



# Toluene Inhalation Causes Early Anxiety and Delayed Depression with Regulation of Dopamine Turnover, 5-HT<sub>1A</sub> Receptor, and Adult Neurogenesis in Mice

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## Abstract

Inhaled solvents such as toluene are of particular concern due to their abuse potential that is easily exposed to the environment. The inhalation of toluene causes various behavioral problems, but, the effect of short-term exposure of toluene on changes in emotional behaviors over time after exposure and the accompanying pathological characteristics have not been fully identified. Here, we evaluated the behavioral and neurochemical changes observed over time in mice that inhaled toluene. The mice were exposed to toluene for 30 min at a concentration of either 500 or 2,000 ppm. Toluene did not cause social or motor dysfunction in mice. However, increased anxiety-like behavior was detected in the short-term after exposure, and depression-like behavior appeared as delayed effects. The amount of striatal dopamine metabolites was significantly decreased by toluene, which continued to be seen for up to almost two weeks after inhalation. Additionally, an upregulation of serotonin 1A (5-HT<sub>1A</sub>) receptor in the hippocampus and the substantia nigra, as well as reduced immunoreactivity of neurogenesis markers in the dentate gyrus, was observed in the mice after two weeks. These results suggest that toluene inhalation, even single exposure, mimics early anxiety- and delayed depression-like emotional disturbances, underpinned by pathological changes in the brain.

**Key Words:** Anxiety, Depression, Dopamine, Neurogenesis, Serotonin 5-HT<sub>1A</sub> receptor, Toluene

## INTRODUCTION

Worldwide, there is particular concern associated with frequently abused solvents that can be easily exposed to the environment, such as toluene, due to their abuse potential and their association with potential neuropathologies and poison risks. Toluene exposure can occur in the workplace; therefore, regulations exist to prevent physiologically adverse effects between 10 to 100 ppm (Cruz *et al.*, 2014). However, people who abuse toluene are exposed to much higher concentrations, up to several thousand ppm in the form of intermittent inhalation (Marjot and McLeod, 1989). Acute neurological symptoms after toluene inhalation include euphoria, headache, and confusion. Chronic exposure to toluene has been reported to cause symptoms similar to alcohol intoxication, memory loss, impaired cognition, dizziness, anxiety, and depression (Lee *et al.*, 2003; Howard *et al.*, 2011). Maternal exposure to toluene

during pregnancy can result in miscarriage in the form of embryopathy or lower birth weights compared to mothers who have not been exposed to toluene (Arnold *et al.*, 1994).

Based on clinical reports, many *in vivo* experiments have investigated the behavioral and neurobiological pathologies caused by toluene exposure. The highly lipophilic nature of toluene enables it to readily cross the blood-brain barrier and become harmful to the nervous system (Cruz *et al.*, 2014). Intraperitoneal (i.p.) injection of toluene (600 or 750 mg/kg) for two weeks in adolescent mice reduced sociability and dopamine (DA) turnover, which was evidenced by a decrease in the ratio of DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) to DA in the nucleus accumbens compared to that in controls (Lin *et al.*, 2010). Single injection (300 mg/kg, i.p.) of toluene damaged the hippocampus and impaired cognitive function such as failure to distinguish novel objects from familiar ones in mice (Tas *et*

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*et al.*, 2013). Toluene inhalation caused a loss of pyramidal cell number in the hippocampal CA1 region (Zhvania *et al.*, 2012) and modulated dentate gyrus granule cell output (Gmaz *et al.*, 2012) in rats, which is associated with learning and memory deficits. Additionally, evidence has demonstrated that toluene could induce anxiety and depression-like behaviors in male Swiss Webster and C57BL/6 mice (Yang *et al.*, 2010; Bowen *et al.*, 2018). Toluene-induced mood-related behavioral changes depend on experimental conditions: toluene injection (500 mg/kg, i.p.) increases depressive and anxiety-like behaviors (Yang *et al.*, 2010; Cruz *et al.*, 2014); toluene inhalation (4,000 ppm) causes anxiolytic-like actions (Paez-Martinez *et al.*, 2003); and toluene exposure (40,000 ppm in air) for 30 days results in a failure of avoidance reactions to aversive conditions (Yang *et al.*, 2010). Most toluene-induced neurobehavioral changes were examined at specific time point, in most cases immediately after exposure, and few studies comparing changes in behaviors over time after toluene inhalation have been conducted.

Although toluene i.p. injection has been widely used to evaluate the effects of toluene exposure in mice rather than inhalation, toluene abuse or exposure occurs mainly via the respiratory tract in humans. Addicts often inhale toluene, known as “sniffing,” at high concentrations. Therefore, it is important to evaluate the neurobehavioral effects in toluene-inhaled animals. The pharmacokinetics of i.p. injections and inhalation differ in many ways, and the brain concentrations and neurobehavioral effects between these routes of administration likely differ as well. Furthermore, the metabolic rates differ between groups administered toluene via respiratory and oral routes (Pyykko *et al.*, 1977).

The aim of this study is to examine whether toluene inhalation could be a contributor to the development of mood disorders in mice. Here we evaluated behaviors related to social interaction, anxiety, and depression in short-term and delayed time frames after toluene-inhalation, and showed that single inhalation of toluene regulated emotional behaviors of mice differently after exposure over time with relevant pathophysiological changes in the brain.

## MATERIALS AND METHODS

### Animal treatment

Male C57BL/6 mice at five weeks of age were obtained from Orient Bio (Orient Bio Inc., Seongnam, Korea) and maintained on a 12 h light, 12 h dark schedule with libitum food and water. Mice were randomly allocated into three experimental groups: control (N=6-8) and two toluene exposure (500 and 2,000 ppm) groups (N=6-8, respectively) and the experiments repeated twice independently. All experiments were conducted with the approval of the animal care and use committee of CHA University (IACUC 170136).

### Materials

Toluene (24451), perchloric acid (244252), DA (H8502), HVA (850217), and DOPAC (H1252) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against antibodies against horseradish peroxidase (HRP)-conjugated anti-mouse IgG (31430) and anti-rabbit IgG (31460) (Thermo Fisher Scientific, Wartham, MA, USA); 5-HT<sub>1A</sub> receptor (gtx104703) (GeneTex, Irvine, CA, USA); doublecortin (4604S) (Cell Signaling

Technology, Danvers, MA, USA); NeuN (EPR12763) (Abcam, Cambridge, UK) and enhanced chemiluminescence kit (WB-KLS0500) were obtained from Millipore (Billerica, MA, USA).

### Experimental procedure

C57BL/6 mice were divided into short-term and delayed effect groups: to assess the short-term effects, 3-4 mice were sacrificed immediately after toluene inhalation and used for neurochemical analysis and immunohistochemical staining, and 12-16 mice were subjected to behavioral experiments according to experimental scheme from 24 h after inhalation; for the delayed effects, brain samples were obtained on the 11th day after toluene inhalation, and behavioral experiments were conducted from two weeks after inhalation (Fig. 1A). Under each condition, mice were divided into three groups and exposed to clean air (control) or toluene at 500 or 2,000 ppm. Their brains were removed immediately after or two weeks toluene exposure. Toluene vapor was generated using a gas generator (Shibata Scientific Technology Ltd, Saitama, Japan). Mice had a habituation time for 30 min in the chamber and laboratory conditions were always the same. The mice were subjected to toluene vapour inhalation for 30 min as they moved freely in the chamber. Toluene concentration was measured using VOCs detector (MiniRAE 3000, RAE systems, CA, USA) during the exposure period (Fig. 1B). Fig. 1A depicts the experimental schedule in detail.

