Effects of dextromethorphan treated at adolescent on behavior and neurochemistry in rats

Dissertation by
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by
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Effects of dextromethorphan treated at adolescent on behavior and neurochemistry in rats

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(Directed by Professor Dong Goo Kim)

Dextromethorphan (DM), the d-isomer of the codeine analog levorphanol, is a highly effective antitussive drug. This drug is widely available in many over-the-counter cough medications. DM is a very safe drug at a recommended dosage. However, episodic and sporadic abuse of high doses of DM (300 mg or more, compared to 30 or 60 mg as an antitussive) has been reported in several countries mostly in adolescents and young adults. However, the behavioral effects of DM have been reported inconsistently.

To elucidate behavioral and neurochemical consequences of DM abuse, DM was administered acutely or chronically in adolescent rats, and alterations in behavior and neurochemistry were assessed during adolescent or aged periods. The following results were obtained:

1. Acute DM decreased nocturnal activity dose-dependently in adolescent rats.

2. Acute DM increased c-Fos expression in the dopaminergic reward pathway and increased mesencephalic tyrosine hydroxylase mRNA expression in adolescent rats.

3. Repetitive DM induced behavioral sensitization in the locomotor activity and the stereotyped behavior, and these phenomena were obvious in female.

4. Hypothalamus NMDAR1 immunoreactivity was increased by repetitive treatment with DM and this was obvious in female.

5. Ten days exposure to DM during adolescent period results in impaired learning and memory and

stress responses with cell loss in the CA1 and CA3 field of hippocampus in male and CA3 in female at old age.

These data indicate that DM has an abuse liability with behavioral sensitization. Furthermore, exposure to DM at adolescent period impairs spatial learning with accelerated cell loss in the hippocampus in aged life. The results suggest that altered NMDA receptor regulation and stress axis contribute to the consequences of DM exposure.

Key Words: dextromethorphan, drug abuse, behavioral sensitization, learning and memory, tyrosine hydroxylase, NMDA receptor, c-Fos, autoshaping, Morris water maze

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<Directed by Professor **Dong Goo Kim**>
Brain Korea 21 Project for Medical Sciences
The Graduate School, Yonsei University
Tie Yuan Zhang

Ⅰ. Introduction

Dextromethorphan (DM), the d-isomer of the codeine analog levorphanol, is a highly effective antitussive drug (Fig. 1). This drug is widely available in many over-the-counter cough medications. DM is a very safe drug at a recommended dosage.[1] However, episodic and sporadic abuse of high doses of DM (300 mg or more, compared to 30 or 60 mg as an antitussive) in adolescents and young adults has been reported in several countries.[2,3]

The psychological symptoms of DM abuse are reported similar to phencyclidine (PCP)-induced psychotomimetic symptoms.[2] Since both DM and PCP bind the N-methyl-D-aspartate (NMDA) receptor and antagonize the action of the excitatory amino acids in a noncompetitive manner,[4] it can be assumed that NMDA antagonistic action of DM plays a role in these adverse reactions.

Recently, interests in DM have been raised again because of its other pharmacological properties than the antitussive effect. In animal studies, DM has neuroprotective, anticonvulsant and analgesic effects.[5] Clinical trials were done or currently are investigated for treatment of degenerative diseases of the central nervous system and other disorders.[5]

