

Strain Differences Influence N-Methyl-D-Aspartate Receptor Subunit Gene Expression in the Olfactory Bulb of an Allergic Mouse Model following Toluene Exposure

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Key Words

N-methyl-D-aspartate receptor • Toluene • Olfactory bulb • Ovalbumin stimulation

Abstract

Objective: Genetic variation is a causative factor in differing sensitivities to environmental chemicals. The present study explored whether differences in mouse strains influence N-methyl-D-aspartate (NMDA) receptor expression in the olfactory bulb after low-level toluene exposure. **Methods:** Ten-week-old male C3H/HeN (H-2^k), BALB/c (H-2^d) and C57BL/10 (H-2^b) mice were exposed to 0, 5, 50 or 500 ppm of toluene for 6 h per day, 5 days per week for 6 weeks. Because individuals with allergic disease are more susceptible to volatile organic compound exposure, the animals of each strain were divided into 2 main groups, a non-allergy (NAG) group and an allergy (AG) group. The AG groups were stimulated with ovalbumin before toluene exposure. The mRNA levels of NMDA receptor subunits (NR1, NR2A and NR2B) in the olfactory bulbs of the NAG and AG groups were examined using real-time RT-PCR. **Results:** In C3H/HeN mice, the expression levels of NR1 and NR2B mRNA decreased significantly in the AG group exposed to 500 ppm of toluene; in the NAG group, however, the NR2A mRNA level increased significant-

ly in mice exposed to 50 ppm while the NR2A and NR2B mRNA levels decreased significantly in mice exposed to 500 ppm of toluene. No significant changes were observed in the NAG groups of BALB/c or C57BL/10 mice. However, in the BALB/c mice, the mRNA levels of NR1, NR2A and NR2B decreased significantly in the AG group exposed to 500 ppm of toluene. **Conclusion:** Mammalian strain differences in NMDA receptor expression after allergic stimulation can be observed in the mouse olfactory bulb after toluene exposure.

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Introduction

Toluene is one of the volatile organic compounds (VOCs) widely used in industry and is present in the indoor air of many homes from the use of common household products and from cigarette smoke [1]. Toluene mainly affects the central nervous system, causing headache, nausea, dizziness, drowsiness, confusion and memory loss [2]. The sensitivity of human responses to toluene exposure in both occupational and home settings varies widely. Different sensitivities to chemical exposure may be due to environmental, genetic and physiological factors. However, there are no data addressing whether some

people are more sensitive to toluene exposure due to their genetic background.

Experimental evidence has indicated that the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors is affected by toluene exposure [3–5]. Expression of the NMDA receptor appears early during development and plays an important role in synaptic transmission, neuronal plasticity and excitotoxicity [6]. NMDA receptors are widely distributed throughout the central and peripheral nervous systems, and NMDA sensitization may be one mechanism underlying the differing sensitivities to chemical exposure [7]. Therefore, in the present study, to determine the role of olfactory function in the induction of chemical sensitivity in 3 different strains of mice in response to chemical exposure, we focused on the expression of the NMDA receptor, a well-known target site of toluene.

Previously, our research group demonstrated that low-level toluene exposure affects memory function-related gene expression in the hippocampus of C3H/HeN mice [8] and aggravates the airway inflammatory responses in ovalbumin (OVA)-immunized C3H/HeN mice by modulating neuroimmune crosstalk [9]. In addition, we have also reported that the intraperitoneal administration of toluene caused a rapid and reversible rise in glutamate and taurine levels in the hippocampus of BALB/c mice [10] and that low-level toluene exposure upregulated the mRNA levels of NMDA receptor subunits and their related transcription factors in the hippocampus of BALB/c wild-type mice, but not in athymic nude mice [11]. Recently, we have shown strain differences in the extracellular amino acid neurotransmitter levels in the hippocampus in 2 congenic mouse strains, C57BL/10 (H-2^b) and B10.BR/Sg (H-2^k), after toluene exposure [12]. From the results of these previous studies, we hypothesized that strain differences may be an important factor explaining differing susceptibilities to environmental chemical exposure. Therefore, in the present study, 3 different mouse strains, namely C3H/HeN (H-2^k), BALB/c (H-2^d) and C57BL/10 (H-2^b), were used to examine whether strain differences influence NMDA receptor expression in the mouse olfactory bulb.