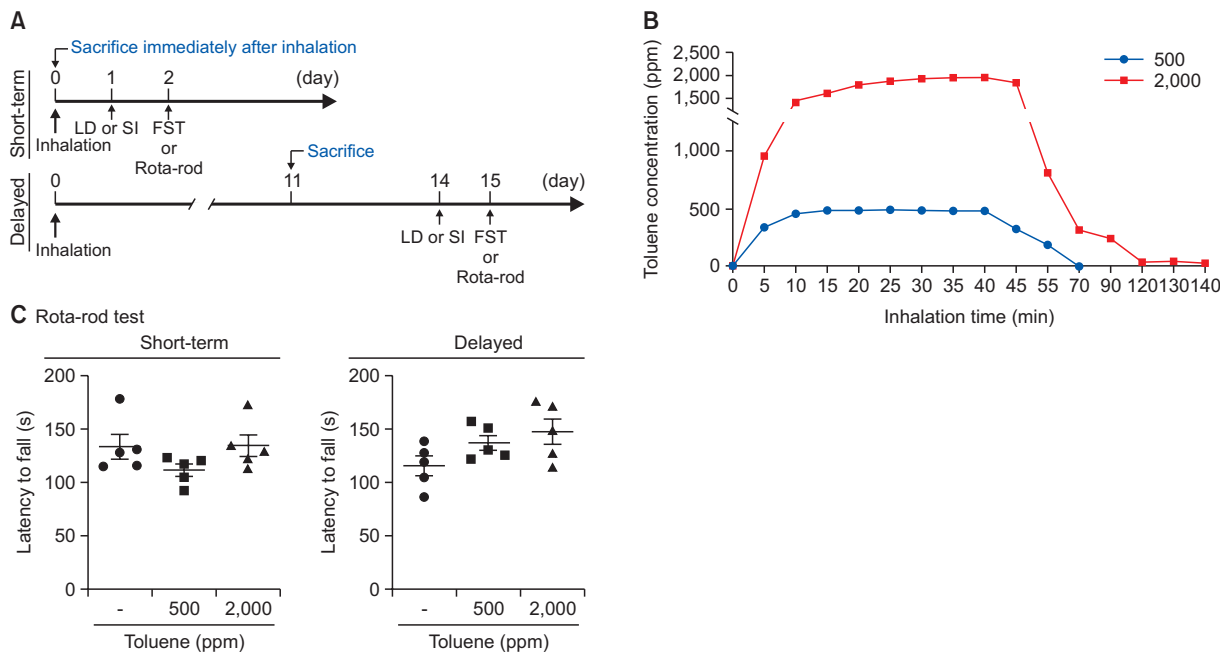
### Behavioral tests

**Light-dark box test:** A cage was divided into two chambers by a partition with a door. One chamber was brightly illuminated by white diodes (390 lux; light chamber, 20 cm×30 cm), and the other chamber was dimly lit (2 lux; dark chamber, 10 cm×30 cm). Each mouse was placed under the passage hole between the two chambers, facing the brighter side, and allowed to move freely between the two chambers for 10 min. The time spent in each chamber was recorded (Han *et al.*, 2015).

**Forced swimming test:** Each mouse was gently placed in a glass cylinder (diameter, 18 cm; height, 26 cm) filled with 15 cm of water at room temperature. The mice were allowed to adapt for 1 min. Immobility time was then measured for 5 min with a stopwatch. At the end of the test, mice were dried with a paper towel and placed in a cage with normal bedding under warm light (Han *et al.*, 2015).

**Social interaction test:** The social interaction (SI) test was performed in a rectangular, three-chambered box (45 cm×30 cm×30 cm). For the sociability test, the test animal was introduced into the middle chamber and allowed to habituate for 5 min, after which a new mouse was introduced into a wire cage in one of the side-chambers, with a dummy in a wire cage in the other side chamber. Next, the test animal was allowed to move freely into all three chambers for 5 min (Fig. 2C). Social interaction tests in the chamber were recorded using EthoVision XT9 video tracking system (EthoVision®, Version 9, Noldus, Wageningen, Netherlands) and SMART video tracking system (Version 2.5.2.1, Harvard Apparatus, MA, USA) (Felix-Ortiz and Tye, 2014).

**Rotarod test:** The rotarod parameters were set to increase from 4 to 40 rpm for 3 min. Mice were placed on the rotating bar towards the wall. The time of movement until mice fell was recorded. Experiments were repeated three times, and the mean value for time to fall was calculated. Measurement



**Fig. 1.** Experimental procedure and effects of toluene motor function of mice. (A) Experimental schedule of toluene inhalation and behavioral evaluation. (B) Toluene concentration was maintained at 500 or 2,000 ppm for 30 min, and the mice were able to move freely and inhale toluene in the chamber. (C) Motor dysfunction was assessed by the rotarod test. After adapting the mouse to the rotarod, the time to fall off the rotating rod was measured. Experiments were performed according to the procedure described in the Methods. The latency to fall from a rotating rod was automatically recorded. Data were obtained from three repeated values. Data are presented as mean  $\pm$  SEM (n=5). LD, light-dark box test; SI, social interaction test; FST, forced swimming test.

times did not exceed 5 min. The rotarod test was performed using a rotarod treadmill (JD-A-07MA5, Jeung Do Bio & Plant Co. Ltd., Seoul, Korea).

### Tissue preparation and high-pressure liquid chromatography and electrochemical detection (HPLC-ECD)

DA and its major metabolites, HVA and DOPAC, were measured in the striatum by HPLC-ECD. Brain tissue blocks were rapidly sacrificed on ice and homogenized with ice-cold 0.4 M perchloric acid with 1 $\times$  PBS, then incubated at  $-80^{\circ}\text{C}$  overnight. After centrifugation at 15,000 rpm for 30 min at  $4^{\circ}\text{C}$ , the supernatant was filtered using an appropriate column (#SC1000-1Kt, Sigma Prep spin column, Sigma-Aldrich). Filtered supernatants were directly injected into the X-bridge column (XBridge<sup>TM</sup> C18 5  $\mu\text{m}$ , Waters Corp., MA, USA). The mobile phase consisted of 85 mM citric acid, 100 mM sodium acetate, 0.9 mM sodium-octyl sulfate, 0.2 mM ethylenediaminetetraacetic acid (EDTA), and 14% methanol at pH 3.8 (Tianzhu *et al.*, 2014). The flow rate was kept constant at 1 mL/min. Chromatographic peak analysis was accompanied by identification of unknown peaks in a sample matched according to retention times.

