[Original]

The Effect of D-Cycloserine on Spatial Learning Performance and Memory Function-Related Gene Expression in Mice Following Toluene Exposure

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Abstract : D-cycloserine (DCS), a partial N-methyl-D-aspartate (NMDA) receptor agonist, is a well-known cognitive enhancer. To investigate the effect of DCS in cognitive function following toluene exposure, eight-week-old male C3H/HeN mice were exposed to filtered air (0 ppm) or 50 ppm toluene for 6 h a day on 5 consecutive days a week for 6 weeks. The day after the day of final exposure, a spatial learning task was performed using a Morris water maze apparatus. During the learning task, some mice were treated with DCS intraperitoneally (20 mg/kg) 30 min before the first trial of the acquisition phase and probe trial. After completion of the spatial learning task, the hippocampus was collected from each mouse to examine memory function-related gene expression using the real-time RT-PCR method. During the acquisition phase, on day 3 and 4, toluene-exposed mice with DCS treatment showed significantly better learning performance than corresponding saline treated groups. Moreover, the toluene-exposed mice with DCS treatment also showed significantly improved memory retention during the probe trial and up-regulation of hippocampal NMDA receptor subunit 2B mRNA expression compared with the saline treated groups. Our findings indicate that a subunit-specific modulation of hippocampal NMDA receptor mRNA expression by DCS contributes to improvement of spatial learning performance in mice following toluene exposure.

Key words : toluene, NMDA receptor, learning performance, hippocampus.

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Introduction

Environmental chemicals are one of the causative factors of neurobehavioral disturbances. Among the environmental chemicals, toluene belongs to a large class of volatile organic compounds, and it targets the central nervous system, especially the hippocampus [1 – 3]. Toluene is one of the well known neurotoxic chemicals affecting memory functions. The occupational exposure limit for toluene is 50 ppm in Japan [4], and the recently updated threshold limit value is 20 ppm in United States [5]. Many reports have indicated that inhalation of low concentrations of toluene induces central neurological abnormalities in humans [6, 7] and a persistent deficit in spatial learning and memory in animals [8, 9]. Learning behavior is one of the higher brain functions, and the hippocampus is one of the brain areas important for learning and memory. Previously, we have reported that 12 weeks exposure of C3H/HeN female mice to 50 ppm toluene caused a significant up-regulation of N-methyl-D-aspartate (NMDA) receptor subunit 2B (NMDA NR2B) expression associated with a simultaneous induction of calcium/calmodulin-dependent protein kinase (CaMK)-IV, cyclic AMP-responsive element binding protein (CREB)-1, and FosB/DeltaFosB in the same hippocampal tissues [10]. Moreover, our laboratory has also showed that NMDA NR2A, CaMKIV, CREB1, and BDNF were significantly up-regulated in the hippocampi of BALB/c wild type mice exposed to 9 ppm of toluene in nose-only exposure chamber for 30 min on 3 consecutive days followed by weekly sessions for 4 weeks [11]. However, the precise mechanisms underlying the neurotoxic action of toluene are still debated.

Ionotropic glutamate receptors consist of the NMDA, alpha-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainite receptor families. Among these receptor families, NMDA receptors are heteromers composed of an NR1 subunit and one or more of the four NMDA receptor subunit 2 (NR2) subunits (NR2A-NR2D) [12,13]. For receptor activation, NMDA receptors not only require a synaptically released neurotransmitter glutamate, but also require the obligatory coagonist, glycine [14,15]. Electrophysiological studies have demonstrated that NMDA receptor activation requires the occupation of two independent glycine sites and two independent glutamate sites [16].

D-cycloserine (DCS) is a broad spectrum antibiotic presently approved as an adjunctive agent in the treatment of susceptible gram positive or negative bacteria and *Mycobacterium tuberculosis*. The antidepressant activities of DCS were first described in depressed tuberculosis patients showing improvement in mood [17]. DCS has a high affinity for the glycine binding site in the NMDA receptor complex and it modulated memory processing in a dose dependent manner [18] and it has attracted interest for its potential to enhance NMDA-mediated learning and memory tasks [19]. The aim of our study was to investigate the role of DCS, a partial NMDA receptor agonist, on the spatial learning performance and NMDA receptor subunit expressions in the hippocampus of mice following toluene exposure. This is the first study to show the effects of DCS on hippocampus-dependent cognitive function following environmental toxic chemical exposure.

