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Effects of Exposure to VOCs on Spatial Learning and Memory Capacity and the Expression of NMDA Receptor in Mice

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Abstract: Volatile Organic Compounds (VOCs) susceptibility is triggered by VOCs present both indoors and outdoors. It has become an important public health concern in both domestic and occupational settings. In the present study, memory and learning capacity, grip strength of mice after exposed to VOCs were investigated. Meanwhile, N-Methyl-D-Aspartate (NMDA) receptor subunit 1, 2A and 2B expressions in hippocampus and oxidative damage levels in brain were analyzed. Results showed no significant changes in grip strength but mice following exposure to high dose VOCs took significantly longer swimming time to reach the platform and made more errors in comparison to the control. The RT-PCR analysis also revealed NR1, NR2A, NR2B decreased in hippocampus. Moreover, VOCs exposure induced oxidative stress in brain. The findings indicate that short-term VOCs inhalation didn't induce damage on physique and motor function of mice but negatively affected learning and memory capacity by decreasing expression of the NMDA receptor through the direct effects on the nervous system. Oxidative damage in brain might be implicated in VOCs-induced neurotoxicity.

Key words: Volatile organic compounds, hippocampus, Morris water maze, oxidative damage, NMDA receptor, learning and memory

INTRODUCTION

Volatile Organic Compounds (VOCs) are organic chemicals that have a high vapor pressure at room-temperature conditions. They are regarded as the main component of indoor air pollutants and important chemicals in the global chemical industry. With the continuous rise of interior decoration and rapid development in chemical industry, VOCs pollution has also increased considerably in both domestic and occupational settings. As the most populous developing country in the world, China is experiencing a serious VOCs-polluted period (Tang *et al*., 2009). Meanwhile, data showed concentration of VOCs in these places varies from 10-50 times of Indoor Air Quality Standard in China (GB/T 18883-2002) (Uchida *et al*., 2004).

VOCs exposure in environment occurs mainly via inhalation. Recent studies showed that VOCs can lead to asthma and the amount of the breath volatile organic compounds can be used for asthma and pulmonary disease diagnosis (Fens *et al*., 2011; Ibrahim *et al*., 2011). Persistent effects have been reported in rats exposed daily to toluene for up to 4 months including motor and cognitive impairment (Berenguer *et al*., 2004). Studies also found that formaldehyde inhibited the learning behavior and the memory of rats and mice, caused oxidative damage in brain simultaneously (Lu *et al*., 2008). However, other studies in rats reported that toluene at daily 6 h for 4 weeks improved performance in the water maze (Von Euler *et al*., 2000). Besides, effects on spatial learning and memory capacity after exposed to the most common indoor VOCs have not been discussed.

In light of these reports, researchers use the Morris water maze and the grip strength meter to evaluate the central nervous toxicity. The Morris water maze is a widely used test of spatial learning and memory capacity for rodents, making it a key technique in the investigation of central nervous system. Studies (D'Hooge and de Deyn, 2001) investigated on the use of the MWM in assessing learning and memory capacity showed that there is relationship between performance in the MWM and both neurotransmitter systems and drug effects (McNamara and Ronald, 1993). It has been proven to be a robust and reliable tool that is strongly correlated with NMDA (N-Methyl-D-Aspartate) receptor function (Moser *et al*., 1998; Bannerman *et al*., 1995) which plays

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a key role in spatial memory and learning capacity, therefore we examined the expression of NR1, NR2A and NR2B in hippocampus. Moreover, locomotor behavior is an important indicator for evaluation of behavioral toxicology. The grip strength meter is devised for the study of the impact on physique and motor function of mice from chemicals which is a useful tool to evaluate damage on nerve, bone and muscle.

Meanwhile, oxidative damage in brain was analyzed because studies have shown that the first response to organic compounds was observed on biochemical parameters (Songur *et al*., 2010). Besides, oxidative damage was one of the most basic and serious effects of VOCs exposure which results from an imbalance between the excessive formations of Reactive Oxygen Species (ROS) and limited antioxidant defenses (Wang *et al*., 2012). ROS are important mediators of cellular injury and play a putative causing in oxidative damage (Matsuoka *et al*., 2010). It can contribute to a variety of diseases and may be present in situations of toxicity (Halliwell, 1997; Kadiiska and Mason, 2000). ROS-initiated oxidative damage can be regulated by cell defense mechanisms including Glutathione (GSH) (Matsuoka *et al*., 2010). As a product of lipid peroxidation, Malondialdehyde (MDA) can be used to evaluate the oxidative damage on lipid (Lu *et al*., 2008). Thus, analysis of ROS, GSH and MDA levels in brain can reflect potential oxidative stress through source, defense system and harmful product after exposed to VOCs.