The olfactory system is the sensory system used to sense smells, detecting volatile, airborne substances and transducing these chemical stimuli into electrical signals. Some evidence suggests that olfactory receptor neurons directly project into the limbic system and are involved in the hippocampal-based spatial learning ability of infant mice [13]. A large cluster of olfactory receptor genes has been mapped to an area close to the human

leukocyte antigen locus on chromosome 6, and the human leukocyte antigen and linked olfactory receptor genes are clustered in haplotypes and are highly polymorphic [14]. In addition, current data from animal models have shown that neuropsychiatric manifestations are intimately associated with smell impairment, autoimmune dysregulation via autoantibodies (anti-NMDA receptor, antiribosomal P) and other mechanisms [15]. High-dose toluene inhalation for 40 days in 4- or 8-week-old rats caused a significant decrease in the number of mitral neurons in the olfactory bulb [16]. Previously, our research group showed that long-term exposure to low-level formaldehyde increased the number of tyrosine hydrolase-immunopositive periglomerular cells in the mouse olfactory bulb [17]. However, limited data are available regarding the effect of VOC exposure on NMDA receptor gene expression in the olfactory system.

In our study, OVA immunization in the allergy (AG) groups was used to create an allergic animal model to examine whether OVA-induced airway hyperresponsiveness exacerbates the susceptibility to VOC exposure compared with that in the non-allergy (NAG) groups. Moreover, our research group previously showed that an allergic condition may act as a stress stimulus on the hypothalamo-pituitary-adrenal axis via cytokines and nerve growth factors [18]. Therefore, another reason for using the NAG and AG groups in the present study was to examine whether an OVA-induced stressful condition would exacerbate the susceptibility to VOC exposure.

Although the mechanism responsible for the differing sensitivities to chemical exposure has not been clarified, the olfactory system may play a critical role in initiating the symptoms of chemical sensitivity [19, 20]. However, little is known about the role of the NMDA receptor in the olfactory bulb following environmental chemical exposure. In the present study, the effect of low-level toluene exposure on NMDA receptor expression in the olfactory bulb of 3 different strains of mice was investigated using real-time RT-PCR.

Individual variations in sensitivity to environmental chemicals also depend on genetic factors. Understanding strain differences in the expression of the NMDA subunits of the glutamate receptor in the olfactory bulb after toluene exposure may be helpful in identifying individuals with an increased susceptibility to environmental chemical exposure. In addition, specific manipulation of NMDA receptor subunit expression in different strains may be therapeutically useful from a clinical aspect in patients with chemical sensitivity syndrome.

Materials and Methods

Animals

Eight-week-old male C3H/HeN (H-2^k), BALB/c (H-2^d) and C57BL/10 (H-2^b) mice were obtained from Japan SLC Inc. (Shizuoka, Japan) and were used at 10 weeks of age. Food and water were given ad libitum. This study was approved by the Ethics Committee for the Use of Experimental Animals at the University of Occupational and Environmental Health, Japan.