A, Exposure chamber

B, Experimental schedule

Fig. 1. A scheme of generation and exposure of toluene. A: Exposure chamber, and B: experimental schedule for toluene exposure and DCS treatment.

Materials and Methods

Animals

Male C3H/HeN mice (7 weeks old) were purchased from Charles River Japan Inc (Tokyo, Japan). At the age of 8 weeks, mice were recruited in the experiments. The mice were housed in cages under controlled environmental conditions (temperature $23 \pm 0.5^\circ\text{C}$, humidity $50 \pm 5\%$, lights on 07:00 – 19:00 h). The experiments were conducted under the control of the Ethics Committee of Animal Care and Experimentation of the National Institute for Environmental Studies, Japan.

Generation of toluene

Toluene vapor was generated by a Permeator Standard Gas Generation System (PD-1B, GASTEC Co., Kanagawa, Japan) and then introduced into acrylic chambers (Fig. 1A). An activated charcoal column was used between the compressor and permeator to remove organic compounds. Inside the chamber, CaCl_2 , NaOH and H_3PO_4 were added to remove water, CO_2 and ammonia, respectively. The airflow rate through the chamber housing the experimental

animals was 2 l/min. The concentration of toluene in the chambers was measured at 30 min intervals using gas chromatography mass spectrometry (Hapsite; Inficon, East Syracuse, NY, USA). The temperature and humidity inside the chambers were checked throughout the exposure period. The toluene concentration in the control chamber was below the lower limit of quantification (1 ppm). The average toluene level in the 50 ppm chamber was 47.6 ± 4.4 ppm.

Exposure of toluene

Mice were allotted into two main groups: mice exposed to filtered air (0 ppm toluene), and mice exposed to 50 ppm toluene in the whole body exposure chamber for 6 h per day, 5 days per week for 6 weeks. Each group contained twenty mice.

Administration of DCS

The mice were further divided into four different groups: 1) mice exposed to control air (0 ppm toluene) without DCS; 2) mice exposed to 50 ppm toluene without DCS; 3) mice exposed to control air (0 ppm toluene) with DCS; 4) mice exposed to 50 ppm toluene with DCS (Fig. 1B). Each group was composed of eight to nine mice. DCS (Sigma-Aldrich, Japan) was dissolved in saline and intraperitoneally injected (20 mg/kg) 30 min before the first trial of the acquisition phase and probe trial. Control mice were given saline only.

Morris water maze (MWM) behavioral test

The animals were exposed daily to control air or toluene from 10:00 h to 16:00 h for 6 h per day, 5 days per week for six weeks. On the day after completing the final exposure, all the animals were allowed to swim freely for 60 s per trial for four trials for adaptation to water, as described previously [20]. On the day after the final exposure, a MWM task was started for 5 days: 4 days for acquisition or training phase, and 1 day probe trial for memory retention. After completion of the probe trial, a visible platform test was performed on all the animals to examine visual acuity and sensorimotor activity.

The maze was a whitish gray circular pool measuring 100 cm in diameter and 30 cm in height and filled with water (temperature fixed at $25 \pm 2^\circ\text{C}$). The maze was located in the laboratory room containing extra maze cues, including a colored ball, figure patterns and posters on the walls. The maze was divided into four quadrants, and the starting point for each training trial was the mid-arc of each quadrant. In the acquisition phase, the mice were given four trials per day on four consecutive days to find a hidden platform (clear transparent Plexiglas platform, 10 cm in diameter) located 1.0 cm below the water surface in the pool. A video camera and tracking system (Muromachi Kikai Co., Ltd. Japan) were used to measure the escape latency, i.e., the time required by the mouse to find and climb on to the platform. In each trial, individual mice were placed in the water facing the wall of the pool at one of the four designated starting points and left to find the hidden platform. During each trial, each mouse was given 60 s to find the hidden platform. If they found the platform, they were allowed to remain on it for 10 s, and then moved from the maze to their home cage. If the mouse did not find the platform within 60 s, it was manually placed on the platform and returned to its home cage after 15 s. The inter-trial

interval was at least 300 s. The platform position remained constant on the four consecutive days. Swimming speed was also recorded throughout the acquisition phase. On day 5, the platform was removed from the pool and a probe trial was performed to assess memory retention. Each mouse was allowed to swim for 60 s in the maze. The time duration that the mice spent in the target quadrant was recorded and averaged. After completion of the probe trial, a visible platform test was performed to examine visual acuity and sensorimotor activity. All the mice were tested in the maze with a visible platform (marked with colored tape) on a new location 1 cm above the water.

