage. The dam and four-to-five mouse pups, littermates of the test pup, were placed in a small wire cage (target location) ($8 \times 10 \times 5$ cm) containing soiled home cage bedding. The round test cage was divided into four sectors and this wire cage was placed in one sector. The start or release points were marked in the other three sectors. Cotton impregnated with lemon oil was fixed on the wall behind the small wire cage to allow memorizable identification of the target location. We used PND 11 neonatal male and female mice for the spatial learning test, because mouse pups at that age have not yet opened their eyes and use only olfactory cues. There were two phases in the study, including the training phase (four trials) and the test phase (one trial), and each trial lasted for 180 s. The examiner guided the pups that failed to reach the target location and placed them at the target location for 1 min to allow for learning. The time to reach the target cage containing the littermates was recorded for each neonatal mouse using a video-assisted computer and software (Muromachi Kikai, Tokyo, Japan). In the training phase, four trials (180 s per trial) were given, with an interval between the trials of 5–15 min, and if the goal was not reached, the pup was guided at 180 s. After the fourth trial, after the pup had spent 1 min in the huddle, the test phase (180 s) was initiated. In the test phase, to examine whether the test pups remembered the target location, the dam and littermate mouse pups was removed from the small

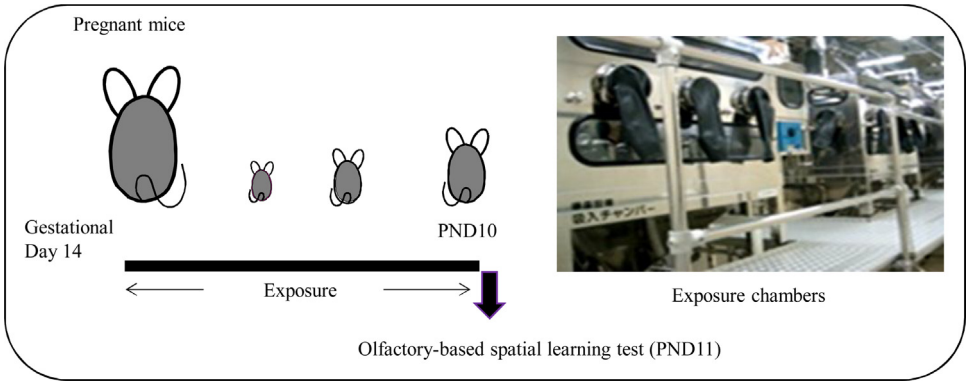

Fig. 1. Experimental schedule and exposure chambers. Pregnant C3H/HeN (TLR4-intact) (n = 24) and C3H/HeJ (TLR4-mutated) (n = 24) mice were allocated to three different groups ($n = 8$ per group), as follows: mice exposed to clean filtered air; mice exposed to DE, and mice exposed to DE-SOA, from gestational day 14 to PND 10 (5 h/day for 5 days) in the exposure chambers (Fig. 1). On PND11, the performance of the neonatal male and female mice (8 male pups and 8 female pups from three groups of C3H/HeN or C3H/HeJ mice) in the olfactory-based spatial learning test was examined. (PND = postnatal day).

wire cage and the ability of the test pups to reach the target location within 180 s was examined.

2.5. Odor discrimination and motor function test

Odor discrimination and motor function tests were performed as described previously (Win-Shwe et al., 2015). Briefly, we used a wire mesh cage (30 × 20 × 10 cm) placed on the tray which was divided into three areas of same size (10 × 20 × 10 cm). In the tray, the home cage bedding was placed under the mesh at the one side of the test cage, the clean bedding was placed under the mesh at the opposite side and the center was kept blank as a neutral area. On the day of odor discrimination and motor function test, PND 11 pups was taken out from the home cage and placed in the center. During two 3 min trials procedure, the time spent by test pup in each of the three areas and the number of crossing between the areas was recorded.

2.6. Quantification of the mRNA expression levels

After the olfactory-based spatial learning test was completed, mice from all groups were sacrificed under deep pentobarbital anesthesia and the hippocampi were collected from all the mice. Samples of the hippocampi were quickly frozen in liquid nitrogen and stored at −80 °C until extraction of the total RNA. Briefly, total RNA extraction from the hippocampi was performed 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). 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 protocol provided by the manufacturer. Next, we examined the mRNA expressions of 18S, NMDA receptor subtype 1 (NR1), NMDA receptor subtype 2A (NR2A) and NMDA receptor subtype 2B (NR2B), proinflammatory cytokines, tumor necrosis factors (TNF)-α, cyclooxygenase (COX)-2, oxidative stress marker, heme oxygenase (HO)-1, and microglial marker, ionizing calcium-binding adaptor molecule (Iba)-1, using a quantitative real-time RT-PCR method and the Applied Biosystems (ABI) Prism 7000 Sequence Detection System (Applied Biosystems Inc., Foster City, CA, USA). The tissue 18S rRNA level was used as the internal control. The primer sequences used in the present study (NR1, NM_008169; NR2A, NM_008170; COX2, NM_011198; HO1, NM_010442; Iba1, NM_019467) were purchased from Qiagen Sample & Assay Technologies. TNF-α primer (forward: 5′-GGTTCCTTTGTGGCACTTG-3′, reverse: 5′-TTCTCTTGGTGACCGG-GAG-3′) was purchased from Hokkaido System Science (Hokkaido System Science, Hokkaido, Japan). Data were analyzed using the comparative threshold cycle method. Then, the relative mRNA expression levels of the memory function-related genes were individually normalized to the 18S rRNA content in the respective samples and expressed as mRNA signals per unit expression of 18S rRNA.

2.7. Statistical analysis

All the data are expressed as the mean ± standard error (S.E.). Olfactory-based spatial learning test was analyzed by two-way analysis of variance (ANOVA). Messenger RNA expression data and odor discrimination were analyzed using a one-way ANOVA with a post-hoc analysis by the Bonferroni/Dunn method. The statistical analyses were performed using the Statcel4software (OMS Publishing Inc., Saitama, Japan). Differences were considered significant at $P < 0.05$.