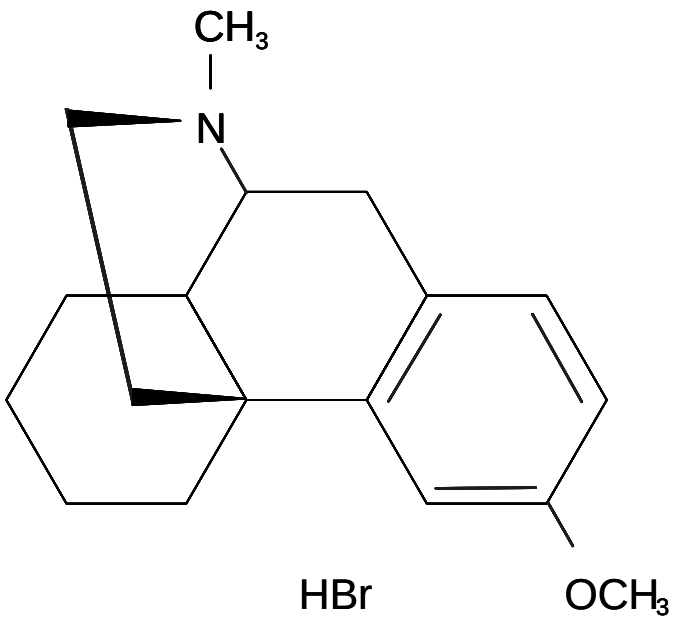

Fig. 1. Chemical structure of dextromethorphan

Chronic administration with high doses of DM is usually recommended for these indications. Therefore, consequences of this schedule of administration are needed to be verified in detail. However, few study addressed on this issue. Changes of locomotor activities,[7,8] stereotyped behavior[9] and impaired learning[10,11] were reported after acute administration. However, the behavioral effects of DM have been reported inconsistently. The effects of DM on the rodent behavior in previous reports might had been masked by the ceiling or floor effect, because all the behavioral tests reported so far were performed during daytime when the baseline locomotor activities maintained at lower level.[12] Actually, Kim et al.[13] showed that the behavioral activities of rats significantly increased during nighttime, compared to daytime. To avoid the possible floor effects of DM on the locomotor activities during daytime, this study was performed during nighttime.

This study also examined whether DM activates the neurons involved in the striatal and mesolimbic reward pathway, because most drugs of abuse activate the natural reward pathway. Since the expression of an immediate early gene c-Fos has been generally used as a marker of neuronal activation by various stimuli, c-Fos immunohistochemistry was performed on the various brain regions after different doses of DM administrations. Dopaminergic neurons localized in the mesencephalon subserve important functions in the action of the drug of abuse. The dopaminergic cell group in the ventral tegmental area (VTA) projects to the nucleus accumbens, and this projection constitutes the mesolimbic pathway which plays important roles in drug addiction and behavioral sensitization. The cell group in the substantia nigra (SN) projects to the striatum, and this projection constitutes the nigrostriatal pathway which is implicated in motor effects associated with drug abuse.[14] Generally, repeated treatments with drugs of abuse increase dopamine release and the gene expression of tyrosine hydroxylase (TH),

the rate-limiting enzyme of dopamine biosynthesis, in the mesolimbic and the nigrostriatal dopaminergic systems.[15,16] However, effects of DM on the mesencephalon dopaminergic systems have not been reported yet although it is currently abused. Therefore, the effects of the high dose of DM were examined on the levels of TH mRNA in the VTA and SN of rats at postnatal day (PND) 28 by using *in situ* hybridization method.

It is not yet known whether DM elicits behavioral sensitization which might account for the psychobiological basis of drug craving.[17] This study determined whether chronic treatment with DM at a high dose elicits behavioral sensitization. Sexual dimorphism was also examined in DM-induced behavioral changes and NMDA receptor regulations.

It has been demonstrated that early life events may permanently affect behavioral and hormonal responses to stress in adulthood.[18] Brief mother deprivation, a mild stress, may attenuate hypothalamic-pituitary-adrenal (HPA) axis response to stress and attenuate spatial learning impairment in aged life. While more severe stressors such as exposure to endotoxin or long time deprivation from the dam may increase response to stress in aged life.[19] Cognitive deficiencies in adulthood have also been reported to be associated with severe stress early in life. For example, repeated exposure to high doses of alcohol in early life impairs spatial learning in water maze test in aged rats[20] with morphological and neurochemical abnormalities. It has also been reported that neuronal loss occurred in the CA1 and CA3 field of the hippocampus, and cerebellum of rats after repeated exposure to high doses of ethanol in early life.[21,22]

Few studies have focused on the consequences of events during adolescent although consequences of early life events were established fairly well. It is possible that permanent changes in brain and behavior would be resulted from events during adolescent because

neurobiological system is still maturating during the period of adolescence. In fact, the prefrontal cortex undergoes major changes during adolescence in humans and other animals.[23] In addition, hormonal levels change dramatically in adolescent. This fluctuation of hormonal levels is believed to play a role in a quicker development of drug dependence in adolescent.[24] In case of DM exposure, NMDA antagonistic property of DM may cause permanent neurobiological changes, because other NMDA antagonists have been reported to result in neuronal apoptosis.[25,26]

It is not yet known whether experience of DM at adolescent would result in long-term or permanent behavioral changes. At present study, DM was administered at adolescent in rats, and their behavioral responses and neurochemical changes were examined at adulthood and old age. This study is the first attempt to examine consequences of excessive consumption of DM in adolescent. The underlying neurobiological mechanisms were also studied.