In view of the above reasons, the present study was designed to investigate the effects of short-term exposure of mice to VOCs on spatial learning and memory capacity. Meanwhile, function-related gene NR1, NR2A and NR2B expression in hippocampus were analyzed to explore the simultaneous changes of NMDA receptor. Moreover, oxidative stress was also assessed as a possible underlying mechanism.

MATERIALS AND METHODS

Animals: Thirty male Kun Ming mice (4-6 weeks old) and standard laboratory rodent diet were purchased from the Experimental Animal Care Center of Dalian Medical University. The mice were housed in individual plastic shoebox cages (n = 3) and left undisturbed for a week in a temperature controlled (22-24°C), moderate humidity (40±5%) colony room with corncob bedding. Food and water were available *ad libitum*. Then, mice were divided into control and two exposure groups (n = 10 each) randomly. All animals were treated following animal welfare guidelines.

VOCs exposure: Formaldehyde, benzene, toluene and xylene were chosen as test substance because they are common and typical volatile organic compounds in both domestic and occupational settings (Bernstein *et al*., 2008; Kum *et al*., 2010; Yeatts *et al*., 2012). Gases vapor were generated by organic solvent (Tianjin, China), diluted with clean air then introduced into a 100 L plexiglass chamber. An Interscan 4160 digital electrochemical analyzer (Interscan Corporation, USA) and Gas Chromatography GC-2010 (Shimadzu Corporation, Japan) were used to monitor the concentrations of formaldehyde and benzene serious separately. Exposure to VOCs of 10-50 times of Indoor Air Quality Standard (GB/T 18883-2002, China) were the current concentration of household and occupational settings (Pandit *et al*., 2001; Uchida *et al*., 2004). Accordingly, 0 (control) 1.0 + 1.1 + 2.0 + 2.0 mg/m^3 (low concentration) 5.0 + 5.5 + 10.0 + 10.0 (high concentration) mg/m^3 formaldehyde + benzene + toluene + xylene levels were applied as the exposure concentrations in the present study. Monitoring data showed that the average VOCs levels (mean±SD) of the low and high group were 6.1±0.6, 30.5±3.34 mg/m^3, respectively correspond to 10, 50 times of Indoor Air Quality Standard (GB/T 18883-2002). Mice were exposed during 15:00-17:00, 2 h day^{-1} for 10 days.

Morris Water Maze (MWM) behavioral test: Morris Water Maze System (Beijing, China) is composed of water maze pool (100 cm in diameter, 22 cm in depth), escape platform (9 cm in diameter), video camera and computer. Water temperature was fixed at 24±2°C. Escape platform was set 0.5 cm below the water surface, 20 cm far vertical to the wall. The maze was divided into four quadrants, escape platform was set in quadrant 1, 2 and the starting point for each training trial was the mid-arc of each quadrant (Win-Shwe *et al*., 2008). Before the formal test, mice (n = 5) were allowed to swim freely during 120 sec trails to enable them to adapt water. Then, the mice were performed in MWM behavioral test for 11 consecutive days after each day's exposure experiment: 10 days for the hidden-platform acquisition training and 1 day for probe trail test to examine memory retention.

The hidden-platform acquisition training began after 30 min of the exposure experiment. The mice were placed in the water facing the wall of the pool separately from four designated starting points. Maximum duration of each trail is 120 sec. The video camera and tracking system were used to measure the escape latency (time required to find the platform). If the mice found the platform, they were allowed to rest on the platform for

10 sec if they could not find the platform within 120 sec then the experimenter guided them to the platform and rest for 20 sec. Meanwhile, the latency was recorded as 120 sec. Then, on day 11, the platform was removed away from the pool. Each mouse was released from a certain point to swim for 120 sec. The video camera and tracking system were used to recorded time, track and swimming speed in each quadrant. The errors made by mice were recorded too. Errors were defined as mice's swimming across the escape platform without stopping on it.