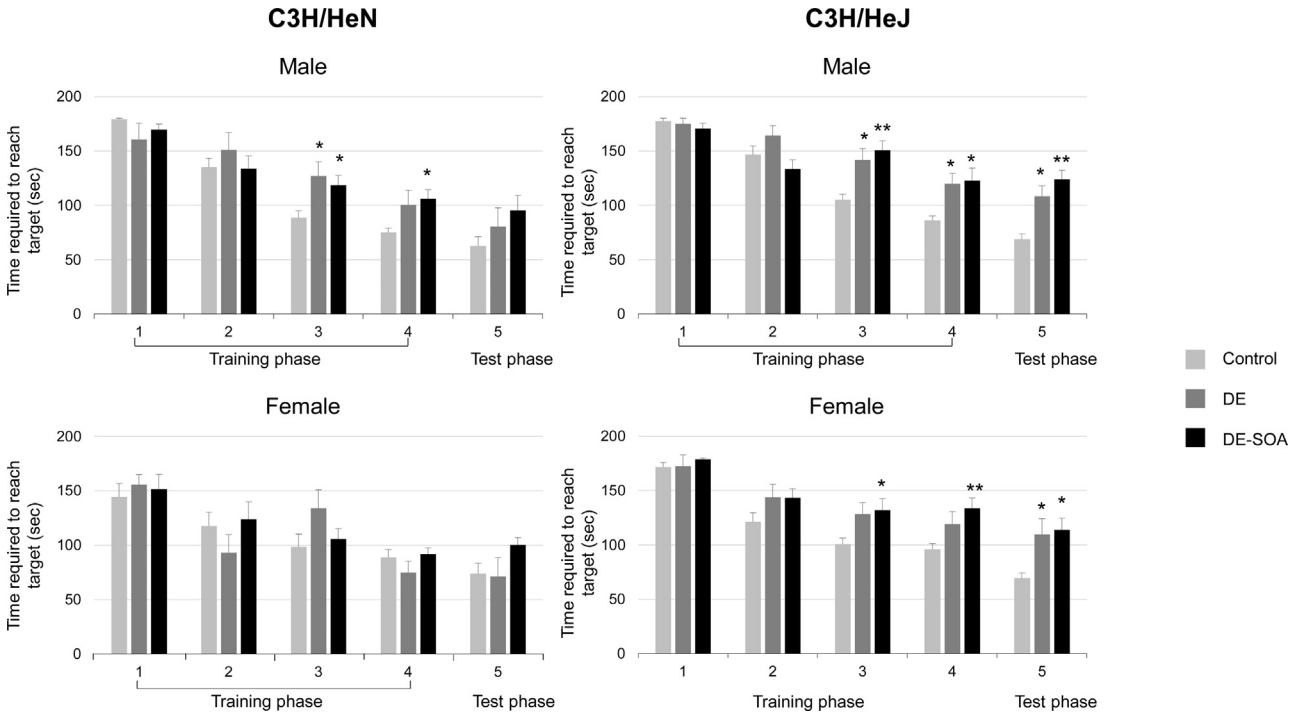

Fig. 2. Effects of DE or DE-SOA on olfactory-based spatial learning ability in male and female C3H/HeN and C3H/HeJ neonatal mice. There were two phases in the study, including the training phase (four trials) and the test phase (one trial), and each trial lasted for 180 s. The time to reach the target cage containing the littermates was recorded for each neonatal mouse using a video-assisted computer and software (Muromachi Kikai, Tokyo, Japan). In the test phase, to examine whether the test pups remembered the target location, the dam and littermate mouse pups was removed from the wire cage and the ability of the test pups to reach the target location within 180 s was examined. Each bar represents the mean ± SE (8 male pups and 8 female pups from three groups of C3H/HeN or C3H/HeJ mice, ** $p < 0.01$ vs. control; * $p < 0.05$ vs. control).

3. Results

3.1. Effects of DE and DE-SOA exposure on the performance of male and female C3H/HeN and C3H/HeJ mice in the olfactory-based spatial learning test

First, we performed two-way ANOVA to examine the effect of strain and exposure in C3H/HeN and C3H/HeJ male and female mice. In strain and exposure factors in male mice, we found that main effect of strain was observed ($F = 8.3477$, $P = 0.0050$), main effect of exposure was observed ($F = 8.4880$, $P = 0.0005$), however, no interaction was observed ($F = 0.6372$, $P = 0.5316$). In strain and exposure factors in female mice, we found that main effect of strain was not observed ($F = 3.0319$, $P = 0.086942$), but main effect of treatment was observed ($F = 6.5055$, $P = 0.0028$), no interaction was observed ($F = 1.5458$, $P = 0.2217$).

Next, we performed two-way ANOVA to examine the effect of sex and exposure in C3H/HeN and C3H/HeJ mice. In sex and exposure factors in C3H/HeN mice, we found that main effect of treatment was observed ($F = 3.4097$, $P = 0.0399$), but main effect of sex was not observed ($F = 0.0443$, $P = 0.8339$), no interaction was observed ($F = 0.2841$, $P = 0.7537$). In sex and treatment factors in C3H/HeJ mice, we found that main effect of treatment was observed ($F = 15.1082$, $P = 3.15E\text{-}06$), but main effect of sex was not observed ($F = 1.2411$, $P = 0.2688$), no interaction was observed ($F = 0.4295$, $P = 0.6524$).