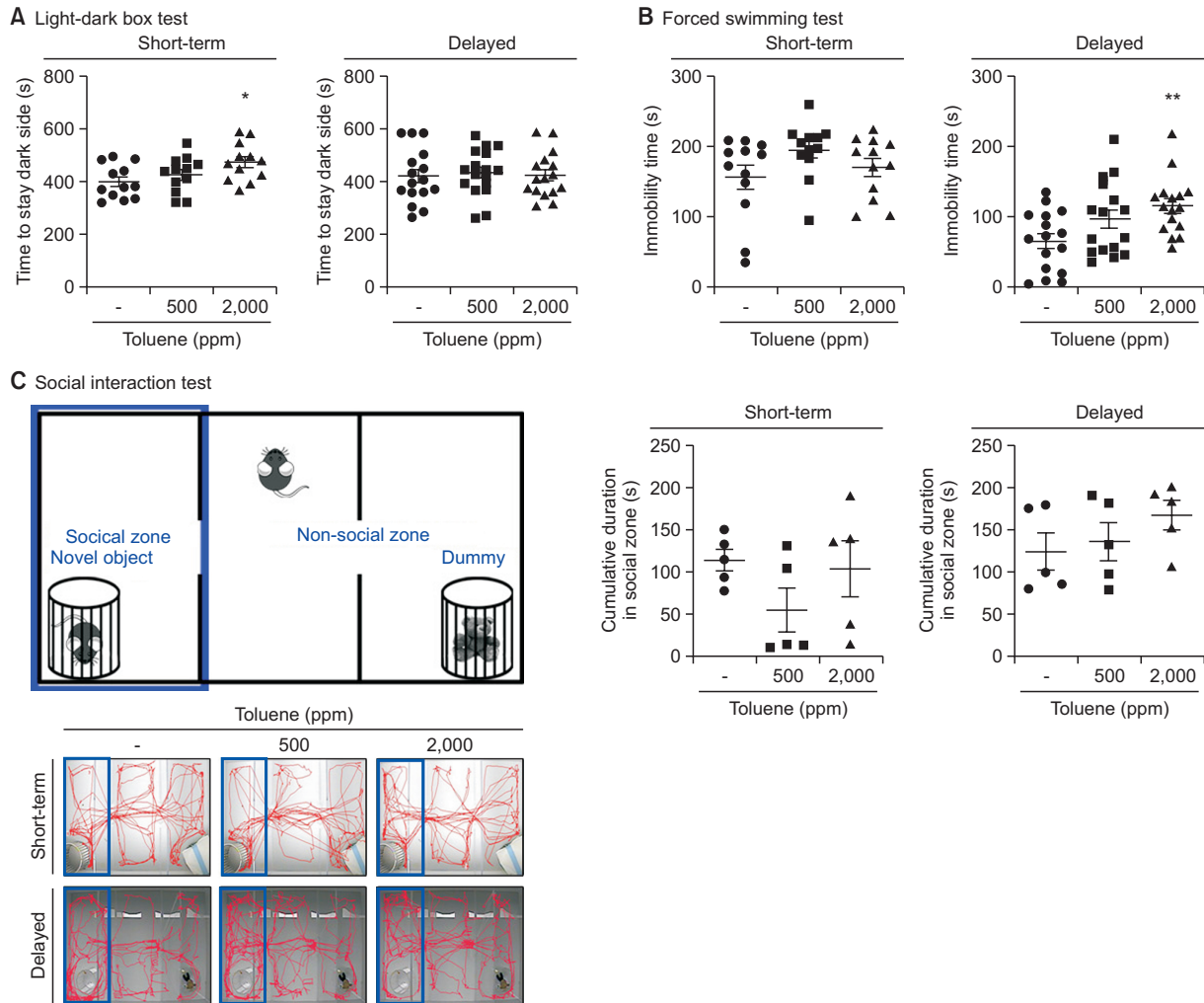
### Western blot analysis

Frozen brain tissues were lysed in RIPA buffer (50 mM Tris-Cl, pH 7.5, 150 mM, NaCl, 1% NP-40, 0.5% DOC, and 0.1% SDS) containing a complete protease inhibitor cocktail and phosphatase inhibitor cocktail (Roche, Basel, Switzerland). Homogenate tissue was incubated at  $-20^{\circ}\text{C}$  overnight and centrifuged at 15,000 rpm for 30 min. Equal amounts of

protein were separated on 8 or 12% SDS polyacrylamide gels and transferred onto polyvinylidene difluoride-nitrocellulose membranes (Millipore). Membranes were incubated overnight at  $4^{\circ}\text{C}$  with suitable primary antibodies followed by the corresponding HRP-conjugated secondary antibodies for 1 h. The target bands were detected using an enhanced chemiluminescence detection kit (Millipore). Data are representative of at least three to four independent experiments and quantified by densitometric analysis.

### Immunohistochemistry

Brains were dissected out and post-fixed overnight at  $4^{\circ}\text{C}$  in 4% Paraformaldehyde followed by dehydrated with two step 15% and 30% sucrose with 1X PBS during 24 h. Then consecutive series of 20  $\mu\text{m}$  coronal sectioned of the brain were sliced using cryomicrotome (CM3050S, LEICA, Wetzlar, Germany) and collected into 12-well plate as floating in cryoprotectant. The collected tissues attached to plate and washed to 0.05 M PBS for 15 min at twice. Sections were incubated for 30 min with 0.05 M PBS+1% BSA+0.2% Triton X-100+0.3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ )+1.5% normal goat serum (NGS) which was followed by twice wash with 0.05M PBS. After rinsing, the sections were incubated with tyrosin (TH) (1:200), doublecortin (DCX) (1:400) and NeuN (1:200) diluted with 0.05 M PBS containing 1.5% NGS at  $4^{\circ}\text{C}$ . After overnight incubation, they were washed twice with 0.05 M PBS for 10 min. After washing, the sections were bathed with second antibodies with 0.05 M PBS+1.5% NGS for 1 h. Immunohistochemical staining was performed with the ABC kit (PK-6100, Vector Laboratories, Inc., CA, USA) for 1 h. After washing, visualization was performed using for 3, 3-diaminobenzidine (DAB; D4293, Sigma-



**Fig. 2.** Effects of toluene exposure on mice behaviors. (A) Anxiety-like behaviors of toluene exposed mice were evaluated using the light-dark box. The time spent in the dark chamber of the light-dark box was calculated for a 10 min session. (B) Depression-like behaviors of toluene inhaled mice were evaluated using the forced swimming test. Time spent immobile over a 5-min period was measured. (C) Diagrammatic representation of the social interaction test chamber. The social interaction test confirmed the sociability of toluene inhaled mouse by measuring time to spent in novel mouse. Test was assessed using EthoVision XT9 and SMART video tracking system. Social-zone cumulative time was measured for 5 min. The blue box indicates the social-zone (novel object chamber). Data are presented as mean  $\pm$  SEM ( $n=5-8$ , repeated twice). \* $p<0.05$  and \*\* $p<0.01$  versus control.

Aldrich) for 10 min and mount sections and coverslipped. The sections were imaged by microscope (DM750, Leica).

### Statistical analysis

Data were analyzed and expressed as the mean  $\pm$  SEM. Comparisons were made using one-way ANOVA with the statistics program GraphPad Prism<sup>®</sup> version 8.00 for Windows (GraphPad software, La Jolla, CA, USA). Replicate numbers and applied tests are annotated in the corresponding figures. Adjustment of p-values for multiple testing (post-hoc analysis) was performed using Dunnett's multiple comparison test. Asterisks indicate statistically significant differences between the compared groups: \* $p<0.05$ , \*\* $p<0.01$ , \*\*\* $p<0.001$  and \*\*\*\* $p<0.0001$ .

## RESULTS

### The effect of toluene inhalation on motor coordination of mice

To evaluate the effects of toluene inhalation on behavior over time, C57BL/6 mice were allowed to inhale toluene and the behavioral experiments were conducted from 24 h (short-term effect) or two weeks (delayed effect) after inhalation. As described in the methods section, behavioral tests were conducted in the order depicted in Fig. 1A. We divided the short-term and delayed effect groups into three subgroups, which were exposed either to clean air (control), or toluene at 500 or 2,000 ppm, respectively. Toluene was inhaled at a constant concentration for 30 min in a free movement chamber that reached a specific concentration within 5% of the error range (Fig. 1B). Toluene inhalation under these conditions did not show significant effects on body weight, even after two weeks



(data not shown). When measuring rota-rod performance of the short-term and delayed effect groups, there were no significant differences in motor function between control and toluene-inhaled mice (500 and 2,000 ppm), respectively (Fig. 1C).