Analysis of memory function-related gene expressions in the hippocampus

To collect the total RNA from the hippocampal samples, we used a previously described protocol [21]. Briefly, total RNA extraction from the hippocampal samples was performed using the BioRobot EZ-1 and EZ-1 RNA tissue mini kits (Qiagen GmbH, Germany). Then the purity of the total RNA was examined and the quantity was estimated using the ND-1000 NanoDrop RNA Assay protocol (NanoDrop, USA). Next we performed first-strand cDNA synthesis from the total RNA using SuperScript RNase H⁻ Reverse Transcriptase II (Invitrogen, USA), according to the manufacturer's protocol. Next we examined the hippocampal mRNA expressions of 18S, NR1, NR2A and NR2B, using a quantitative real-time RT-PCR method and the Applied Biosystems (ABI) Prism 7000 Sequence Detection System (Applied Biosystems Inc., USA). The tissue 18S rRNA level was used as the internal control. Data were analyzed using the comparative threshold cycle method. Then the relative expression levels of NMDA NR1, NR2A and NR2B mRNAs were individually normalized to the 18S rRNA content in the respective samples and expressed as mRNA signals per unit 18S rRNA expression.

Statistical Analysis

The results of each group were calculated and expressed as the mean $\pm$ standard error (SE). Body weight and various organ weights were analyzed using a one-way analysis of variance (ANOVA). Data from swimming speed, acquisition phase, probe trial, and mRNA expressions of NMDA receptor subunits were analyzed using a two-way ANOVA followed by a Bonferroni *post hoc* test. When the main effect was significant, specific measures between groups were analyzed by Bonferroni's multiple comparison tests. A *P* value < 0.05 was considered statistically significant. The statistical analysis was performed using GraphPad Prism software (version5; GraphPad Software, Inc., San Diego, CA).

Results

Body weight and organ weights

To determine the general toxicity of toluene, we measured the body and organ weights of male mice at the time of sampling. Apart from the spleen, we found that the body weight and relative organ weights of the brain, thymus, lung, liver, kidney and testis were not significantly different among the control and the toluene-exposed groups with or without DCS treatment (Table 1).

Table 1. Body weight, weight of various organs after 6 weeks toluene exposure

Group	Body weight (g)	Spleen (mg)	Lung (mg)	Thymus (mg)	Liver (mg)	Kidney (mg)	Testis (mg)	Brain (mg)
Control	30.15 ± 1.20	71.7 ± 2.1	45.4 ± 0.8	31.0 ± 2.4	1352.3 ± 64.0	230.1 ± 8.3	82.0 ± 2.6	448.4 ± 4.6
C + DCS	29.78 ± 0.33	71.2 ± 1.9	49.4 ± 2.6	35.2 ± 2.2	1368.9 ± 19.0	232.8 ± 2.0	80.8 ± 1.5	448.6 ± 2.9
Toluene	30.05 ± 0.91	78.8 ± 4.8	54.1 ± 2.8	34.6 ± 1.8	1399.7 ± 69.1	242.7 ± 13.4	77.7 ± 4.8	451.8 ± 3.1
T + DCS	30.19 ± 0.72	88.3 ± 2.4 *	51.1 ± 1.3	31.5 ± 2.5	1378.4 ± 27.9	242.9 ± 4.9	88.3 ± 3.5	451.5 ± 7.6

Data are presented as mean ± standard error, *: $P < 0.05$ vs control, C = control, T = toluene

Fig. 2. Swimming speed in the control and toluene-exposed mice with or without DCS treatment. Each bar represents the mean ± S.E. ($n = 8 \sim 9$). □: DCS (-), ■: DCS (+)

Swimming speed

The swimming speed was measured for each mouse throughout the acquisition phase and we did not observed any significant difference between the control and toluene-exposed mice with or without DCS treatment (Fig. 2).