Ⅱ. Materials and Methods

1. Animals

The animals were supplied from the Division of Laboratory Animal Medicine, Yonsei University College of Medicine. Animals were cared for in a SPF barrier area, where the temperature (22 ±1℃) and humidity (55%) were constantly controlled with a 12:12 hr light:dark cycle (lights on 07.00 hr). Food (PMI Feeds, Inc., IN, USA) and tap water (membrane filtered purified water) were available *ad libitum*. For the behavioral studies, Sprague-Dawley rats were bred and the offspring were reared in a controlled manner to minimize and to standardize unwanted environmental stimulation from *in utero* life.

Nulliparous female and proven breeder male Sprague-Dawley rats were used for breeding. Twelve hours after confirming delivery, pups were divided by sex, and weighed. Litters which had 5 males and 5 females or more were used, and pups were culled to 5 males and 5 females in a litter. Littermates were distributed over the saline and the DM groups. The mean body weights of groups were not different because rats were assigned by Latin Square method according to the order of body weights within a litter on PND 28.

2. Drugs

Dextromethorphan HBr, was purchased from Sigma Chemical Co. (Catalog No. D9684, St. Louis, MO, USA). All other chemicals were of analytical reagent grade. For the behavioral studies, DM was dissolved in physiological saline and injected intraperitoneally in a volume of 2 ml/kg. For the immunohistochemical and Western blot analyses, monoclonal mouse anti-NMDAR1 (Catalog No. 60021A, BD PharMingen, San Diego, CA, USA), polyclonal anti-c-Fos primary antibody raised in rabbits (Oncogene Research Product, Cambridge, MA, USA), biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA, USA), goat anti-mouse IgG HRP conjugate (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), ABC kit (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA, USA), TH cDNA probe (a 1.6-kb Pst1 restriction fragment of a rat TH cDNA,[27] random primed kit (Roche Diagnostics GmbH, Mannheim, Germany), ^{35}S-α-dATP (NEN Life Science Products, Inc. Boston, MA, USA) were used.

3. Behavioral measurements

(1) Locomotor activity

A rat was placed into the activity chamber (43.2 cm wide, 43.2 cm long, 30.5 cm high, MED Associates, Georgia, VT, USA), and its ambulatory and rearing activities were measured. The transparent acryl chamber was equipped with 2 horizontal planes (2.5 cm, for ambulation count, and 12.5 cm, for rearing count, above the floor) of 16 infrared photocell-detector pairs in each x, y dimensions spaced 2.54 cm apart. The rats were allowed to acclimatize to the activity chamber for 30 min. They were put back into the chamber immediately after injection, and ambulatory and rearing activity counts were recorded automatically. Chambers were cleaned with alcohol (70%) after each use to prevent the influence of a previously tested rat.

(2) Stereotyped behavior

Sniffing and repetitive head movement were measured as the stereotyped behaviors and rated using the method of Iyo et al.,[28] 0, not observed; 1,questionable; 2, slightly observed; 3, moderately observed; 4, intensely observed; 5, almost continuously observed.

(3) Ataxia

Ataxia was rated by the modified method of Sturgeon et al.,[29] 0, inactive or in-place activity, coordinated movement; 1, unusual, awkward or jerky movement, loss of balance during rearing, occasional falling on side; 2, awkward-jerky movements, moderate rate of falling on side while rearing or moving about; 3, frequent falling on back and/or side while moving, partial impairment of antigravity reflexes. Scores of stereotypic behavior and ataxia were taken for 1 min at the end of every 10 min period after injection. Scores were cumulated for statistical analyses.

(4) Autoshaping with 4 sec delay reinforcement

Prior to the test, rats were allowed at least 7 days to acclimate to the housing conditions. Body weights of the animals were gradually reduced to 85% of their free-feeding weights and maintained at that level with a restricted feeding schedule. Water was available *ad libitum*, throughout the experiment. All testing occurred between the hours of 9:30 a.m. and 1 p.m. Rats were tested in standard operant chambers (ENV-007, MED Associates, Georgia, VT, USA), enclosed in sound-attenuating cubicles with built-in C.C.T.V. systems. Behavioral boxes were 30.5 cm high, 29 cm wide and 24 cm long, with grid floors of stainless steel bars (4.8 mm diameter, spaced 1.6 cm apart). Each box was equipped with a retractable lever (ENV-112B, MED Associates, Georgia, VT, USA), food dispenser (ENV-203, MED Associates, Georgia, VT, USA) for the delivery of 45 mg food pellets (Lot number 28612A2, P. J. Noyes Company, INC., Lancaster, NH, USA) and a speaker for the introduction of white masking noise. The boxes were controlled and data were collected by the MED-PC program for IBM computers (MED Associates, Georgia, VT, USA). During each autoshaping trial the lever was extended into the chamber for 15 sec on a random interval schedule ranging from 22 to 68 sec, with an average interval of 45 sec. One food pellet was delivered 4 sec after the lever had retracted whether or not the rat made a lever touch response. A daily session consisted of 12 trials and the procedure continued up to the 15th session. The extended lever touch was defined as the correct lever touch and the maximum number of correct lever touches in a session was 12. In this learning task, animals learned to associate and then touch a lever, which was intermittently extended into an operant chamber and retracted, in conjunction with delivery of food reinforcement. Lever retracted when rats made a lever touch response or after 15 sec. One 45 mg of food pellet was delivered after a 4 sec delay whether or not rats made a lever touch

response.