Exposure to Toluene

Toluene vapor was generated using an organic solvent gas generator (Shibata Scientific Technology Ltd.) and was diluted with clean, filtered air to achieve the desired gas concentrations, then introduced into a stainless steel and glass chamber as described previously [21]. The toluene concentration in the chamber was monitored using gas chromatography (Model 353B, GL Sciences, Tokyo, Japan). The average toluene levels (mean $\pm$ SD) in the 5-, 50- and 500-ppm chambers were 4.9 ± 0.6 , 49.5 ± 4.4 and 496.7 ± 17.9 ppm, respectively. Each group of mice ($n = 6$) was exposed to a filtered air control (0 ppm) or 5, 50 or 500 ppm of toluene for 6 h (from 10:00 to 16:00 h) per day, 5 days per week for 6 weeks. The AG groups of mice were stimulated with OVA on days 0, 7, 21 and 42 approximately 1 h before toluene exposure; the NAG groups only received saline. The AG groups were injected with 10 μ g of OVA plus 2 mg of alum intraperitoneally on day 0 and 10 μ g of OVA only on day 7. Each of these mice was then challenged with nebulized OVA as a booster once every 3 weeks (days 21 and 42) during the exposure period, as described previously [22].

Quantification of Brain mRNA Expression Levels

One day following the final toluene inhalation, the mice were sacrificed under deep pentobarbital anesthesia and the olfactory bulbs were collected from each group of the 3 strains of mice and frozen quickly in liquid nitrogen, then stored at -80°C until total RNA extraction. The expression levels of the NMDA receptor subunits NR1, NR2A and NR2B as well as that of 18S mRNA in the olfactory bulb were estimated using a quantitative real-time PCR analysis performed using the ABI Prism 7000 Sequence Detection System (Applied Biosystems Inc., USA), as described previously [23]. Briefly, total RNA was isolated from the tissue samples using the BioRobot EZ1 RNA tissue mini-kit (Qiagen GmbH, Germany). First-strand cDNA was then synthesized from 500 ng of total RNA using SuperScript RNase H Reverse Transcriptase II (Invitrogen, USA), in accordance with the manufacturer's protocol. For use as standards, we diluted the olfactory bulb cDNA samples serially by several fold. Briefly, reactions were prepared in a final volume of 25 μ l containing 5 μ l of each of the 5-fold diluted cDNA samples or each of the diluted standards, 12.5 μ l of 2 $\times$ Master mix (Quantitect SYBR Green PCR Kit, Qiagen), 2.5 μ l of 10 μ M primer and 5 μ l of RNase-free H₂O. The SYBR Green I-based PCR was performed using a dissociation protocol and the following conditions: initial cycle of enzyme activation for 15 min at 95°C , followed by 20–35 cycles of amplification (20 cycles for the 18S rRNA and 35 cycles for the other genes) comprising an initial denaturation step at 94°C for 15 s, annealing at 56 – 59°C for 30 s and elongation at 72°C for 30 s. The tissue 18S level was examined for use as an internal control. Primers (NR1: ID_14810; NR2A: ID_14811; NR2B: ID_14812) were purchased from Qia-

gen. The data were analyzed using the comparative threshold cycle method. Then, the relative expression levels of each mRNA were individually normalized according to the 18S rRNA content in the respective samples, and the mRNA levels were expressed as mRNA signals per 18S rRNA.

Statistical Analysis

All results are expressed as the mean $\pm$ standard error (SE). Statistical analysis was performed using the StatMate II statistical analysis system for Microsoft Excel, version 5.0 (Nankodo Inc., Tokyo, Japan). Dose response data were analyzed using a one-way analysis of variance with a Bonferroni-Dunn post hoc analysis. A value of $p < 0.05$ was considered statistically significant.

Results

mRNA Expression Levels of NMDA Receptor Subunits in the Olfactory Bulbs of the NAG Mice Groups

The NAG C3H/HeN mice exposed to 500 ppm of toluene showed significantly decreased expression of NR2A and NR2B mRNAs ($p < 0.05$, $p < 0.01$ vs. 0 ppm), while the mice exposed to 50 ppm of toluene exhibited an increase in NR2A mRNA (fig. 1). In contrast, no significant change was observed in NAG BALB/c mice (fig. 2) or NAG C57BL/10 mice (fig. 3).

mRNA Expression Levels of NMDA Receptor Subunits in the Olfactory Bulbs of the AG Mice Groups