Learning performance in the control and toluene-exposed mice with or without DCS treatment

Two-way ANOVA (treatment × day) of escape latency revealed that the main effect of treatment and the main effect of day were significant; however, the interaction of treatment versus day was not significant. Further analysis of the Bonferroni posttests showed that during the acquisition phase, there was no difference in escape latency between the control and toluene-exposed groups with or without DCS treatment on day 1 and 2. However, on day 3 and 4, the toluene-exposed

Fig. 3. A: Learning performance in acquisition phase, and B: memory retention in probe trials in the control and toluene-exposed mice with or without DCS treatment. Each bar represents the mean $\pm$ S.E. ($n = 8\sim 9$) (*: $P < 0.05$, compared with the corresponding saline treated group). □: DCS (-), ■: DCS (+)

mice with DCS treatment showed significantly better learning performance than the saline treated groups (Fig. 3A). Moreover, two-way ANOVA (DCS $\times$ toluene) of memory retention revealed that the main effect of DCS was significant; however, the main effect of toluene and the interaction of DCS versus toluene were not significant. Further analysis of the Bonferroni posttests showed that the toluene-exposed mice with DCS treatment had improved memory retention during the probe trial than saline treated groups (Fig. 3B). However, DCS did not affect spatial learning ability and memory function-related gene expression in the control mice.

Fig. 4. Memory function-related gene NMDA receptor subunit. A: NR1, B: NR2A and C: NR2B mRNA expression in the hippocampus of the control and toluene-exposed mice with or without DCS treatment. Each bar represents the mean $\pm$ S.E. ($n = 8\sim 9$) (*: $P < 0.05$, compared with the corresponding saline treated group). $\square$: DCS (-), $\blacksquare$: DCS (+)

Memory function-related gene expression in the hippocampus of the control and toluene-exposed mice with or without DCS treatment

Experimental evidence has indicated that the NMDA subtype of glutamate receptors is the most prominent ligand-gated ion channel to be affected by toluene exposure [22]. Thus, we examined the mRNA expression of NMDA subunits NR1, NR2A and NR2B in the hippocampus of the control and toluene-exposed mice with or without DCS treatment. Two-way ANOVA (DCS $\times$ toluene) of memory function-related gene expressions revealed that the main effect of DCS was significant; however, the main effect of toluene and the interaction of DCS versus toluene were not significant. Further analysis of the Bonferroni posttests showed that the mRNA expression of either NR1 or NR2A was not different between the control and toluene exposed groups and within groups after DCS treatment (Fig. 4A, B). In contrast, DCS treatment upregulated the expression of NR2B mRNA in the toluene exposed mice but not in the control mice (Fig. 4C).

Discussion

Toluene is widely used in industrial, commercial and household products [23], and wide applications of toluene in both industrial settings and many consumer products have thus rendered the human central nervous system readily exposed to this noxious chemical, both inside and outside the home. The occupational exposure limit for toluene is 50 ppm in Japan [4], and the recently updated threshold limit value is 20 ppm in the United States [5]. Many reports have indicated that inhalation of low concentrations of toluene induces central neurological abnormalities in humans [6, 7] and a persistent deficit in spatial learning and memory in animals [8, 9]. However, to the best of our knowledge, the manipulation of the NMDA receptor subunit while monitoring learning performance following toluene exposure has not been investigated yet. Therefore, in the present study, we showed for the first time, using a partial NMDA agonist DCS, the role of the hippocampal NMDA receptor expression in the spatial learning performance following volatile organic compound exposure in a mouse model.

The major findings of our present study were that DCS improved the spatial learning performance simultaneously with up-regulation of the NMDA receptor subunit NR2B mRNA expression in the hippocampus of mice exposed to toluene, but not in the control mice. We observed that DCS treatment enhanced learning performance in day 3 and day 4 during the acquisition or training phase and memory retention in mice exposed to 50 ppm of toluene. However, toluene exposure did not affect body and organ weights, spatial learning ability and memory function-related gene expression in the mice hippocampus.