Determination of grip strength: The grip strength (Shandong Academy of Medical Sciences, China) is usually used to evaluate the impact on muscle and locomotor activity from chemicals. After the exposure experiment of each day, another 5 targeted mice from each group were subjected to the grip strength task to be performed on 10 consecutive days. Each mouse had 3 tests per day with interval of 5 sec.

Measurements of ROS, MDA and GSH in brain: Mice were killed immediately after the final experiment. Brains were collected in ethanol on ice box. Hippocampus was isolated and stored at -80°C for RT-PCR. The rest part of the brain was homogenized in ice-cold 0.9% NaCl to produce 1:9 homogenates. Homogenates were centrifuged at 2500 r min^{-1} for 10 min at 4°C. The supernatant liquid was collected for measurements of MDA, GSH and total protein. ROS were analyzed using the DCFH-DA probe following the instructions of ROS kit. DCFH-DA reacts with ROS in brain to form the fluuorescent DCF and observed using a Hitachi F-4500 fluorescence spectrophotometer. Specific operations were carried out according to the instructions of the kit from Nanjing Jiancheng Bioengineering Institute.

RT-PCR analysis: Total RNA was collected from the hippocampus samples using the Takara RNAiso Plus kit (TaKaRa Company, Dalian, China) according to the manufacturer's protocol. Before Reverse Transcription (RT) of RNA to cDNA, the purity of total RNA was tested by measuring OD260, 280 and 320. If total RNA samples producing an OD260/280 nm value between 1.8 and 2.2 then it can be used for cDNA synthesis.

Next, researchers performed first-strand cDNA synthesis from the total RNA using Biometra Tpersonal Thermocycler (Biometra, German). Reverse Transcription reaction tubes were placed on ice box. The 5 μL total RNA was used as template and was reverse transcribed to cDNA using PrimeScript™ RT reagents kit (TaKaRa Company, Dalian, China) in a final reaction volume of 10.0 μL. Then, incubate at 37°C for 15 min, 85°C for 5 sec to inactivate the enzyme. Finally, β-actin, NR1, NR2A, NR2B expressions were examined by using a RT-PCR Method (Applied Biosystems Inc., USA). The primers for the RT-PCR were synthesized by the TaKaRa Biotechnology Company (Dalian, China). The primer sequences were shown in Table 1. The total volume of the reaction mixture was 20 μL. The process include holding and cycling stage, 40 cycles of amplification consist of initial denaturation at 95°C for 10 sec, annealing at 60°C for 20 sec, elongation at 95°C for 5 sec. The relative quantification of gene transcription among was analyzed by using the $2^{-\Delta\Delta CT}$ Method (Livak and Schmittgen, 2001).

Table 1: Sequences of primer used in RT-PCR

Genes	Primer (5'-3')	Length (bp)
β-Actin	Forward: GAA GAT CCT GAC CGA GCGT	249
	Reverse: CCA CAG GAT TCC ATA CCC AA	
NR1	Forward: GCT GTA CCT GCT GGA CCG CT	219
	Reverse: GCA GTG TAG GAA GCC ACT AT	
NR2A	Forward: GCT ACG GGC AGA CAG AGA AG	257
	Reverse: GTG GTT GTC ATC TGG CTC AC	
NR2B	Forward: GCT ACA ACA CCC ACG AGA AGAG	314
	Reverse: GAG AGG GTC CAC GCT TTCC	

Statistical analysis: Data were expressed as the mean±Standard Deviation (SD). Two-way Analysis of Variance (ANOVA) was performed to analyze the escape latency and the grip strength. The results of RT-PCR and oxidative damage were tested for significance using Student's t-test (Origin 7.5). A $p<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Effect of exposure to VOCs on spatial learning and memory ability: As shown in Fig. 1, escape latency of different groups showed a gradually decrease trend during 10 days training. But escape latency of the control group decreased fastest. A two-way ANOVA (day x treatment) of the escape latency revealed the effects of day ($F = 22.42$, $p<0.01$) and VOCs exposure ($F = 16.77$, $p<0.01$) and no effect of the day versus treatment ($F = 1.68$, $p>0.05$). A further multiple comparison indicated that from the 4th day, the high concentration group showed a statistically significant difference compared with the control group ($p<0.05$ or $p<0.01$). Also, the number of errors made by mice in each group decreased during 10 days experiment. Mice in the VOCs exposed groups made a greater number of total mistakes than the control during performance of the MWM test and this was evident from day 3 (Fig. 2).