The AG C3H/HeN mice exposed to 500 ppm of toluene showed significantly decreased expression of NR1 and NR2B mRNAs in the olfactory bulbs ($p < 0.01$ vs. 0 ppm; fig. 1). However, AG BALB/c mice exposed to 500 ppm of toluene showed significant decreases in NR1, NR2A and NR2B mRNAs ($p < 0.05$, $p < 0.01$ vs. 0 ppm; fig. 2). In the AG C57BL/10 mice, only the NR2A mRNA level decreased significantly and dose-dependently in mice exposed to 5, 50 or 500 ppm of toluene ($p < 0.05$ vs. 0 ppm; fig. 3). These results indicate that allergic stimulation influences the host's response to external stimuli and that among the 3 strains, the C3H/HeN mice seemed to be more sensitive to toluene exposure than the BALB/c mice or the C57BL/10 mice. The C57BL/10 mice seemed to be less sensitive to toluene exposure than the other 2 strains. The expression of the NMDA receptor subunits in the olfactory bulbs of the 3 strains of mice after 6 weeks of toluene exposure is summarized in table 1.

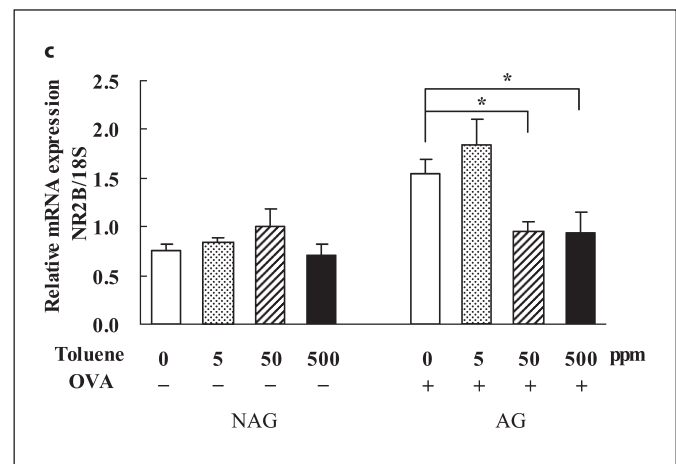
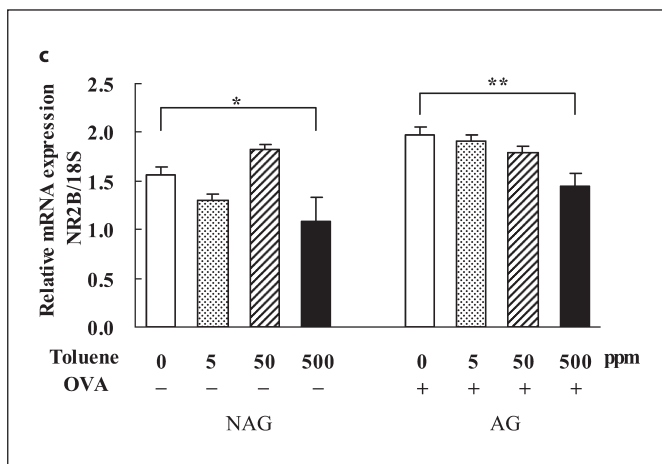
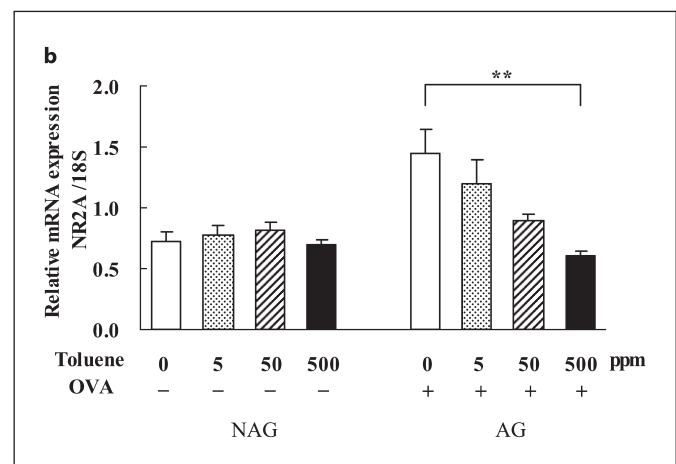
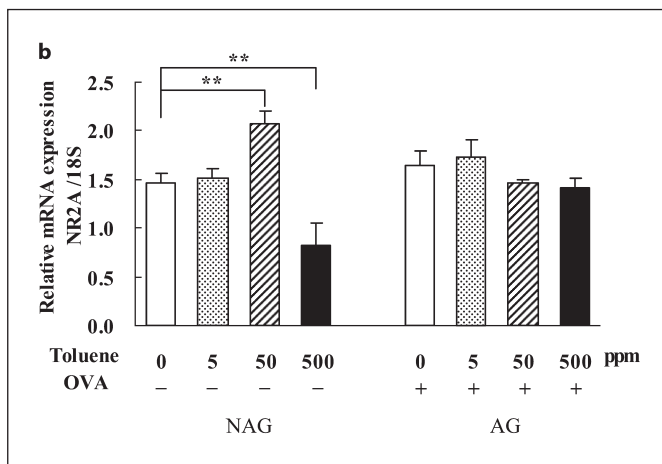
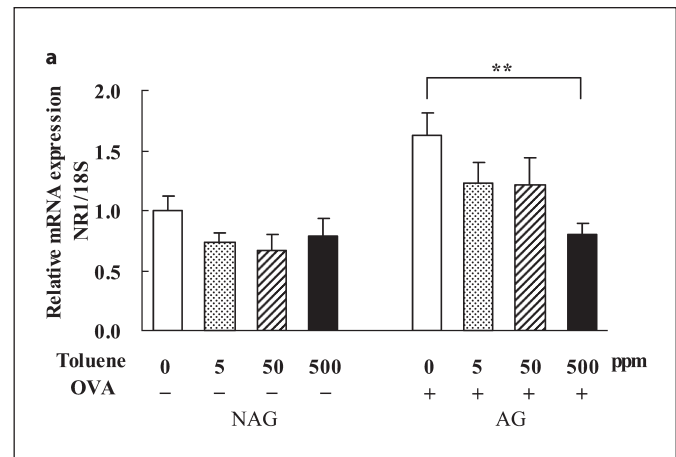
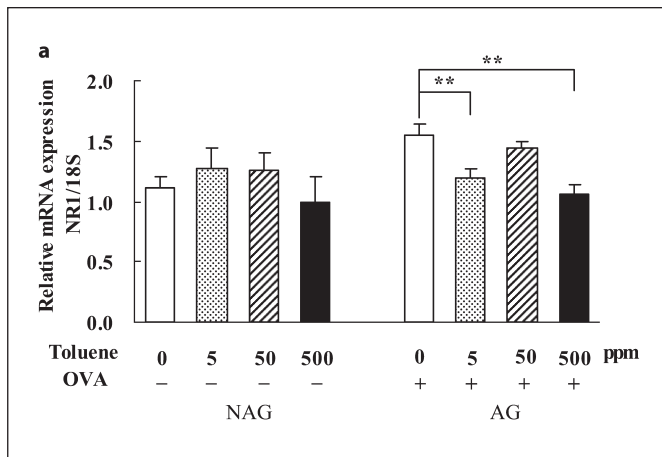


Fig. 1. Expression of NMDA receptor subunits in the olfactory bulb of C3H/HeN mice. NR1 (a), NR2A (b) and NR2B (c) mRNA expression in NAG and AG groups of C3H/HeN mice following exposure to 0, 5, 50 or 500 ppm of toluene for 6 weeks. Each bar represents the mean $\pm$ SE (n = 6). * $p < 0.05$, ** $p < 0.01$.