### **Toluene induces anxiety- and depression-like behaviors depending on time after exposure**

To evaluate the effects of toluene inhalation on emotional behaviors in mice, we first examined anxiety-like behavior using the light-dark box test, which is one of the most widely used tests to measure unconditioned anxiety responses in rodents (Arrant *et al.*, 2013). The light-dark box test is based on the report that was initially described by Crawley and Goodwin (1980). They used a device consisting of a small dark safety compartment (1/3) and a large light avoidance compartment (2/3), and showed that anxiolytic agent caused a significant increase in residence time in the white box area of mice, which suggesting that more time spent in the dark side is indicative of greater anxiety. As shown in Fig. 2A, 24 h after inhalation of 2,000 ppm toluene, mice stayed in the dark side for a longer time compared to the control group ( $F_{2,33}=3.637$ ,  $p<0.05$ ). However, there was no significant differences in the time spent in the dark side between control and toluene-inhaled mice after two weeks ( $F_{2,45}=0.0895$ ,  $p=0.09145$ ).

Next, we examined whether toluene inhalation increased depression-like behaviors. The forced swimming test is used to evaluate the despair behavior of mice by measuring the immobility time in a container filled with water (Yankelevitch-Yahav *et al.*, 2015). As shown in Fig. 2B, toluene inhalation did not cause significant changes in immobility time of mice in the short-term effect groups at any concentration ( $F_{2,33}=1.909$ ,  $p=0.1642$ ). However, immobility time was significantly increased by toluene 2,000 ppm inhalation in the delayed effect group ( $F_{2,45}=4.873$ ,  $p<0.01$ ).

Inhalation addiction could induce social deficits or increase sociability (Zachrisson *et al.*, 2017); thus, we conducted a social interaction test. We placed mice in a three-chambered box as described in the methods section, and measured how much time the mice spent in the social zone, measured using EthoVision and SMART video tracking programs. As social recognition is critical for relationship formation, a decrease in time spent in the social zone implies social deficits. As shown in Fig. 2C, there were no significant differences in time spent in the social zone between toluene-inhaled and control mice in both the short-term and delayed effect groups. These results demonstrate that anxiety-like behavior occurs within a relatively short period, while depressive behavior gradually develops over time after toluene inhalation. In addition, it was noted that single inhalation of toluene did not affect social activity of mice.

### **Toluene inhalation reduces DA metabolites DOPAC and HVA in the striatum**

Because one of the most important neurotransmitters involved in the regulation of emotional behaviors is DA (Zarindast and Khakpai, 2015), we examined the effect of toluene on the amounts of DA and its metabolites DOPAC and HVA in the striatum of mice brains. Fig. 3A shows that the striatal amounts of DA metabolites DOPAC ( $4.53 \pm 0.16$ ,  $p<0.0001$  in the 500 ppm group;  $3.64 \pm 0.34$ ,  $p<0.0001$  in the 2,000 ppm inhalation group) and HVA ( $2.16 \pm 0.04$ ,  $p<0.0001$  in the 500 ppm group;  $1.82 \pm 0.10$ ,  $p<0.0001$  in the 2,000 ppm inha-

tion group) were significantly reduced in the mice immediately after toluene inhalation. These decreases in DA metabolites were also detected more than 10 days after inhalation (Fig. 3B); significant reductions in DOPAC ( $8.63 \pm 0.50$ ,  $p<0.01$  in the 2,000 ppm inhalation group) and HVA ( $5.53 \pm 0.16$ ,  $p<0.01$  in the 500 ppm group;  $4.0 \pm 0.18$ ,  $p<0.0001$  in the 2,000 ppm inhalation group) were observed in toluene-inhaled mice. The amount of DA also tended to decrease compared to the controls over time, but not significantly.

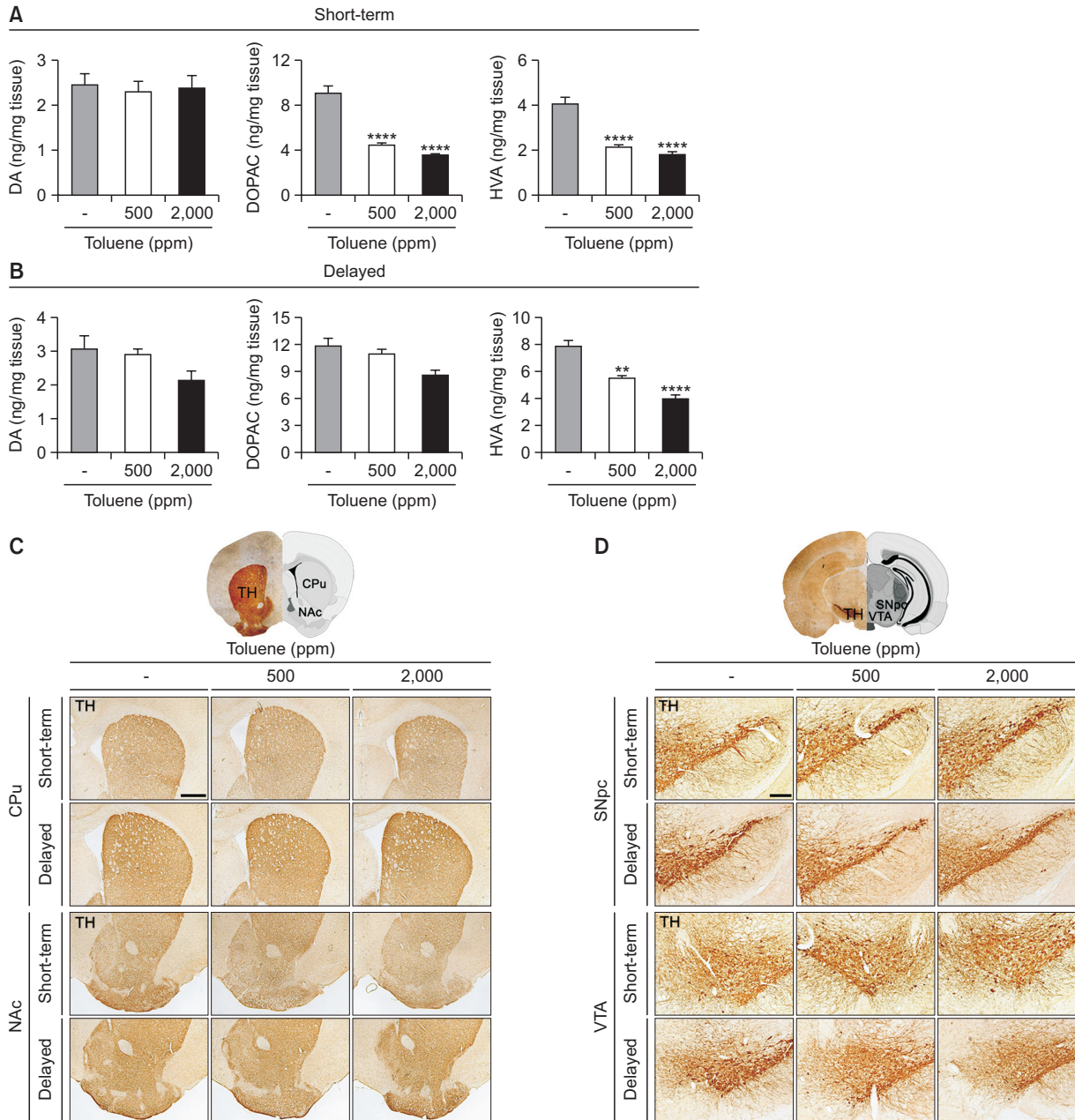
To verify the possibility that DA-producing neuronal cells were being degenerated by toluene inhalation, we performed immunohistochemistry to identify TH in the striatum and substantia nigra (SN). As shown in Fig. 3C, there were no significant changes in the immunoreactivity of TH in either regions both in the short-term and delayed toluene exposure groups. These data indicate that toluene continuously inhibits DA turnover for a long time without significant cytotoxicity to DAergic neurons.