According to two-factor ANOVA, our results indicate that the exposure effect of DE and DE-SOA was occurred in both C3H/HeN and C3H/HeJ neonatal mouse pups. There was no sex difference in both strains. Strain difference was observed and exposure effect was more prominent in C3H/HeJ group. In this study, we found that both male and female TLR4-mutated C3H/HeJ mice exposed to DE and DE-SOAs took significantly longer times, as compared to the corresponding control mice exposed to clean air, to reach the target location in training phases 3 and 4 and the test phase of the spatial learning test. On the other hand, both male and female TLR-4 intact C3H/HeN mice exposed to DE, DE-SOAs showed similar pattern of male and female C3H/HeJ mice in the test phase. These results suggest that the adverse effect of DE or DE-SOA exposure of decreasing the spatial learning ability based on olfactory cues may involve TLR4 signaling (Fig. 2).

3.2. Odor discrimination and motor function test

We performed assessment of olfactory and motor deficits in each group of C3H/HeN and C3H/HeJ male and female mouse pups. We found that all pups from the control and exposure groups spent more time in the home cage bedding area compared to the clean bedding area within groups and there was no difference of time spent in home cage bedding between the control and the exposure groups. We also found that locomotion, expressed as a number of crossing, was not different between groups (Figs. 3 and 4).

3.3. Effects of DE and DE-SOA on the hippocampal expressions of the NMDA receptor subunits NR1 and NR2B in male C3H/HeN and C3H/HeJ mice

Spatial learning and memory requires NMDA receptor-mediated synaptic currents and long-term potentiation in the CA1 neurons of the hippocampus (Tsien et al., 1996). Thus, we examined the mRNA expression levels of the NMDA receptor subunits NR1 and NR2 in the hippocampus, and found significantly higher mRNA expression levels of the NMDA receptor subunits NR1 and NR2B in the hippocampi of the C3H/HeN male mice exposed to DE/DE-SOAs (Fig. 5).

3.4. Effects of DE and DE-SOA exposure on the mRNA expression levels of the inflammatory markers TNF-α and COX2 in the hippocampi of male C3H/HeN and C3H/HeJ mice

We found that the mRNA expression levels of the inflammatory marker COX2 were significantly higher in the DE-/DE-SOA-exposed

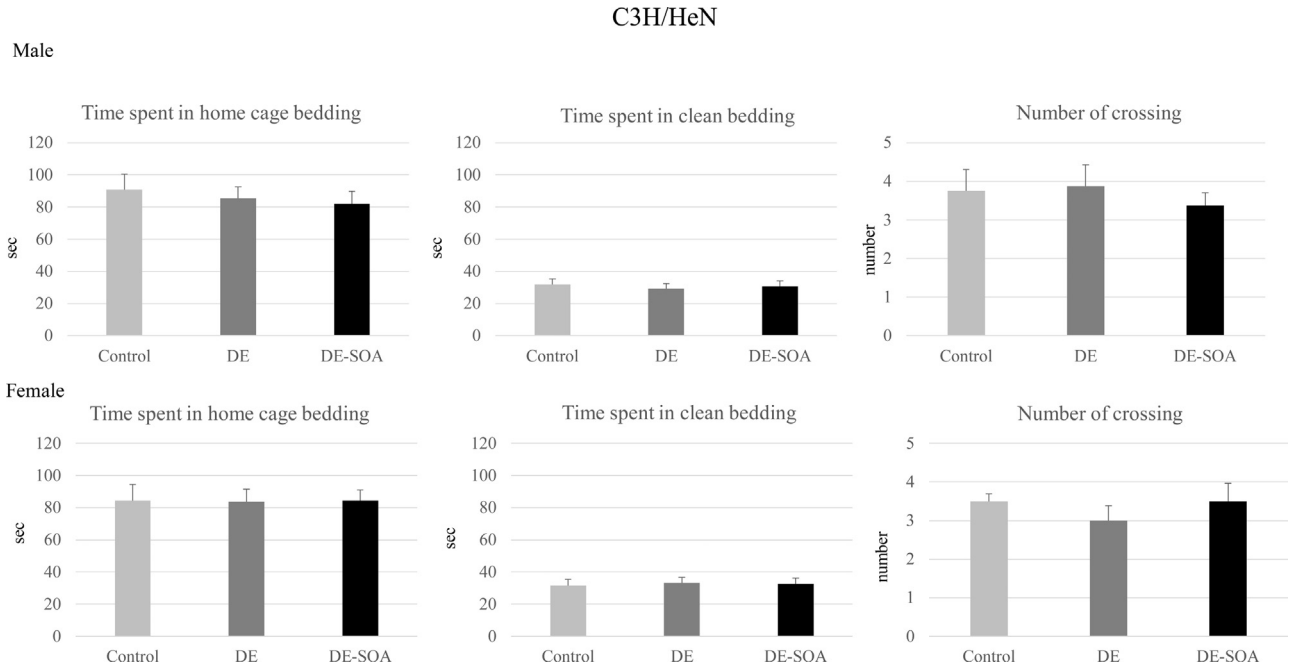

Fig. 3. Odor discrimination and motor function test in PND 11 C3H/HeN male and female neonatal mice. (n = 8 in each group). A wire mesh cage (30 × 20 × 10 cm) placed on the tray was divided into three areas of same size (10 × 20 × 10 cm). In the tray, the home cage bedding was placed under the mesh at the one side of the test cage, the clean bedding was placed under the mesh at the opposite side and the center was kept blank as a neutral area. On the day of odor discrimination and motor function test, PND 11 pups was taken out from the home cage and placed in the center. During two 3 min trials procedure, the time spent by test pup in each of the three areas and the number of crossing between the areas was recorded.