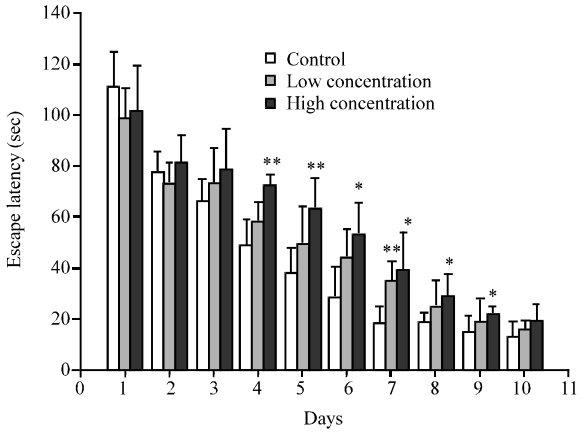

Fig. 1: Escape latency of mice (n = 5) exposed to VOCs at different concentrations during MWM test (*p<0.05; **p<0.01, compared to the control)

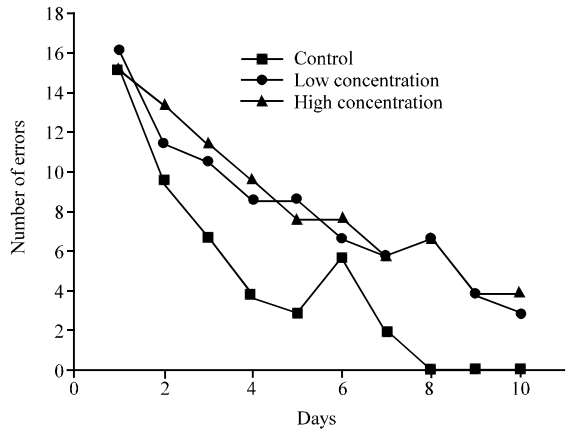

Fig. 2: Number of errors made by mice (n = 5) of different groups during MWM test

The probe trail test was used to examine the spatial memory of animal. On day 11, time spent in target quadrant of different is shown in Fig. 3a. Mice in the control spent more time in quadrant 1 than other groups. A Student's t-test showed a significant decrease between the control and high concentration group ($p<0.01$). The swimming tracks spent in each quadrant during probe trails are shown in Fig. 3b. In quadrant 1, trails in the control were more intensive than other groups. Mice in low concentration group switched their search areas frequently and aimlessly in different quadrants. However, mice in high concentration group even spend much more time exploring in the wrong quadrant. Meanwhile, swimming speed in target quadrant of the control was the slowest although, there were differences between each group Fig. 3c. This indicates that mice in the control spent more time and explored carefully in the target quadrant.

Effect of exposure to VOCs on grip strength: The grip strength was performed on 10 consecutive days. A two-way ANOVA analysis of the grip strength revealed there were significant effects of day ($F = 25.25$, $p<0.01$) and no main effects of VOCs treatment ($F = 2.85$, $p<0.05$) and no interaction of the day versus treatment ($F = 0.85$, $p>0.05$). Figure 4 shows the performance on each day. A further statistical analysis showed no significant changes between the control and the VOCs exposed groups ($p>0.05$). The results indicate that short-term exposure of mice to VOCs does not induce damage on physique and motor function of mice.

Oxidative damage in mice brain: Results showed a dose-effect relationship between ROS fluorescence, MDA, GSH levels and VOCs concentration. In low concentration group, the changes in MDA and GSH levels didn't show any significance but ROS fluorescence increased significantly ($p<0.05$). However, in high concentration group, ROS fluorescence (Fig. 5a) and MDA levels Fig. 5b increased significantly ($p<0.01$) while GSH levels showed a prominent decrease ($p<0.01$) (Fig. 5c). Moreover, ROS fluorescence, MDA levels increased by 1.62, 1.80 times while GSH levels showed a 1.44 times decrease as compared to the control after exposed to high concentration VOCs.