Fig. 2. Expression of NMDA receptor subunits in the olfactory bulb of BALB/c mice. NR1 (a), NR2A (b) and NR2B (c) mRNA expression in NAG and AG groups of BALB/c mice following exposure to 0, 5, 50 or 500 ppm of toluene for 6 weeks. Each bar represents the mean $\pm$ SE (n = 6). * $p < 0.05$, ** $p < 0.01$.

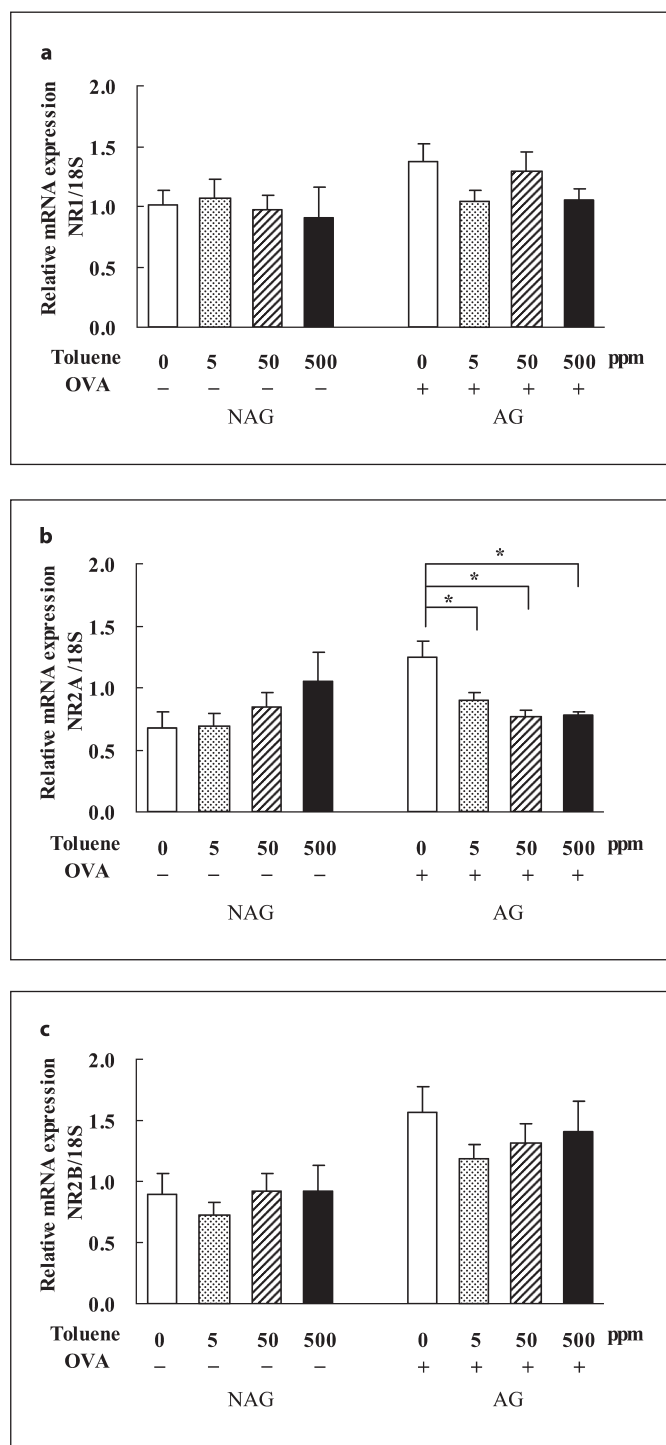


Fig. 3. Expression of NMDA receptor subunits in the olfactory bulb of C57BL/10 mice. NR1 (**a**), NR2A (**b**) and NR2B (**c**) mRNA expression in NAG and AG groups of C57BL/10 mice following exposure to 0, 5, 50 or 500 ppm of toluene for 6 weeks. Each bar represents the mean $\pm$ SE ($n = 6$). * $p < 0.05$.