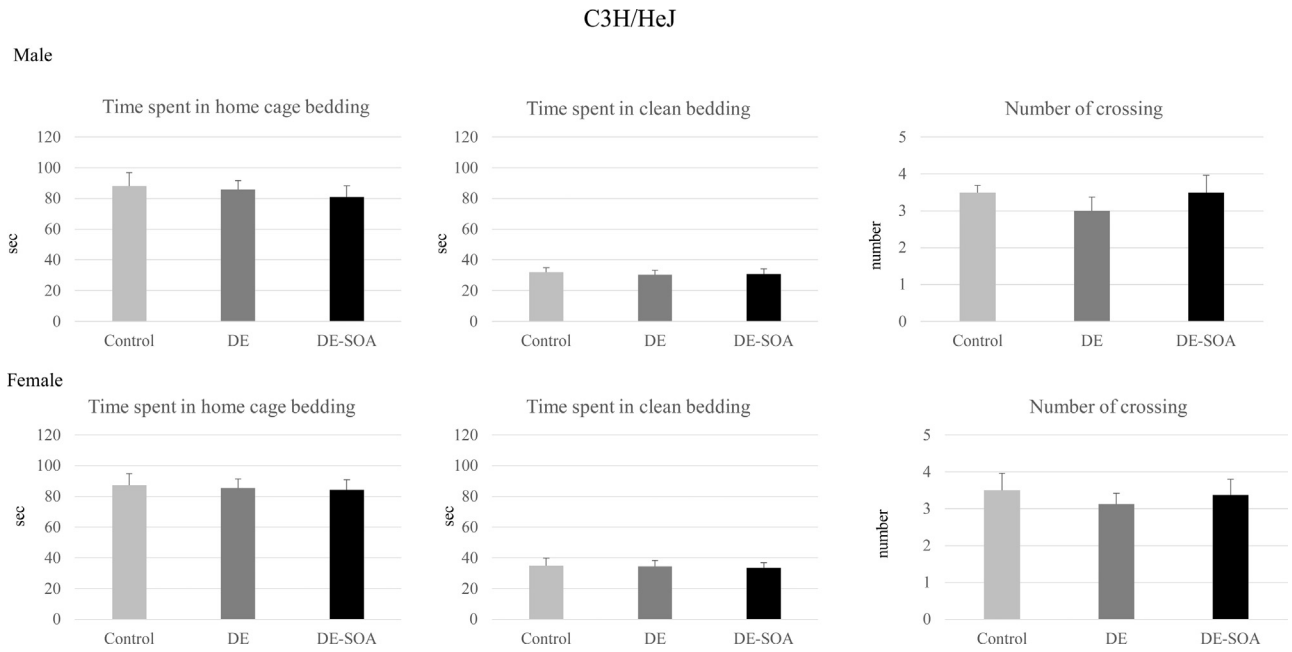

Fig. 4. Odor discrimination and motor function test in PND 11 C3H/HeJ male and female neonatal mice. (n = 8 in each group). Experimental schedule is same as Fig. 3.

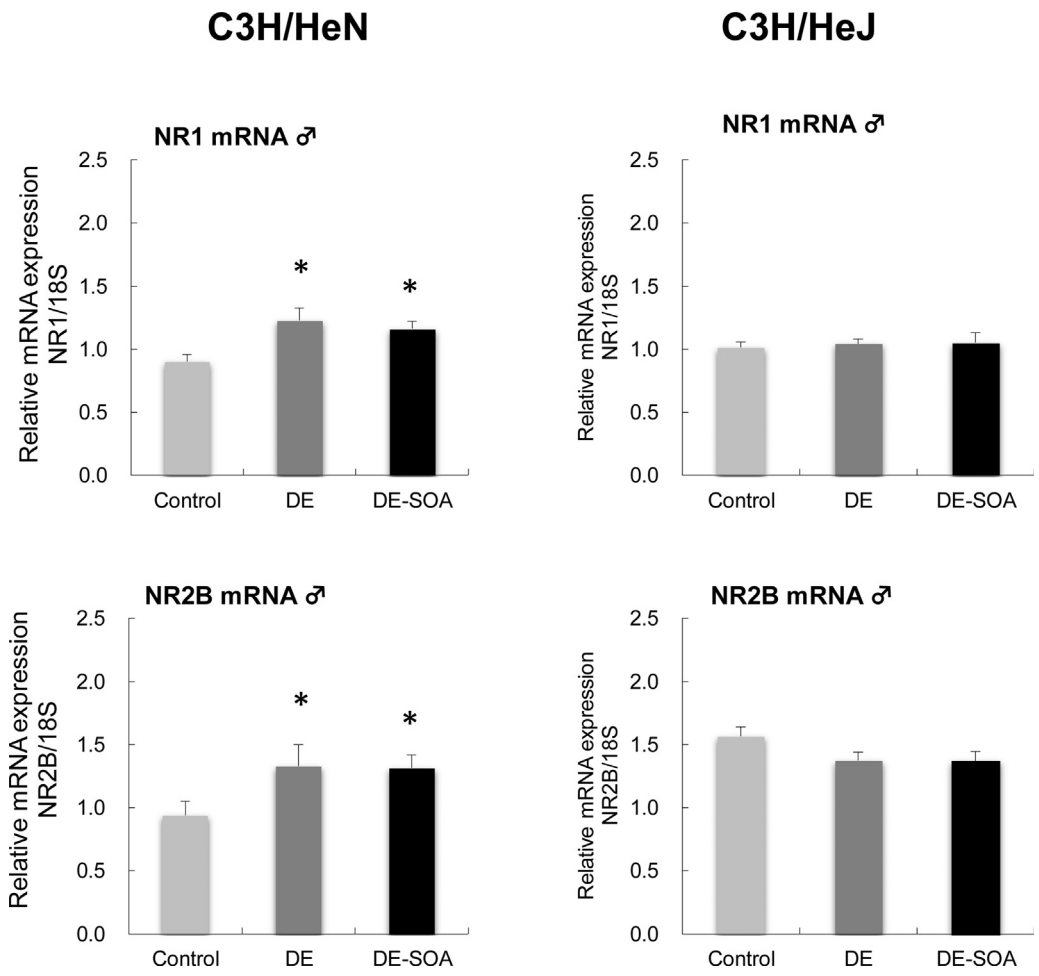

Fig. 5. Effects of DE or DE-SOA on mRNA expressions of NMDA receptor subunit NR1 and NR2 B in the hippocampi of C3H/HeN and C3H/HeJ neonatal male mice. Messenger RNA expression was examined by real-time RT-PCR method. Each bar represents the mean ± SE ($n = 8$, * $p < 0.05$ vs. control).
NMDA NR1 = N-Methyl-D-Aspartate receptor subtype 1.
NMDA NR2B = N-Methyl-D-Aspartate receptor subtype 2B.