Expression of NR1, NR2A and NR2B in hippocampus after VOCs exposure: The results of RT-PCR in this study showed that the expression of NR1, NR2A and NR2B subunits increased initially in low concentration group, then decreased significantly in higher concentration ($p<0.05$ or $p<0.01$, compared to the control) (Fig. 6). Furthermore, NR1, NR2A and NR2B in hippocampus reduced by 27.4, 29.9 and 33.1%, respectively as compared to the control after exposed to high concentration VOCs. This indicates that expression of NMDA receptors were blocked after exposed to higher levels VOCs.

VOCs exposure has been associated with interior decorating which is becoming increasingly popular in China. In addition, current Occupational Exposure Limits (OELs) for benzene and formaldehyde are 6 and 0.5 mg/m^3 (MOH, 2007) but increasing demands for organic solvents in chemical industry strengthen the concentration in occupational settings. Studies have shown that the concentrations of several VOCs in some occupational environment were far higher than the limit (Fan *et al*., 2006; Zhang *et al*., 2007).

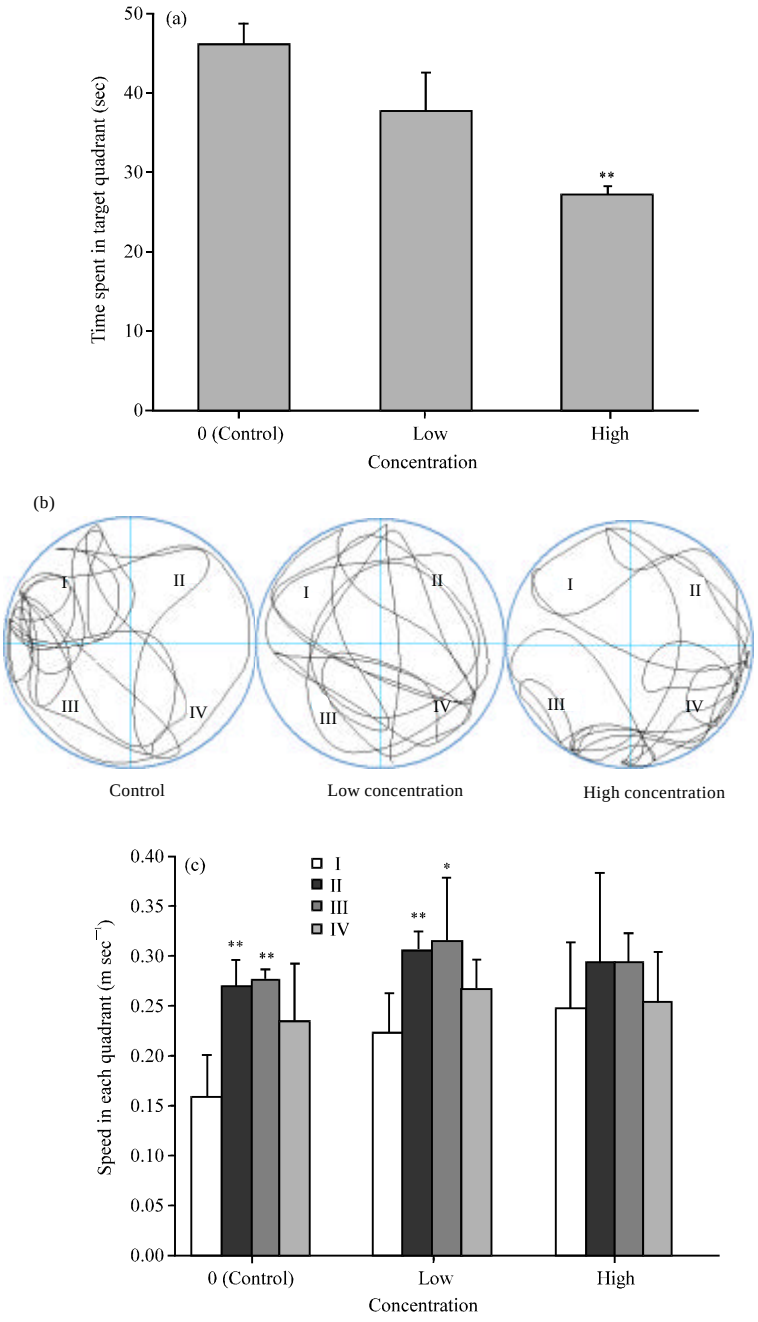

Fig. 3: a) Swimming time, b) track and c) speed spent in the target quadrant during probe trails (n = 5). (*p<0.05; **p<0.01, compared to the control)