Discussion

Our findings clearly indicate that strain differences influence the gene expression of NMDA receptor subunits in the mouse olfactory bulb following toluene exposure. Toluene exposure alone downregulated NR2A and NR2B mRNAs in the olfactory bulb of NAG C3H/HeN mice, but not in BALB/c and C57BL/10 mice. In C3H/HeN mice, the expression of NR2A and NR2B mRNAs was significantly decreased in mice exposed to 500 ppm of toluene, but the expression of NR2A mRNA was significantly increased in mice exposed to 50 ppm of toluene. The induction of a compensatory response may be one possible cause of the increased NR2A mRNA level in NAG C3H/HeN mice. Co-exposure to toluene and OVA stimulation downregulated the NR1 and NR2B mRNAs but not NR2A mRNA in AG C3H/HeN mice and also downregulated NR1, NR2A and NR2B mRNAs in BALB/c mice, but only downregulated NR2A mRNA in C57BL/10 mice. Regarding the C3H/HeN mice, a preliminary study involving exposure to toluene for 3 weeks showed that the expression of NR1 mRNA decreased significantly in immunized mice exposed to 5 ppm of toluene ($p < 0.05$ vs. 0 ppm control) but not in nonimmunized mice. Moreover, in that study, the NR2B mRNA level decreased significantly in nonimmunized mice exposed to 5 ppm of toluene ($p < 0.05$ vs. 0 ppm control) but not in immunized mice (unpublished data). Taken together, these results suggest that the effect of toluene exposure on NMDA receptor expression may depend on the type of animal strain, the type of subunit affected, the OVA immunization status and the dose of chemicals to which the animal is exposed. Based on our present study, we suggest that C3H/HeN mice may be highly susceptible, BALB/c mice may be moderately susceptible and C57BL/10 mice may be resistant to the effects of toluene exposure on NMDA receptor subunit composition in the olfactory bulb.

Individuals with different genetic backgrounds have different sensitivities to toxic chemical exposure [24]. Because of genetic polymorphisms, some people are more susceptible to chemical exposure than others. The adverse health effects of chemicals depend on the chemicals' toxicities and how people are exposed to the chemicals, as well as individual susceptibility. Recently, our research group reported strain differences in the extracellular glutamate levels in the hippocampus of 2 MHC-congenic mice strains, C57BL/10 (H-2^b) and B10.BR/Sg (H-2^k), following toluene exposure [13]. Therefore, we suggest that genetic variation in different strains of ani-

Table 1. Summary of the expression of NMDA receptor subunits in the olfactory bulb of 3 strains of mice after 6 weeks of toluene exposure

Mouse strain	NMDA receptor expression					
	NR1		NR2A		NR2B	
	OVA (-)	OVA (+)	OVA (-)	OVA (+)	OVA (-)	OVA (+)
C3H/HeN (H-2 ^k)	→	↓	↑ ↓	→	↓	↓
BALB/c (H-2 ^d)	→	↓	→	↓	→	↓
C57BL/10 (H-2 ^b)	→	→	→	↓	→	→

→ = Not significant; ↓ = significantly decreased at 500 ppm of toluene; ↑ = significantly increased at 50 ppm of toluene.

mals plays a role in individual susceptibility to toxic chemicals.