### **Toluene upregulates serotonin 1A (5-HT<sub>1A</sub>) receptors**

Evidences indicate that the modulation of serotonin (5-HT) neurotransmission is linked to anxiety and depression, and the main inhibitory serotonin receptor 5-HT<sub>1A</sub> is involved in those mood disorders (Lim *et al.*, 2018b). Moreover, 5-HT<sub>1A</sub> receptors attenuated the psychostimulant-induced increase in limbic forebrain DA levels (Przegalinski and Filip, 1997). Because toluene inhalation causes early anxiety-like behaviors and delayed depressive-like behaviors (Fig. 2B), as well as reduced DA turnover (Fig. 3A, 3B), we wanted to examine if the 5-HT<sub>1A</sub> receptor is regulated by toluene inhalation in mice. We first measured the expression levels of 5-HT<sub>1A</sub> receptors in the hippocampus. As shown in Fig. 4A, 5-HT<sub>1A</sub> receptor expression tended to increase, but not significantly in the immediately after toluene exposure. However, significant upregulation of 5-HT<sub>1A</sub> receptor was detected in the hippocampus of mice in delayed effect group after 2,000 ppm toluene inhalation ( $1.19 \pm 0.063$ ;  $p<0.01$ ) (Fig. 4B). Under the same conditions, we also evaluated 5-HT<sub>1A</sub> receptor expression in the SN. As shown in both Fig. 4C and 4D, similar, but more significant changes in the expression of 5-HT<sub>1A</sub> receptor were detected in the SN both under short-term ( $1.79 \pm 0.032$ ;  $p<0.05$  in 2,000 ppm) and delayed ( $3.11 \pm 0.67$ ;  $p<0.05$  in 2,000 ppm) time after exposure. Together, these results indicate that 5-HT<sub>1A</sub> receptor was significantly upregulated in the hippocampus and SN of toluene-inhaled mice over time.

### **Toluene reduces neurogenesis in the dentate gyrus two weeks after inhalation**

Recent studies have demonstrated that a decreased rate of dentate gyrus (DG) neurogenesis is linked with the pathology of depression (Djavadian, 2004; Yau *et al.*, 2011; Lim *et al.*, 2018a). Therefore, we asked if toluene inhalation might affect DG neurogenesis over time, by monitoring DCX and NeuN protein levels. As shown in Fig. 5A, immature neuronal marker DCX levels were not changed relatively short-term after toluene inhalation, but significantly decreased delayed period after inhalation ( $0.72 \pm 0.05$ ;  $p<0.05$  in 2,000 ppm). In addition, the reduced immunoreactivity of NeuN neuronal marker was observed in the DG of mice in delayed effect group after inhalation (Fig. 5B). These results suggest that toluene inhalation induces delayed reduction of neurogenesis.

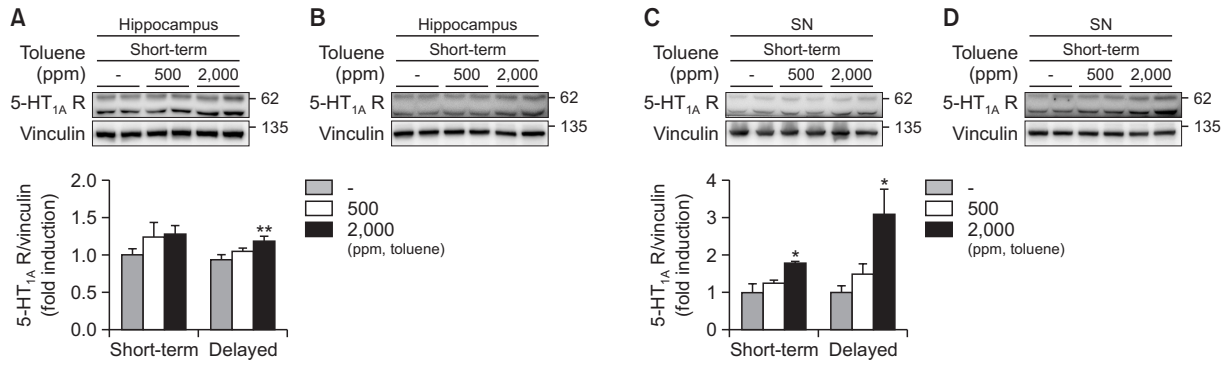


**Fig. 3.** Effects of toluene on DA and its metabolites in the striatum and TH immunoreactivity in the striatum and SN. (A, B) DA and its metabolites DOPAC and HVA were measured in the striatum using HPLC/ECD, immediately after (A) or two weeks after (B) toluene inhalation. (C, D) Representative photomicrograph demonstrating TH-immunostained sections at  $\times 4$  magnification of the (C) striatum CPu and NAc and  $\times 10$  magnification of the (D) SNpc and VTA regions of SN in toluene inhaled and control mice. Coronal section schematics modified from Allen Mouse Brain Atlas (reference atlas version 1 (2008)) showing the location of CPu, NAc, SNpc and VTA. Data are represented as mean ( $\pm$  S.E.M.) to control mice ( $n=3-4$ ).  $**p<0.001$  and  $****p<0.00001$  versus control. Scale bar: 500  $\mu\text{m}$  ( $\times 4$ ) and 200  $\mu\text{m}$  ( $\times 10$ ). DA, dopamine; DOPAC, 4-dihydroxyphenylacetic acid; HVA, homovanillic acid; CPu, caudoputamen; NAc, nucleus accumbens; SN, substantia nigra; SNpc, substantia nigra pars compacta; VTA, ventral tegmental area.

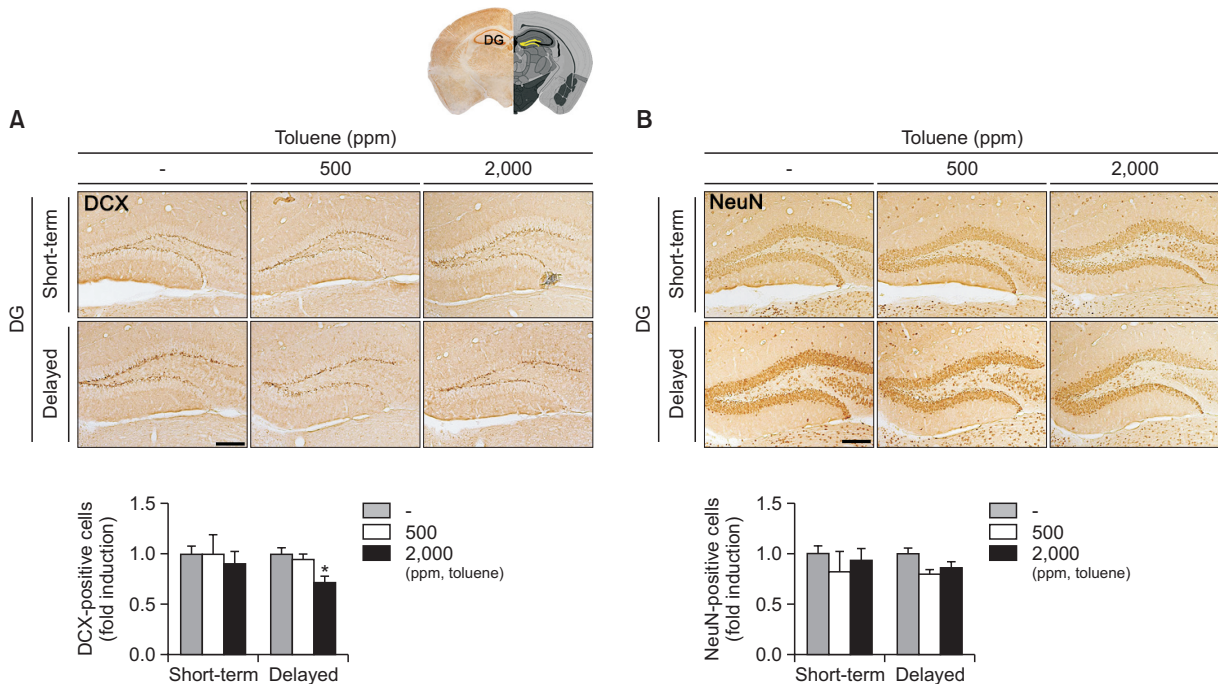
## DISCUSSION

Clinical reports in humans and *in vivo* experiments have demonstrated neurobehavioral disturbances after toluene inhalation, but there are few reports on the changes in behavioral patterns over time and the underlying neurobiological pathologies. In this study, we show that toluene inhalation, even