Human brain is inherently susceptible to injury caused by toxic chemicals especially indoor organic compounds. Deleterious effects of organic solvents on the central nervous system are attributed to the incorporation of their molecules into the neural cell membrane which disturb ion transfer and NMDA receptor expression (Malek *et al*., 2003) inducing deviations in neurotransmitters (Win-Shwe and Fujimaki, 2010) causing pathological symptoms like dizziness, depression and memory impairment (Echeverria *et al*., 1991; Kishi *et al*., 1993). The Morris water maze test reflects the relationship between performance of mice and both neurotransmitter systems and drug effects (McNamara and Ronald, 1993). MWM performance has been linked to NMDA receptor

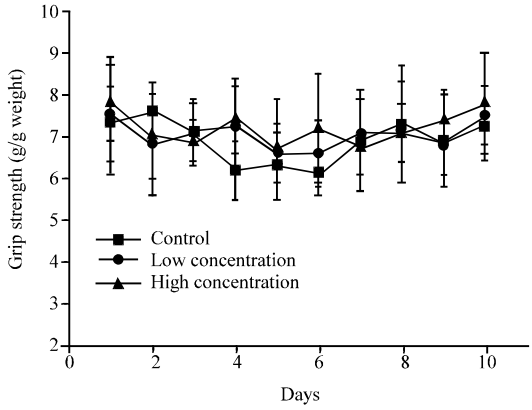

Fig. 4: Grip strength of mice (n = 5) during the exposure period. The grip strength is in average, the unit is g/g weight

function, making it a widely used technique to assess hippocampus dependent learning and memory capacity in rodents (Vorhees and Williams, 2006; Van Dam *et al*., 2006).

The present behavioral studies show that the inhalative exposure of mice to VOCs negatively affects their performance in the MWM test. Mice exposed to VOCs needed longer swimming time to find the platform. And this was accompanied by greater numbers of mistakes. Malek's study also showed that rats exposed to 5.4 ppm formaldehyde at daily 2 h for 10 consecutive days took significantly longer time to reach the finish and made significantly more errors in comparison to the control (Malek *et al*., 2003). However, Euler's findings indicated that 80 ppm toluene exposed rats showed a longer time in the correct quadrant in a Morris swim maze which is contradictory to the present result that mice in the control spent more time and explored carefully in the target quadrant. Animal species and types of pollutants were speculated as to the possible reason of different results (Von Euler *et al*., 2000). Meanwhile, the grip strength didn't show any significance during the experimental period. Since, the Morris Water Maze System is usually used to investigate changes in memory and learning behavior in animals, researchers can conclude that under investigational conditions VOCs inhalation didn't induce damage on physique and motor function of mice but negatively affected learning and memory capacity by the direct effects on the nervous system.

The NMDA receptor in the hippocampus is composed of NR1, NR2 (A, B, C, D) and NR3 (A, B) subunits (Cull-Candy *et al*., 2001) which plays important roles in the learning and memory processes of animals.