TLR 4-intact male C3H/HeN mice as compared to the levels in the control mice exposed to clean air and TLR 4-mutated male C3H/HeJ mice. However, there were no significant differences in the expression levels of TNF-α mRNA among the control and exposed groups, or between C3H/HeN and C3H/HeJ mice. These results imply that the adverse effects of DE and DE-SOA exposure on the spatial learning ability is mediated by TLR4 receptor signaling, through increased expressions of inflammatory markers in the hippocampus (Fig. 6).

3.5. Effects of DE and DE-SOA exposure on the mRNA expression levels of the oxidative stress marker HO1 and microglial marker Iba1 in the hippocampi of male C3H/HeN and C3H/HeJ mice

The mRNA expression levels of the microglial marker Iba1 were found to be significantly higher in TLR 4-intact male C3H/HeN mice exposed to DE and DE-SOAs, whereas no significant differences in the expression levels of Iba1 mRNA were observed among the three different exposure groups of TLR 4-mutated male C3H/HeJ mice. On the other hand, no significant differences in the mRNA expression levels of HO1, the oxidative stress marker, were observed among the three exposure groups or between the male C3H/HeN or the C3H/HeJ mice. These results suggest that an increase in the number of microglia in the hippocampus underlies the impaired spatial learning ability mediated by TLR4 in mice exposed to DE/DE-SOAs (Fig. 7).

4. Discussion

The major finding of the present study was that male and female of C3H/HeN and C3H/HeJ neonatal mice exposed to DE and DE-SOAs took longer times, as compared to the corresponding control mice exposed to clean air, to reach their target location in training phases 3, 4, and the test phase of the olfactory-based spatial learning test. However, according to two-way ANOVA results, prominent effect was observed in C3H/HeJ mouse pups. Thus, learning based on olfactory cues was impaired in the DE-/DE-SOA-exposed animals, possibly mediated by the TLR receptors. No sex difference effect was observed in olfactory-based spatial learning test. In C3H/HeN mice, we found significant changes of neuroimmune markers in the male mice, but not in the female mice. Sexual dimorphism in the expression of TLR4 receptor in murine macrophages was reported (Marriott et al., 2006). In addition, it has been demonstrated that gender differences in murine airway responsiveness and LPS-induced inflammation via TLR4 (Card et al., 2006). Our present study shows that sexual dimorphic effects of DE and DE-SOA on neuroimmune markers in the hippocampus of in C3H/HeN mice. We suggest that sex

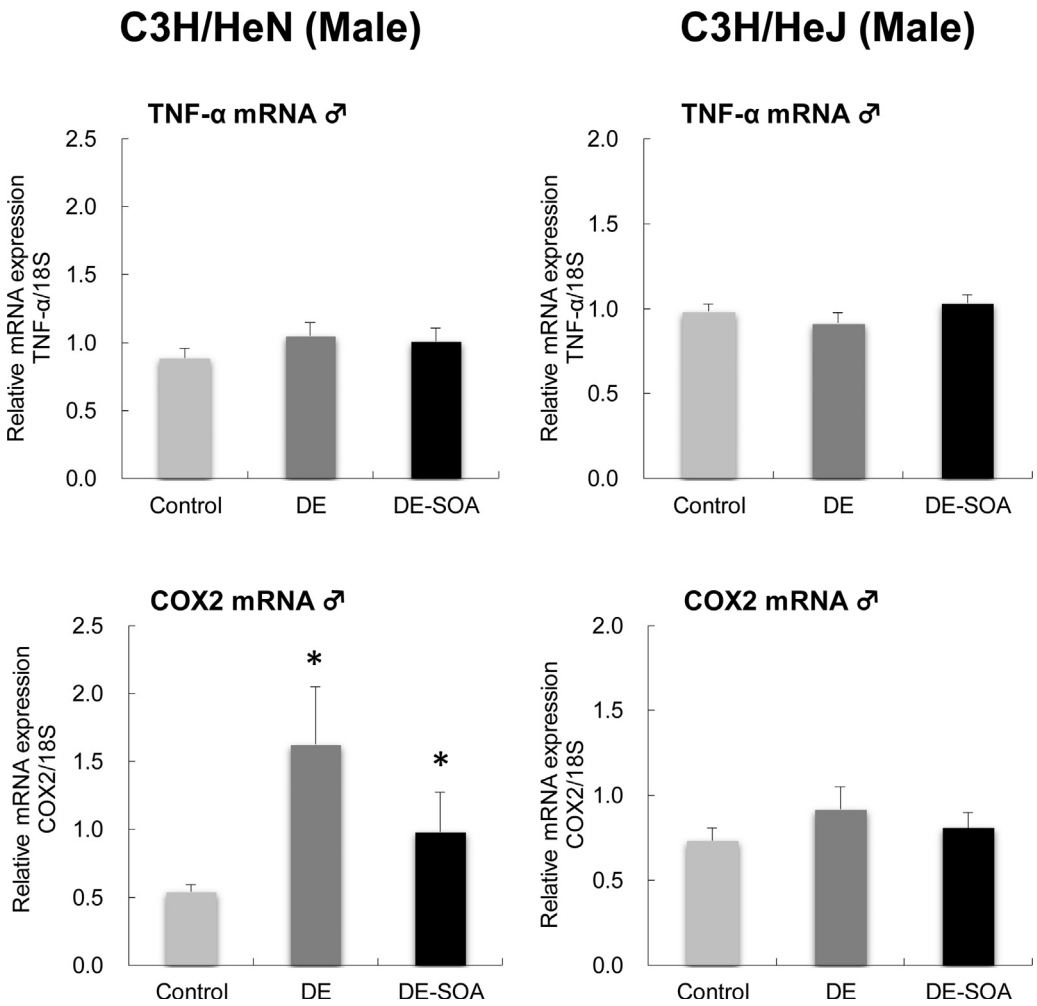

Fig. 6. Effects of DE or DE-SOA on mRNA expressions of inflammatory marker TNFα and COX2 in the hippocampi of C3H/HeN neonatal male mice. Messenger RNA expression was examined by real-time RT-PCR method. Each bar represents the mean ± SE ($n = 8$, * $p < 0.05$ vs. control).
TNF-α = tumor necrosis factor alpha; COX2 = cyclooxygenase 2.