a single exposure, induces different forms of neurobehavioral changes over time. Anxiety-like behaviors are increased relatively shortly after toluene inhalation (Fig. 2A), and restored over time. Conversely, depression-like behaviors are significantly increased two weeks after toluene inhalation (Fig. 2B). None of the experimental conditions affected motor coordination that could interfere with performance on other behavioral



**Fig. 4.** Effects of toluene inhalation on the expression of 5-HT<sub>1A</sub> receptor in the hippocampus and SN. Mice were sacrificed after (A, C) a short-term or (B, D) a two-week delay after toluene or clean air exposure, and the expression levels of 5-HT<sub>1A</sub>R were analyzed using western blot analysis. Each band was quantified by densitometry and represented as a graph. Data are presented as mean ± SEM relative to control (n=3-4). \**p*<0.05 and \*\**p*<0.01 versus control. 5-HT<sub>1A</sub> receptor, serotonin 1A receptor.



**Fig. 5.** Histological analysis of neurogenesis markers in the dentate gyrus (DG). Representative photomicrograph demonstrating of (A) DCX and (B) NeuN-immunostained sections at ×20 magnification of the DG regions of hippocampus in toluene inhaled and control mice. Mouse Brain Atlas showing the location of DG. Data are presented as mean ± SEM relative to control (n=3-4). \**p*<0.05 versus control. Scale bar: 100 μm. DG, dentate gyrus; DCX, doublecortin.

tests. Moreover, toluene inhalation leads to persistent effects on the nervous system, such as decreases in DA turnover and adult neurogenesis.

In this study, a single exposure to toluene caused a significant increase in anxiety-like behaviors 24 h after inhalation (Fig. 2A). Reports on the behavioral effects of toluene are inconsistent, depending on the experimental conditions. Some evidences have demonstrated that toluene produced anxiolytic-like reactions; Beasley *et al.* (2010) reported that anxiety-like behaviors of mice were decreased immediately after a brief exposure to toluene. Another group reported that toluene exerted anxiolytic-like reactions in mice that showed

decreased cumulative time in burying behavior and increased retention time in the open arm in the elevated plus maze test (Lopez-Rubalcava *et al.*, 2000). In contrast, opposing results have also been reported: anxiety-like behaviors are increased in mice 24 h after prolonged exposure to toluene, which might be a sign of withdrawal (Bowen *et al.*, 2018), and chronic toluene exposure increased anxiety in mice in the burying behavior test (Arrant *et al.*, 2013). In the present study, anxiety-like behavior was detected 24 h after toluene inhalation, but not after two weeks. When monitoring DA and its metabolites in the striatum immediately after toluene inhalation, there were significant reductions in DOPAC and HVA, suggestive of di-



minished DA turnover. It is suggested that DA balance is vital not only for motor coordination but also mental wellbeing, including anxiety modulation in different parts of the brain (Zarrindast and Khakpai, 2015). Evidence shows that the nigrostriatal DAergic system is also involved in anxiety (Erro *et al.*, 2012; Zarrindast and Khakpai, 2015). It has been reported that the decreases in DA and its metabolites, or a reduced DOPAC/DA ratio are associated with anxiety-like behaviors (Chiavegatto *et al.*, 2009; Thiemann *et al.*, 2009). Of course, studies related to toluene addiction demonstrate that toluene exposure in animals leads to increases in DA release in brain regions including the striatum (Stengard *et al.*, 1994), VTA (Riegel *et al.*, 2007), and prefrontal cortex (Gerasimov *et al.*, 2002). However, there are reports of opposite effects of toluene on DA dynamics, as well. Beckley and Woodward (2011) showed that toluene differentially modulates excitatory and inhibitory synaptic transmission, associated with the generation of retrograde signalling molecules such as endocannabinoids. In fact, interactions of DAergic systems with cannabinoid systems in several brain regions such as the amygdala, NAc, and striatum have been suggested to be involved in the regulation of anxiety-like behavioral responses (Terzian *et al.*, 2011). Previous reports demonstrate that toluene inhalation alters DA neurotransmission depending on whether mice were exposed once or repeatedly (Apawu *et al.*, 2015): short-term exposure potentiates striatal DA release, whereas delayed exposure attenuates it. Our study showed that striatal DA turnover decreased immediately after toluene inhalation and continued to decrease for up to almost two weeks (Fig. 3A, 3B). Over time, not only DA metabolites but also DA itself tended to decrease. Based on the results showing that toluene does not affect TH-immunoreactivity in the nigrostriatal pathway (Fig. 3C, 3D), the reduction in DA and its metabolites is likely due to functional inhibition rather than DA neuronal damage.

Unlike anxiety-like behaviors, depression-like behaviors were significantly increased two weeks after toluene inhalation (Fig. 2B). Although our results reflect clinical findings that workers who are chronically intoxicated from toluene show depressive symptoms during weekend relief from work-related toluene exposure (Lee *et al.*, 2003), there are limited reports on whether toluene directly induces depression-like behaviors. Yang *et al.* (2010) reported that toluene induces depression-like behaviors in adult mice. They exposed mice to toluene using i.p. injections and showed that toluene induced depression-like behaviors in the short-term (1 and 4 days after administration). On the other hand, Cruz *et al.* (2009) demonstrated that toluene had anti-depressant effects 30 min after inhalation. Our findings suggest that the risk of depression could increase in the long term, even if exposure to toluene is not repeated. 5-HT plays a crucial role in the regulation of emotion; therefore, dysregulation of 5-HT homeostasis is involved in the development of anxiety and depression. Multiple serotonin receptor subtypes are expressed in serotonergic neurons and are associated with the complexity of 5-HT neurotransmission by regulating serotonergic neuronal activity (Dale *et al.*, 2016). Among them, there is growing evidences demonstrating the involvement of the main inhibitory serotonergic receptor 5-HT<sub>1A</sub> receptor in anxiety and depression. 5-HT<sub>1A</sub> presynaptic auto-receptors negatively regulate serotonergic neuronal activation (Pineyro and Blier, 1999), and post-synaptic 5-HT<sub>1A</sub>R mediate serotonergic neurotransmission with inhibitory signalling (Yamamura *et al.*, 2011). Excessive

increases in 5-HT<sub>1A</sub> auto-receptors are implicated in depression by inhibiting serotonergic neuron signals (Albert *et al.*, 2011). In patients attempting suicidal behaviors, 5-HT<sub>1A</sub> auto-receptor concentration was higher in the dorsal raphe nucleus (Stockmeier *et al.*, 1998; Boldrini *et al.*, 2008). In this study, we showed for the first time that toluene inhalation increased the expression of 5-HT<sub>1A</sub>R in the hippocampus as well as in the SN. Notably, toluene-induced 5-HT<sub>1A</sub>R upregulation was more significant in delayed effect group, which was also when depression-like behaviors of mice were detected. Further studies are underway to determine which signalling events are responsible for the upregulation of 5-HT<sub>1A</sub>R in the hippocampus and SN of toluene-inhaled mice.