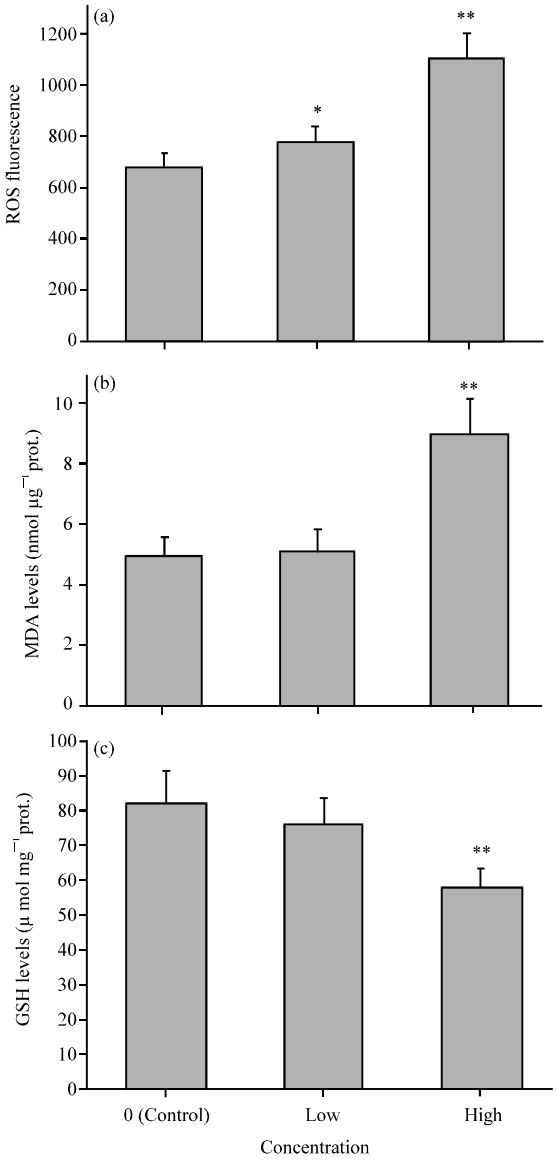

Fig. 5: Effects of VOCs exposure on, a) ROS, b) MDA and c) GSH levels in mice brain (n = 5) (*p<0.05; **p<0.01, compared to the control)

A functional NMDA receptor is constructed from combinations of an obligatory NR1 subunit and different NR2 subunits (Millan, 2005). Studies have shown that knockout mice lacking either NR1 or NR2B subunit of the NMDA receptor show cognitive deficits and severely impaired performances of the MWM task (Clayton *et al*., 2002; Rondi-Reig *et al*., 2006). NR2B subunit is also involved in hippocampus long-term potentiation which is critical in memory formation (Tang *et al*., 1999; Nakazawa *et al*., 2004). While NR2A subunit is involved in the glutamatergic neurotransmission (Vitanova, 2011).

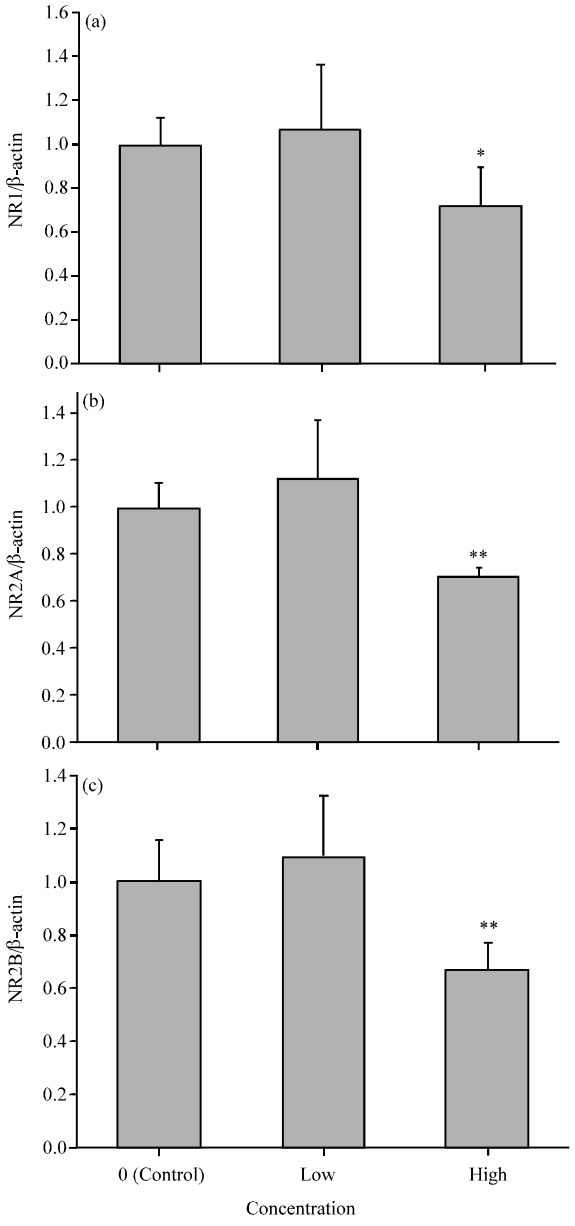

Fig. 6: Effects of VOCs exposure on the, a) NR1, b) NR2A and c) NR2B expression in hippocampus (n = 5) (*p<0.05; **p<0.01, compared to the control)