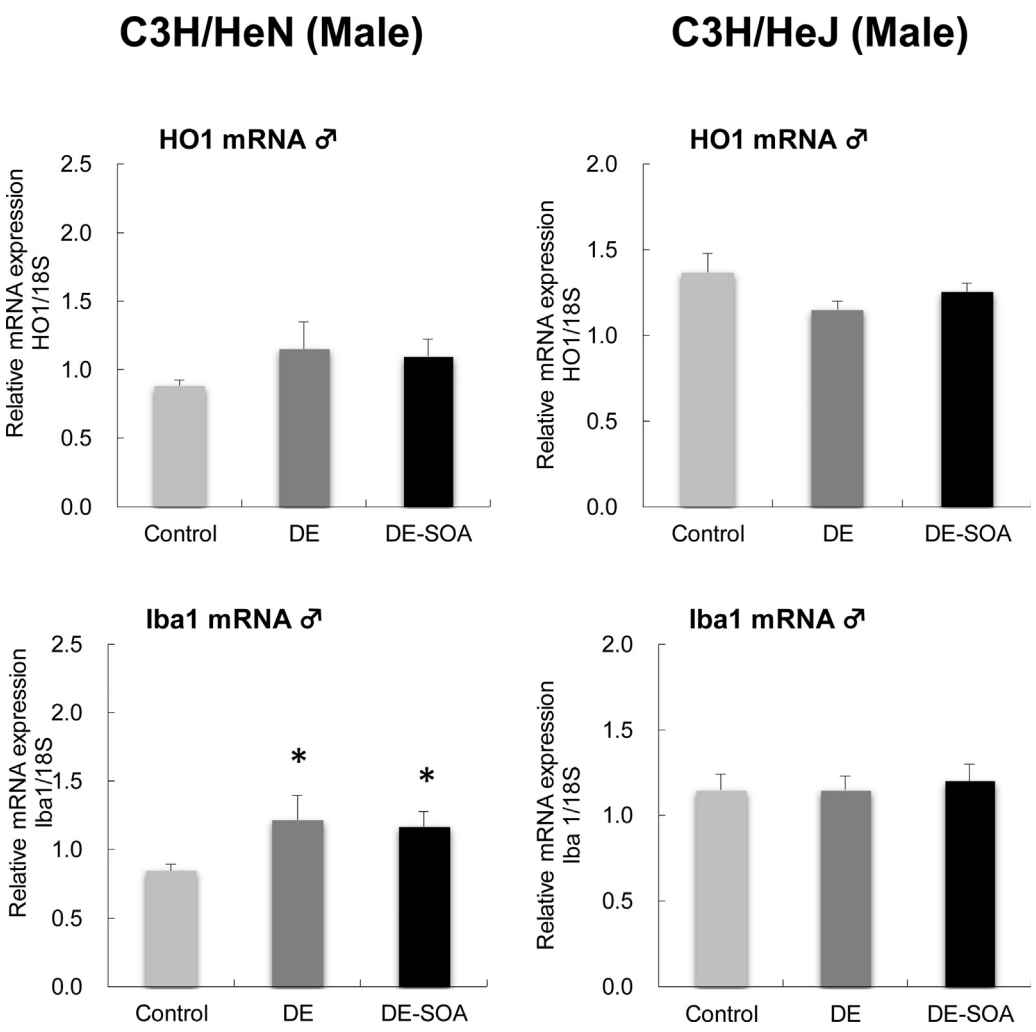

Fig. 7. Effects of DE or DE-SOA on mRNA expressions of HO1 and microglial marker Iba1 in the hippocampi of C3H/HeN neonatal male mice. Messenger RNA expression was examined by real-time RT-PCR method. Each bar represents the mean ± SE ($n = 8$, * $p < 0.05$ vs. control).
HO1 = heme oxygenase 1.
Iba1 = ionizing calcium-binding adaptor molecule 1.

hormones may play a role in gender difference in TLR4-mediated neuroimmune function (Figs. 6 and 7).

TLRs are transmembrane proteins with extracellular domains containing leucine-rich repeat motifs, which are evolutionarily conserved to recognize pathogen-associated molecular patterns (PAMPs) in bacteria, viruses, fungi and parasites (Koga and Mor, 2010). A previous study demonstrated that mice exposed to ozone and lipopolysaccharide developed asthma as a result of the activation of TLR4 expressed on the surfaces of inflammatory cells (Hollingsworth et al., 2007). Another study suggested that genetic polymorphisms in TLRs may explain the link between particulate matter, passive smoking, NO_2 and childhood asthma (Esposito et al., 2014). Similarly, TLR2 (−/−) and TLR4 (−/−) mice showed less ozone-induced airway hyperresponsiveness and neutrophilia than wild-type mice (Williams et al., 2007). Although TLR4 activation is known to be a first-line response of the innate immune system, whether and how environmental pollutants influence TLR4 signaling in the immature brain remain unclear.