(5) Morris water maze test

A circular water maze (Panlab s.l., Barcelona, Spain) measuring 200 cm in diameter and 40 cm in height was used. A circular platform with 15 cm in diameter and 30 cm in height was located in the center of one quadrant of the water tank. The platform was submerged 1 cm beneath the surface of water and 42.5 cm apart from the edge. The temperature of the water was 22±1℃. Non-toxic, black tempera paint powder (Funstuff, Reeves and Poole Group, Toronto, Canada) was used to make the water opaque so the platform could not be seen. The movements of the rats within maze were recorded by a video tracking system (Smart, Panlab s.l., Barcelona, Spain). A variety of posters as cued stimuli were located on the walls of the test room. To assess the spatial learning ability, rats randomly placed in one of quadrants, facing edge and 5 cm apart from edge. These rats were given 60 sec to find the escape platform. If they found the platform they were allowed to stay at the platform for 15 sec, after which they were removed from the maze and placed under a heat lamp (60 Watt, 20 cm above) for 5 min. Then they were put in the maze for the next trial. If a rat did not find the platform within 60 sec, it would be guided to find the platform and stayed there for 15 sec. There were 4 trials each day. After 9 days training, a probe test was completed on the first trial of the 10th day. The platform was moved out from the tank. The rats were placed opposite side to the position of the platform and they were allowed to swim in the tank for 60 sec. The animal performance in each quadrant was recorded to measure the spatial learning ability without the influence of chance encounters with the platform. After the probe trial, the platform was replaced to the original position and the animals were retrained from the 2nd to the 4th trial as well as the 11th day. After retraining, the reversal training was completed for four days to assess the rat's ability to learn a new

platform position. The platform was moved to the opposite quadrant of the maze, and the training method was the same as in the initial training period. On the 16th day, three cued trials were done to test sensory-motor impairment. In this trial, the platform was raised above the surface of water 1 cm, a red cross shape of 2-dimensional flag with 18 cm in height was placed on the platform. The panels of the flag were 5 cm in length and 4 cm in height. The location of the platform was moved to different quadrant in each trial. Three trials were tested. The method was the same as in the initial training.

4. Neurochemical studies

(1) Immunohistochemistry

Rats were transcardially perfused with heparinized isotonic saline (0.9% NaCl, 0.5% $NaNO_2$) briefly after overdose of sodium pentobarbital, then followed by ice-cold paraformaldehyde (4% paraformaldehyde, 0.1 M phosphate buffer, pH 7.2). Brains were immediately dissected out and postfixed for 6 hours in the same fixative then cryoprotected with 30% sucrose solution for 24 hours prior to sectioning. Brains were coronally sectioned in 40 μm thickness with a sliding microtome (MICROM Laborgeräte GmbH, Walldorf, Germany). Free-floating tissue sections were washed twice for 15 min in 0.1 M sodium phosphate buffered saline (PBS), then permeabilized in 0.2% Triton, 1% bovine serum albumin (BSA) in PBS for 30 min. After washing twice in PBS-BSA, sections were incubated overnight with diluted primary antibody. Sections were washed twice in PBS-BSA and incubated for 1 hour with biotinylated secondary antibody (1:200 dilution), then bound secondary antibody was amplified with the ABC kit. Antibody complexes were visualized with 0.05% of

diaminobenzidine for 5 min. Immunostained sections were mounted on gelatine -coated slides in 0.05 M phosphate buffer, air dried, dehydrated through graded ethanol to xylene, and coverslipped. Results were analyzed by using the light microscope with the MCID imaging system (MCID, Imaging Research Inc., Ontario, Canada).