Dysregulation of adult neurogenesis is also representative of depression. Neurogenesis markers such as DCX and BrdU tended to decrease in the DG of mice with depression behaviors, where there was suppressed proliferation and production of new neurons (Schoenfeld and Cameron, 2015; Siopi *et al.*, 2016). Several studies have demonstrated the effects of toluene on neurogenesis: acute high-level toluene exposure (7,000 ppm) was shown to cause significantly decreased hippocampal neurogenesis in rats, which persisted until 8 days after exposure (Yoon *et al.*, 2016); the immunoreactivity of Ki-67 and DCX in the DG of adult hippocampus was acutely reduced between 0 and 24 h after toluene injection (500 mg/kg, i.p.) and increased gradually from two days after injection (Seo *et al.*, 2010); and prenatal toluene exposure resulted in abnormal neuronal proliferation and migration in rat somatosensory cortices (Gospe and Zhou, 2000). The results observed in this study are consistent with those reports, especially in showing that even single exposure can lead to long-term suppression of neurogenesis with increased depressive behavior.

Collectively, the results shown here suggest that toluene inhalation, even if only once, might contribute to the development of depressive disorders; toluene cause anxiety-like behaviors in the short-term, and could be a risk factor for developing depression in the long term. In addition, toluene induces neuropathological changes in the DAergic and serotonergic system over time after inhalation, accompanied by persistent inhibition of neurogenesis. Because many inhalants including toluene are commonly found in most home and relatively low cost, they are readily available to young children and adolescents. The experience of inhalation serves as a “gateway” for the use of other types of drugs abuse in adulthood (Salmanzadeh *et al.*, 2020). In addition, the present results showing that single toluene inhalation can cause long-term pathological changes in the brain suggest that toluene inhalation might be a potential risk factor for the development of mood disorders.

## CONFLICT OF INTEREST

There is no conflict of interest.

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## REFERENCES

- Albert, P. R., Le Francois, B. and Millar, A. M. (2011) Transcriptional dysregulation of 5-HT1A autoreceptors in mental illness. *Mol. Brain* **4**, 21.
- Apawu, A. K., Mathews, T. A. and Bowen, S. E. (2015) Striatal dopamine dynamics in mice following acute and repeated toluene exposure. *Psychopharmacology (Berl.)* **232**, 173-184.
- Arnold, G. L., Kirby, R. S., Langendoerfer, S. and Wilkins-Haug, L. (1994) Toluene embryopathy: clinical delineation and developmental follow-up. *Pediatrics* **93**, 216-220.
- Arrant, A. E., Schramm-Sapota, N. L. and Kuhn, C. M. (2013) Use of the light/dark test for anxiety in adult and adolescent male rats. *Behav. Brain Res.* **256**, 119-127.
- Beasley, T. E., Evansky, P. A., Gilbert, M. E. and Bushnell, P. J. (2010) Behavioral effects of subchronic inhalation of toluene in adult rats. *Neurotoxicol. Teratol.* **32**, 611-619.
- Beckley, J. T. and Woodward, J. J. (2011) The abused inhalant toluene differentially modulates excitatory and inhibitory synaptic transmission in deep-layer neurons of the medial prefrontal cortex. *Neuropsychopharmacology* **36**, 1531-1542.
- Boldrini, M., Underwood, M. D., Mann, J. J. and Arango, V. (2008) Serotonin-1A autoreceptor binding in the dorsal raphe nucleus of depressed suicides. *J. Psychiatr. Res.* **42**, 433-442.
- Bowen, S. E., Hannigan, J. H., Davidson, C. J. and Callan, S. P. (2018) Abstinence following toluene exposure increases anxiety-like behavior in mice. *Neurotoxicol. Teratol.* **65**, 42-50.
- Chiavegatto, S., Izidio, G. S., Mendes-Lana, A., Aneas, I., Freitas, T. A., Torrao, A. S., Conceicao, I. M., Britto, L. R. and Ramos, A. (2009) Expression of alpha-synuclein is increased in the hippocampus of rats with high levels of innate anxiety. *Mol. Psychiatry* **14**, 894-905.
- Crawley, J. and Goodwin, F. K. (1980) Preliminary report of a simple animal behavior model for the anxiolytic effects of benzodiazepines. *Pharmacol. Biochem. Behav.* **13**, 167-170.
- Cruz, S. L., Rivera-Garcia, M. T. and Woodward, J. J. (2014) Review of toluene action: clinical evidence, animal studies and molecular targets. *J. Drug Alcohol Res.* **3**, 235840.
- Cruz, S. L., Soberanes-Chavez, P., Paez-Martinez, N. and Lopez-Rubalcava, C. (2009) Toluene has antidepressant-like actions in two animal models used for the screening of antidepressant drugs. *Psychopharmacology (Berl.)* **204**, 279-286.
- Dale, E., Pehrson, A. L., Jeyarajah, T., Li, Y., Leiser, S. C., Smagin, G., Olsen, C. K. and Sanchez, C. (2016) Effects of serotonin in the hippocampus: how SSRIs and multimodal antidepressants might regulate pyramidal cell function. *CNS Spectr.* **21**, 143-161.
- Djavadian, R. L. (2004) Serotonin and neurogenesis in the hippocampal dentate gyrus of adult mammals. *Acta Neurobiol. Exp. (Wars.)* **64**, 189-200.
- Erro, R., Pappata, S., Amboni, M., Vicidomini, C., Longo, K., Santangelo, G., Picillo, M., Vitale, C., Moccia, M., Giordano, F., Brunetti, A., Pellicchia, M. T., Salvatore, M. and Barone, P. (2012) Anxiety is associated with striatal dopamine transporter availability in newly diagnosed untreated Parkinson's disease patients. *Parkinsonism Relat. Disord.* **18**, 1034-1038.
- Felix-Ortiz, A. C. and Tye, K. M. (2014) Amygdala inputs to the ventral hippocampus bidirectionally modulate social behavior. *J. Neurosci.* **34**, 586-595.
- Gerasimov, M. R., Schiffer, W. K., Marsteller, D., Ferrieri, R., Alexoff, D. and Dewey, S. L. (2002) Toluene inhalation produces regionally specific changes in extracellular dopamine. *Drug Alcohol Depend.* **65**, 243-251.
- Gmaz, J. M., Matthews, B. A. and McKay, B. E. (2012) Toluene inhalation modulates dentate gyrus granule cell output *in vivo*. *Neurotoxicol. Teratol.* **34**, 403-412.
- Gospe, S. M., Jr. and Zhou, S. S. (2000) Prenatal exposure to toluene results in abnormal neurogenesis and migration in rat somatosensory cortex. *Pediatr. Res.* **47**, 362-368.
- Han, A., Yeo, H., Park, M. J., Kim, S. H., Choi, H. J., Hong, C. W. and Kwon, M. S. (2015) IL-4/10 prevents stress vulnerability following imipramine discontinuation. *J. Neuroinflammation* **12**, 197.
- Howard, M. O., Bowen, S. E., Garland, E. L., Perron, B. E. and Vaughn, M. G. (2011) Inhalant use and inhalant use disorders in the United States. *Addict. Sci. Clin. Pract.* **6**, 18-31.
- Lee, Y. L., Pai, M. C., Chen, J. H. and Guo, Y. L. (2003) Central neurological abnormalities and multiple chemical sensitivity caused by chronic toluene exposure. *Occup. Med. (Lond.)* **53**, 479-482.
- Lim, J., Bang, Y. and Choi, H. J. (2018a) Abnormal hippocampal neurogenesis in Parkinson's disease: relevance to a new therapeutic target for depression with Parkinson's disease. *Arch. Pharm. Res.* **41**, 943-954.
- Lim, J., Bang, Y., Choi, H. H., Han, A., Kwon, M. S., Liu, K. H. and Choi, H. J. (2018b) LRRK2 G2019S induces anxiety/depression-like behavior before the onset of motor dysfunction with 5-HT1A receptor upregulation in mice. *J. Neurosci.* **38**, 1611-1621.
- Lin, B. F., Ou, M. C., Chung, S. S., Pang, C. Y. and Chen, H. H. (2010) Adolescent toluene exposure produces enduring social and cognitive deficits in mice: an animal model of solvent-induced psychosis. *World J. Biol. Psychiatry* **11**, 792-802.
- Lopez-Rubalcava, C., Hen, R. and Cruz, S. L. (2000) Anxiolytic-like actions of toluene in the burying behavior and plus-maze tests: differences in sensitivity between 5-HT(1B) knockout and wild-type mice. *Behav. Brain Res.* **115**, 85-94.
- Marjot, R. and McLeod, A. A. (1989) Chronic non-neurological toxicity from volatile substance abuse. *Hum. Toxicol.* **8**, 301-306.
- Paez-Martinez, N., Cruz, S. L. and Lopez-Rubalcava, C. (2003) Comparative study of the effects of toluene, benzene, 1,1,1-trichloroethane, diethyl ether, and flurothyl on anxiety and nociception in mice. *Toxicol. Appl. Pharmacol.* **193**, 9-16.
- Pineyro, G. and Blier, P. (1999) Autoregulation of serotonin neurons: role in antidepressant drug action. *Pharmacol. Rev.* **51**, 533-591.
- Przegalinski, E. and Filip, M. (1997) Stimulation of serotonin (5-HT)1A receptors attenuates the locomotor, but not the discriminative, effects of amphetamine and cocaine in rats. *Behav. Pharmacol.* **8**, 699-706.
- Pyykko, K., Tahti, H. and Vapaatalo, H. (1977) Toluene concentrations in various tissues of rats after inhalation and oral administration. *Arch. Toxicol.* **38**, 169-176.
- Riegel, A. C., Zapata, A., Shippenberg, T. S. and French, E. D. (2007) The abused inhalant toluene increases dopamine release in the nucleus accumbens by directly stimulating ventral tegmental area neurons. *Neuropsychopharmacology* **32**, 1558-1569.
- Salmanzadeh, H., Ahmadi-Soleimani, S. M., Pachenari, N., Azadi, M., Halliwell, R. F., Rubino, T. and Azizi, H. (2020) Adolescent drug exposure: a review of evidence for the development of persistent changes in brain function. *Brain Res. Bull.* **156**, 105-117.
- Schoenfeld, T. J. and Cameron, H. A. (2015) Adult neurogenesis and mental illness. *Neuropsychopharmacology* **40**, 113-128.
- Seo, H. S., Yang, M., Song, M. S., Kim, J. S., Kim, S. H., Kim, J. C., Kim, H., Shin, T., Wang, H. and Moon, C. (2010) Toluene inhibits hippocampal neurogenesis in adult mice. *Pharmacol. Biochem. Behav.* **94**, 588-594.
- Siopi, E., Denizet, M., Gabellec, M. M., de Chaumont, F., Olivo-Marin, J. C., Guilloux, J. P., Lledo, P. M. and Lazarini, F. (2016) Anxiety- and depression-like states lead to pronounced olfactory deficits and impaired adult neurogenesis in mice. *J. Neurosci.* **36**, 518-531.
- Stengard, K., Hoglund, G. and Ungerstedt, U. (1994) Extracellular dopamine levels within the striatum increase during inhalation exposure to toluene: a microdialysis study in awake, freely moving rats. *Toxicol. Lett.* **71**, 245-255.
- Stockmeier, C. A., Shapiro, L. A., Dilley, G. E., Kolli, T. N., Friedman, L. and Rajkowska, G. (1998) Increase in serotonin-1A autoreceptors in the midbrain of suicide victims with major depression-postmortem evidence for decreased serotonin activity. *J. Neurosci.* **18**, 7394-7401.
- Tas, U., Ekici, F., Koc, F., Sogut, E., Ayan, M., Kuloglu, T., Arici, A. and Ozyurt, B. (2013) Acute cardiotoxic effects of high dose toluene: an experimental study. *Anadolu Kardiyol. Derg.* **13**, 3-8.
- Terzian, A. L., Drago, F., Wojtak, C. T. and Micala, V. (2011) The dopamine and cannabinoid interaction in the modulation of emotions and cognition: assessing the role of cannabinoid CB1 receptor in neurons expressing dopamine D1 receptors. *Front. Behav. Neurosci.* **5**, 49.
- Thiemann, G., Watt, C. A., Ledent, C., Molleman, A. and Hasenohr, R. U. (2009) Modulation of anxiety by acute blockade and genetic