The NMDA receptors are heteromeric protein complexes of different subunits (NR1_{a-h}, NR2A-D, NR3) that confer distinct biophysical properties [24]. The activation of NMDA receptors requires, in addition to the binding of amino acid neurotransmitter glutamate to NR2 and membrane depolarization, the binding of glycine or D-serine to the glycine site of NR1 [25,16]. Because the D-serine antagonist D-amino acid oxidase (DAO) suppresses NMDA currents and blocks hippocampal long-term potentiation (LTP) [26], this suggest that D-serine is the major endogenous co-agonist in the hippocampus and may play a role in the regulation of LTP and long-term depression (LTD).

Regarding DCS as a cognitive enhancer, previous studies have demonstrated that DCS attenuates scopolamine-induced learning and memory deficits [27] and cognitive performance following traumatic brain injury in rat models [28]. Furthermore, recent studies have shown that DCS promotes episodic-like memory [29] and accelerates the extinction of cocaine-induced condition place preference in a mouse model [30]. Experimental evidence has indicated that the NMDA receptor hypofunction mediated by low D-serine is a specific causative factor in some types of schizophrenia [31, 32]. In addition, schizophrenic behavior in rodents treated with an NMDA receptor antagonist can be partially ameliorated by D-serine [33, 34]. Duffy and colleagues demonstrated that D-serine does not improve the acquisition of spatial tasks in wild-type mice, but allows acquired information to be utilized more effectively under changing conditions [35]. Consistent with these findings, we observed that DCS did not affect working memory and memory retention in control mice with a normal brain, or enhanced memory

retention during a probe trial in toluene-exposed mice with central neurobiological changes.

To understand the possible mechanisms of the action of toluene, our laboratory has done many supportive experiments. We have firstly indicated that the extracellular levels of neurotransmitter glutamate, a ligand for the NMDA receptor, in the mice hippocampus were rapidly and reversibly increased within 30 min after an acute single toluene administration (300 mg/kg, i.p.) in a dose-dependent manner, and returned to the basal level by 1h [36]. Recently, using an *in vivo* microdialysis study and schedule-controlled operant behavioral task, we also showed that the extracellular glutamate level was increased throughout the learning performance in the control mice while the extracellular glutamate level was decreased throughout the learning performance in mice after acute toluene administration [37]. In addition, to confirm the neurotoxic effect of toluene, we have already measured brain toluene levels by the Solid-phase microextraction technique and reported the hippocampal toluene levels following inhalation exposure [38] and intraperitoneal administration [36]. Moreover, in our previous study, we found a significant up-regulation of NMDA receptor subunit NR2B expression associated with a simultaneous induction of CaMKIV, CREB1 and FosB/ Δ FosB in the hippocampus of mice exposed to 50 ppm toluene for 12 weeks, but not for 6 weeks [10]. The present results, which are consistent with our previous findings, indicate that the effect of toluene exposure was not observed in a 6 week exposure period; however, the remarkable effects of DCS on the spatial learning performance and the NMDA receptor subunit NR2B mRNA expression in the hippocampus were observed in mice exposed to toluene.

In conclusion, we suggest that agonism of the glycine site on the NMDA receptor appears to facilitate learning performance in a spatial task following environmental toxic chemical exposure. Further study is needed to clarify the intracellular mechanism of the neurotoxic effect of toluene at the transcription level.

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トルエン曝露したマウスの空間学習行動と記憶機能関連遺伝子発現へのD-シクロセリンの影響

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要 旨： N-methyl-D-aspartate (NMDA) 受容体のアゴニストD-シクロセリンの投与が, トルエン曝露による空間認識障害の改善に効果があるか否かC3H/HeNマウスを用いて検討した. モリス水迷路を用いての空間学習試験で, 水中の踏み台を捜す訓練習得段階ではトルエン曝露(50 ppm, 6時間/日, 6週間)をうけD-シクロセリン投与したマウスで投与(20 mg/kg)しないマウスより学習効果がよくなった. 踏み台を取り除いて観察するプローブトライアルでは, やはりトルエン曝露をうけD-シクロセリン投与したマウスにおいて投与しないマウスより保持機能の改善, およびRT-PCRによるNMDA receptor subunit 2B (NR2B) 遺伝子発現の増加がみられた. 本研究結果は, トルエン曝露がNMDA受容体機能に影響を与え, D-シクロセリン投与がNR2B遺伝子を介して空間学習機能回復に貢献している可能性を示唆している.

キーワード： トルエン, NMDA受容体, 学習行動, 海馬.

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