In this study, C3H/HeN mice were selected because these mice are known to have enhanced neuroinflammation, when compared to other background strains, such as CD1, SJL and C57 (Höfling et al., 2014). In our previous study, we exposed male C3H/HeN and C3H/HeJ mice to 0, 5, 50 or 500 ppm of toluene, one of the volatile organic compounds, for 6 weeks, and examined the mRNA expression levels of molecules of the TLR4-related signal transduction pathway in the mouse hippocampi (Win-Shwe et al., 2011); we found that the relative mRNA expression levels of TLR4 and NF-κB activating protein were markedly increased in the C3H/HeN mice, but not C3H/HeJ mice, exposed to toluene. In addition, the mRNA expression level of heat shock protein 70, a possible endogenous ligand for TLR4, was also increased in the C3H/HeN mice exposed to toluene. These results suggest a possible role of TLR4 in the neurotoxic effects of toluene in mice.

In the present study, we used postnatal day 11 mice, which could still not open their eyes, so the learning and memory for the target was based on olfactory cues alone. As we attempted to identify the effects of exposure to DE and DE-SOA in early neonatal life, we used the olfactory-based spatial learning test, and demonstrated that TLR4-mutated male and female mice exposed to DE and DE-SOAs suffered from a deficit in spatial learning ability in early life; on the other hand, this deficit was also observed in the TLR-intact mice, but not prominent as in C3H/HeJ mice. We concluded that the impaired spatial learning ability in neonatal mice following exposure to DE and DE-SOAs is mediated by TLR receptors.

In a previous related study, we found that adult male mice showed sub-unit specific changes in the expression levels of the receptor subunits of NMDA, a learning and memory function-

related gene, in the hippocampus. Higher brain functions, such as long-term memory and maternal behavior were shown also to be affected in the animals exposed to DE-SOAs (Win-Shwe et al., 2014). The NMDA receptors are heterotetrameric protein complexes composed of two NR1 and two NR2 subunits with different isomeric forms (NR1 a-h, NR2A-D, NR3) expressing distinct biophysical properties (Carroll and Zukin, 2002; Laube et al., 1998). Binding of L-glutamate to the NR2 subunit and glycine to the NR1 subunit results in stimulation of the NMDA receptors (Clements and Westbrook, 1991). NMDA receptors and their subunits are known to be involved in synaptic plasticity and long-term potentiation, which are important for information encoding and storage throughout the brain, which in turn, are essential for learning and memory (Hunt and Castillo, 2012; Tsien et al., 1996). In the present study, the mRNA expression levels of the NMDA receptor subunits NR1 and NR2B were higher in the hippocampi of the male C3H/HeN mice exposed to DE and DE-SOAs. Thus, we found that impaired spatial learning ability induced by exposure to DE and DE-SOAs in the neonatal mice was accompanied by upregulation of the NR1 and NR2B subunits of the NMDA receptor. Consistent with our previous reports, we showed that nanoparticle-rich diesel exhaust exposure impaired the hippocampus-dependent spatial learning ability and that the novel object recognition ability was related to the increased NMDA receptor expressions in mice (Win-Shwe et al., 2008). However, the mRNA expression levels of the NR2A subunit of the NMDA receptor decreased after three months of exposure to DE-SOAs (Win-Shwe et al., 2014). These results suggest that exposure to DE and DE-SOAs may alter the composition or functions of the NMDA receptors, probably by interaction with other regulatory proteins or by alteration of the receptor localization or post-translational modification, which in turn affects learning and memory. Further studies are needed to explore the possible alterations in synaptic plasticity and long-latent potentiation under the condition of NMDA receptor subunit upregulation in the neonatal hippocampus.

In the present study, SOAs formed by oxidative reactions of either chemicals on the surfaces of the particles or particle-free volatile products were used. Primary particles may also be easily converted to SOAs. In this present work, we studied the effects of exposure to nanoparticle-rich diesel exhaust by exposing the mice to high doses of DE and DE-SOAs, because in our previous studies in mice, we found that while moderate doses had no effect on the brain functions, high dose-DE exposure significantly affected the learning and memory functions (Win-Shwe et al., 2012a, 2008, 2012b). A previous study stated that 10 $\mu g/m^3$ elevation in fine particulate air pollution was associated with an approximately 6% increase in the risk of cardiopulmonary mortality and 8% increase in the risk of lung cancer mortality (Pope et al., 2002). Increased plasma viscosity and altered blood rheology due to inflammatory processes in the lung was observed in both men and women exposed to air pollution (Peters et al., 1997). In a study conducted to determine the effect of inhalation of SOAs derived from oxidation of toluene, increased levels of heme-oxygenase-1, endothelin-1 and matrix metalloproteinase-9 were observed, without pulmonary inflammation, in ApoE$^{-/-}$ mice after 7 days' exposure (McDonald et al., 2012). With regard to human studies in the nervous system, a study suggested that air pollution exposure should be considered as a risk factor for Alzheimer's disease as well as Parkinson's disease, owing to the knowledge that accumulation of amyloid β42 and α-synuclein starts in childhood in subjects residing in areas with high levels of air pollution (Calderon-Garciduenas et al., 2008). Another study found a general cortical stress response in the EEG in humans after DE exposure (Cruts et al., 2008). In one of our studies we found that a single intranasal instillation of DE-SOA into mice was associated with a marked increase in the mRNA expression levels of proinflammatory cytokines, their transcription factors and neurotrophins in the lung tissues (Win-Shwe et al., 2013). However, there is still much to be explored with regard to the effects of DE and DE-SOA exposure on the higher brain functions of the neonatal brain, especially learning and memory functions.

In this study, we measured the mRNA expression levels of TNFα and COX2 in the hippocampi of the neonatal mice. No significant differences in the TNFα levels were found among the different exposure groups in either the C3H/HeN or C3H/HeJ mice, or indeed between these two groups of mice, suggesting that TNFα may have no role in the effects of DE/DE-SOA exposure, or that it may not be activated by air pollutant- induced TLR4 activation. However, the observation of increased COX2 expression only in TLR4-intact, and not TLR4-mutated, mice following exposure to DE/DE-SOA suggested that the increased expression of COX2 in the hippocampi of mice following DE/DE-SOA exposure is probably induced by TLR4 activation. COX is the enzyme that converts arachidonic acid to prostaglandins, and is responsible for the formation of prostanoids, including thromboxane and prostaglandins, which are involved in the inflammatory cascade. COX2 is an inducible form of cyclooxygenase released at the site of inflammation (Win-Shwe et al., 2015).