The NMDA receptors are composed of the obligatory NR1 subunit and at least 1 copy of NR2A, NR2B, NR2C or NR2D. The presence of a specific NR2 subunit reportedly determines the properties of these receptors, including agonist affinity, magnesium sensitivity, deactivation kinetics, modulation by polyamines and channel conductance [25, 26]. Cruz et al. [3, 4] have shown that inhibition by toluene of the activity of recombinant NMDA receptors expressed in *Xenopus* oocytes occurs in a subunit-dependent manner, with NR1/NR2B receptors being significantly more sensitive to toluene than NR1/NR2A receptors. In addition, our previous study demonstrated that chronic exposure of mice to 50 ppm of toluene for 12 weeks caused a significant upregulation of the NR2B subunit, which was associated with the simultaneous induction of CaMKIV, CREB1 and FosB/ΔFosB in the hippocampus of C3H/HeN mice [8]. We suggest that the upregulation of hippocampal NMDA receptor expression found in our previous study might have arisen from compensatory responses resulting in the induction of neuroprotection. On the other hand, the expression level of NR2B, but not of NR1 or NR2A, was significantly reduced in cultured cerebellar granular neurons from rat pups exposed to toluene on postnatal days 4–7 [27]. Williams et al. [28] demonstrated that exposure to 8,000 ppm of toluene for 10 days (30 min/day) increased the NR1 and NR2B protein levels in the medial prefrontal cortex and decreased the levels of the NR1 subunit in the substantia nigra compacta. They also reported that the NR2B subunit levels within the nucleus accumbens were significantly higher after toluene exposure. Taken together with our findings, these

results suggest that toluene differentially alters the levels of specific NMDA receptor subunits in a regionally selective manner. However, how toluene causes the downregulation of the NMDA receptor subunits (NR1, NR2A and NR2B) in the olfactory bulbs of the 3 strains of mice examined in this study remains unclear. One possibility is that toluene directly alters the subunit configuration of the NMDA receptors in the mouse olfactory bulb, disrupting the neuronal sensitivity to toluene. Another possibility may be a decrease in the number of mitral neurons in the olfactory bulb after toluene intoxication [16].

Regarding OVA immunization, our previous study indicated that the number of corticotropin-releasing hormone-immunoreactive neurons and adrenocorticotrophic hormone-immunoreactive cells and adrenocorticotrophic hormone mRNA expression levels were significantly elevated in AG mice exposed to 80 ppb of formaldehyde but not in NAG mice [18]. This result is consistent with our present findings, which showed significantly different results between AG and NAG mice exposed to low levels of toluene. The expression of the NMDA receptor subunits NR1 and NR2B was downregulated in the AG groups of the C3H/HeN and BALB/c mice but not in the C57BL/10 mice. In contrast, the expression of NR2A was downregulated in the AG groups of the BALB/c and C57BL/10 mice but not in the C3H/HeN mice. The expression of all 3 NMDA receptor subunits remained unchanged in the NAG groups of the BALB/c and C57BL/10 mice. We suggest that chemical sensitivity may also be associated with the immune status of individuals. BALB/c mice have been reported to be Th2-dominant [29], while C57BL/10 mice are generally Th1-dominant. In our previous study, we showed that nasal exposure to low-level toluene modu-

lates the mRNA expression of proinflammatory cytokines such as IL-1 β and CCL3 as well as that of the NMDA receptor subunit in the olfactory bulb of C3H/HeN mice with OVA sensitization, but not in nonsensitized mice [30]. It may be suggested that neuroimmune crosstalk plays an important role in sensitized mice exposed to environmental chemicals. Under this hypothesis, strain differences do not affect chemical sensitivity directly and require an activated neuroimmune system to respond to environmental chemicals. Although the symptoms of chemical sensitivity do not appear to be the result of an allergic reaction, we suggest that, at least in part, allergic conditions may reduce the threshold for susceptibility to toluene exposure at low levels that do not affect normal healthy individuals.

In summary, this is the first report of mammalian strain differences modulating NMDA receptor subunit expression in the mouse olfactory bulb following toluene exposure in the presence or absence of allergic stimulation. Our findings clearly demonstrated that not only genetic factors but also allergic conditions influence the susceptibility of individuals to VOC exposure. Further studies are needed to clarify the mechanism of NMDA receptor modification at cellular and molecular levels after low levels of environmental chemical exposure.

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