In the study, NR1, NR2A and NR2B expression initially increased then decreased in hippocampus after exposed to different concentrations of VOCs. The up-regulation of those functional gene in low concentration group consistent with Lu and Tong's studies (Lu *et al*., 2008; Tong *et al*., 2012) indicating that acute organic compounds treatment at low concentration increase NR1, NR2A and NR2B expression. Interestingly, NR1, NR2A and NR2B expression decreased significantly in high concentration group when compared to the control. Some reports also support this phenomenon. For example, down-regulation of NR1, NR2A and NR2B expression was associated with significant memory decline after 30 days of chronic excess formaldehyde treatment (Tong *et al*., 2012). A decrease in NR1 and NR2B expression has been found in Alzheimer's disease patients (Hynd *et al*., 2004; Amada *et al*., 2005).

Taken together the results of MWM test and NMDA receptor expression, researchers speculate that neuronal damage or blockade of the NMDA receptor initially occurs when exposed to low level VOCs which affects performance of mice in MWM task. Then, the expressions of NR1, NR2A and NR2B subunits increase to compensate. Thus, the transmitter receptors would increase to compensate the deficit when synaptic transmission is inhibited by short-term low level VOCs exposure. But in high concentration group, NR1, NR2A and NR2B expression decreased significantly, consistent with the significant longer escape latency and greater number of mistakes during performance of the MWM test. This phenomenon demonstrates that severe impairment to cognitive capacity occurs when exposed to high level VOCs.

To further explore the possible mechanisms of VOCs-induced neurotoxicity, researchers performed ROS, MDA and GSH analysis in mice's brain. The balance of oxidation and antioxidation is essential for normal biological functioning of the cells and tissues (Aly *et al*., 2009). ROS form as a product of the normal metabolism of oxygen and play important roles in cell signaling and homeostasis (Devasagayam *et al*., 2004). However, ROS levels can increase dramatically when exposed to toxic gases such as formaldehyde, toluene and xylene (Qujeq *et al*., 2004). This may result in significant damage to cell membrane function, gene expression and cause lipid peroxidation. Malondialdehyde (MDA) is one of the most important products of lipid peroxidation which can be used as a key marker to evaluate the oxidative stress (Doreswamy *et al*., 2004). Glutathione (GSH) can clear out excess free radicals, therefore plays critical roles in anti-oxidation in organs (Kurata *et al*., 1993). In the present study, there are dose-effect relationships between ROS fluorescence, MDA, GSH levels and VOCs concentration. Also, the 1.62 times increase in ROS indicated impairment of cell activities in brain. The prominent 1.44 times decrease in GSH and 1.80 times increase in MDA demonstrated occurrence of oxidative stress in the brain as well as damage on neuronal cells. They are signals of reduction in anti-oxidation function and enhancement in lipid peroxidation. These effects only occurred at higher level of VOCs, consistent with the results of

MWM test and NMDA receptor expression and implied that high level of VOCs have obvious negative effects on the brain. Thus, researchers speculate that oxidative stress in brain is one of the possible mechanisms of deficit in spatial learning and memory capacity of mice.

CONCLUSION

The present behavioral and biochemical data suggested that VOCs exposure associated with significant learning and memory decline and oxidative stress in brain. But the grip strength didn't show any significance. Meanwhile, down-regulation of NR1, NR2A and NR2B subunits in hippocampus had better relevance with the results of MWM test and oxidative parameters which indicated deterioration in spatial learning and memory. Finally, researchers conclude that short-term VOCs inhalation didn't induce damage on physique and locomotor function of mice but negatively affected learning and memory capacity by decreasing expression of the NMDA receptor through the direct effects on the nervous system. Oxidative damage in brain might be implicated in VOCs-induced neurotoxicity. The results also reflect that much more attention should be paid to new interior decorating rooms and occupational settings which lie at higher VOCs concentration.

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