- deletion of the CB(1) cannabinoid receptor in mice together with biogenic amine changes in the forebrain. *Behav. Brain Res.* **200**, 60-67.
- Tianzhu, Z., Shihai, Y. and Juan, D. (2014) Antidepressant-like effects of cordycepin in a mice model of chronic unpredictable mild stress. *Evid. Based Complement. Alternat. Med.* **2014**, 438506.
- Yamamura, S., Abe, M., Nakagawa, M., Ochi, S., Ueno, S. and Okada, M. (2011) Different actions for acute and chronic administration of mirtazapine on serotonergic transmission associated with raphe nuclei and their innervation cortical regions. *Neuropharmacology* **60**, 550-560.
- Yang, M., Kim, S. H., Kim, J. C., Shin, T. and Moon, C. (2010) Toluene induces depression-like behaviors in adult mice. *Toxicol. Res.* **26**, 315-320.
- Yankelevitch-Yahav, R., Franko, M., Huly, A. and Doron, R. (2015) The forced swim test as a model of depressive-like behavior. *J. Vis. Exp.* **(97)**, 52587.
- Yau, S. Y., Lau, B. W., Tong, J. B., Wong, R., Ching, Y. P., Qiu, G., Tang, S. W., Lee, T. M. and So, K. F. (2011) Hippocampal neurogenesis and dendritic plasticity support running-improved spatial learning and depression-like behaviour in stressed rats. *PLoS ONE* **6**, e24263.
- Yoon, J. H., Seo, H. S., Lee, J., Moon, C. and Lee, K. (2016) Acute high-level toluene exposure decreases hippocampal neurogenesis in rats. *Toxicol. Ind. Health* **32**, 1910-1920.
- Zachrisson, L., Ruchkin, V., Stickley, A. and Kuposov, R. (2017) Inhalant use and mental health problems in russian juvenile delinquents. *Subst. Use Misuse* **52**, 1616-1623.
- Zarrindast, M. R. and Khakpai, F. (2015) The modulatory role of dopamine in anxiety-like behavior. *Arch. Iran. Med.* **18**, 591-603.
- Zhvanina, M. G., Chilachava, L. R., Japaridze, N. J., Gelazonia, L. K. and Lordkipanidze, T. G. (2012) Immediate and persisting effect of toluene chronic exposure on hippocampal cell loss in adolescent and adult rats. *Brain Res. Bull.* **87**, 187-192.