We also measured the mRNA expression levels of the oxidative stress marker HO1 in the hippocampus in this study. HO1 is a stress protein induced in response to a variety of oxidative stresses, which has been shown to have a protective effect in the face of oxidative challenges (Choi and Alam, 1996; Le et al., 1999). Neither the C3H/HeN, nor the C3H/HeJ mice in any exposure group showed increase in the hippocampal expression levels of HO1. These results indicate that neither DE nor DE-SOA induces oxidative stress, that HO1 does not protect against the deleterious effects of DE/DE-SOA exposure, and that the expression levels of HO1 are not affected by the interaction of TLR4 with DE or DE-SOA. Recent study indicates that prolong exposure to high dose of vehicle exhaust increased anxiety and depression-like behavior and triggered impaired memory function in adult male SD rats (Salvi et al., 2017). They suggest that elevated levels of free radicals in the brain regions such as prefrontal cortex, hippocampus and amygdala are associated with these behavioral and cognitive deficit.

Furthermore, we also measured the mRNA expression levels of Iba1, which is a microglial marker (Jeong et al., 2013). C3H/HeN mice exposed to DE and DE-SOA showed significantly higher hippocampal expression levels of Iba1 as compared to the control group, suggesting an ongoing inflammatory process associated with major immune cell microglial activation in the hippocampi of these mice. The microglial activation may possibly be mediated by TLR4 activation, since TLR4-mutated mice exposed to DE/DE-SOAs failed to show any increase of Iba1 expression in the hippocampus.

In our recent study, pregnant BALB/c mice were exposed to clean air, DE, or DE-SOA from gestational day 14 to PND 10 in exposure chambers. On PND 11, the preweaning mice were examined by the olfactory-based spatial learning test and investigated the expressions of neurological and immunological markers. In that study, the mice exposed to DE or DE-SOA took a longer time to reach the target as compared to the mice exposed to clean air in developmental period. The expression levels of neurological markers such as the NMDA receptor subunits NR1 and NR2B, and of immunological markers such as TNF-α, COX2, and Iba1 were significantly increased in the hippocampi of the DE-SOA-exposed preweaning mice as compared to the mice exposed to clean air in developmental period. Our results indicate that DE-SOA exposure brain developmental period may affect the olfactory-based spatial learning behavior in preweaning mice by modulating the expressions of memory function–related pathway

genes and inflammatory markers in the hippocampus (Win-Shwe et al., 2015). Our present results studied in C3H/HeN mice are consistent with previous results studied in BALB/c mice.

In the present study, although olfactory bulb might be associated with olfactory-based learning function, we focus to study the hippocampus which is critically associated with spatial learning ability in neonatal animal model (Wiedenmayer et al., 2000). Actually, we have examined the mRNA expression level of cyclic AMP (c-AMP) signaling pathway genes such as adenylyl cyclase type 3 (AC3) and olfactory-specific G protein (GOLF) presented on sensory neurons and proinflammatory markers in the olfactory bulb of BALB/c male PND11 neonatal mice. We found that expression level of neurological markers such as AC3, GOLF and immunological markers such as TNF-α, COX2 and Iba1 were increased significantly in olfactory bulb of DE-SOA exposed neonatal mice compared to the control mice. We suggest that developmental exposure to diesel engine exhaust origin SOA may affect c-AMP signaling pathway genes and the inflammatory markers in the olfactory bulb of neonatal mice (unpublished data).

In summary, it is well established that TLR4, a pathogen-associated molecular pattern (PAMP) receptor, initiates the innate immune response within the central nervous system (Rivest, 2009). Our results demonstrated that the potential toxic substances in the DE and DE-SOA may act as PAMPs and may exert their deleterious effects on the brain via TLR4, which upregulates the expression of the inflammatory marker COX2 and neuronal immune cell microglial marker Iba1 to induce neuroinflammation and enhances NMDA receptor subunit expression, which result in the neonatal mice taking a longer time to reach the target location in the spatial learning test. Limitation of our study is we could not say exactly which substances in the DE or DE-SOA are toxic to brain and we did not trace translocation of these potential toxic substances to the brain. We used ozone to generate DE-SOA by oxidative reaction. We have checked the ozone level and did not find added ozone in the DE-SOA chamber. This indicates that almost all added ozone are used for oxidative process and the effects of DE-SOA may not be due to ozone. Therefore, we suggest that potential toxic substances included in DE-SOA may affect olfactory-based spatial learning activity directly and indirectly by modulating neuroimmune biomarkers in the neonatal mice. Further studies are necessary for more thorough and detailed exploration of the neuroimmune mechanisms underlying the learning and memory disability observed in neonatal mice following in utero and early neonatal exposure to environmental pollutants.

5. Conclusion

We conclude here, our results showed that appearance of similar effect of DE or DE-SOA on olfactory-based spatial learning ability in two strains of mouse pups. However, strain difference was observed between C3H/HeN and C3H/HeJ groups and treatment effect was more prominent in C3H/HeJ mouse pups. We suggest that DE or DE-SOA induced inflammatory signal through TLR4 in TLR4 intact C3H/HeN mice and that signal and alteration in neuroimmune markers in the brain are critical for body homeostasis and at least in part, may protect from cognitive deficit. In contrast, no inflammatory signal and no induction of neuroimmune markers in TLR4-mutated C3H/HeJ mice and that triggers more prominent cognitive deficit in those mice. It is possible that involvement of neuroimmune system activation and TLR4 signaling in the olfactory-based spatial learning impairment in neonatal mice induced by exposure to environmental pollutants.

Conflicts of interest

The authors declare that they have no conflict of interest to report in relation to this work.

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