(2) Western blot analysis

Rats were decapitated and brains were removed rapidly. Tissues were homogenized in 1 ml of Solution A (0.32 M sucrose, 1 mM $NaHCO_3$, 1 mM $MgCl_2$, 0.5 mM $CaCl_2$) by using a glass homogenizer. Homogenates were centrifuged at 4℃ at 1,400 g for 10 min. The pallets were washed with 0.5 ml of Solution A, and centrifuged at 710 g for 10 min. Supernatants from above were collected and centrifuged again at 710 g for 10 min to remove debris. The membrane fraction was isolated by centrifugation at 13,800 g for 20 min. Pellets were resuspended in 100 μl of 100 mM Tris, pH 6.8 containing 2% sodium dodecyl sulfate (SDS), 100 M phenylmethylsulfonyl fluoride, 2 g/ml leupeptin, and 1 mM EDTA. Protein contents of the samples were determined by the Bradford assay. Samples containing 40 g of protein were subjected to 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically to nitrocellulose membranes. The membranes were blocked in 3% nonfat dry milk in 25 mM Tris-buffered saline/Tween 20, 0.1% v/v (TBST) 1 hour. The NMDAR1 subunit (116 kDa) was immunolabeled with a primary antibody (mouse anti-NMDAR1, diluted 1:2,000) overnight at 4℃. Blots were rinsed three times in TBST and incubated in goat anti-mouse IgG at 1:2,000 dilution in 3% milk in TBST. Immunoreactivity was visualized by using enhanced chemiluminescence (ECL, Renaissance, Boston, MA, USA). Immunoreactivity was

quantified by measuring the optical density of specific bands using the MCID imaging system.

(3) *in situ* hybridization

For *in situ* hybridization, sections were collected in 20 ml glass scintillation vials containing ice-cold 2x saline-sodium citrate (SSC) (0.3 M NaCl, 0.03 M sodium citrate). The SSC was pipetted off, and sections were suspended in 1 ml of prehybridization buffer (50% formamide, 10% dextran sulfate, 2 x SSC, 1 x Denhardt's solution, 50 mM 1,4 dithiothreitol (DTT), and 0.05% denatured salmon sperm DNA). Following two hours of prehybridization at 48°C, Tyrosine hydroxylase (TH) cDNA probe labeled with ^{35}S-α-dATP using a random primed kit was added to the vials (1 x 10^7 cpm/vial), and hybridized overnight at 48°C. Following hybridization, the sections were washed at 15-min intervals in decreasing concentrations of SSC (2 x, 2 x, 1 x, 0.5 x, 0.25 x, 0.125 x, 0.125 x) at 48°C. The tissue sections were then mounted on gelatin-subbed slides, air-dried, and apposed to Hyperfilm-βmax (Amersham International plc. CEA AB, Sweden) for 24 - 48 hours. Films were developed in Kodak D-19 and then fixed in Kodak rapid fixer. Tissue sections from different groups were hybridized within the same vial, and exposed to film together on the same microscope slide. To minimize experimental bias tissue sections from different rats were identified by punctures made in the brain tissue during sectioning. Thus, *in situ* hybridization was carried out on representative members of each experimental group at the same time under the identical conditions, allowing for a direct comparison of mRNA expression.

Images of the VTA and SN were digitized from autoradiographic films using MCID image analysis system (MCID, Imaging Research Inc., Ontario, Canada). Six sections (plate #43 from the atlas of Paxinos and Watson[30]) per rat were used for quantification. Messenger

RNA expression levels were determined by quantifying the mean relative optical density of pixels (mRNA pixels) of the area of interest in each section. For the quantification of mRNA pixels, the optical density of the background was subtracted from the pixel value of the area of interest. The mRNA pixel values were averaged across six sections from each individual rat, and the average mRNA value for each rat was then averaged across all rats within the experimental groups. The results were analyzed by using the light microscope with the MCID imaging system.

(4) Cresyl violet staining

For cresyl violet staining, brains were coronally sectioned in 20 μm thickness with a sliding microtome. Free-floating tissue sections were mounted on gelatin coated slides and dehydrated by a series of ethanol baths (50%, 75%, 95%, 100%) for 2 min each, then immersed in chloroform ethanol solution (1:1) for 20 min, rehydrated by a series of decreased concentration of ethanol baths (100%, 95%, 75%, 50%, 0%) and immersed in 0.125% cresyl violet dye for 30 sec. Dehydrated again as above and final rinsed in xylene for 20 min and covered slip.

Images of the hippocampus were captured at 200 X magnification using MCID image analysis system. The dorsal hippocampal section (plate #32, bregma -3.6mm from the atlas of Paxinos and Watson[30]) per animal was used for quantification. Cell densities were quantified by visual counting of neurons in the screen of the computer. The neurons were counted over 800 μm length in the CA1 subfield and 600 μm length in CA3 respectively. The cell counts were expressed as the number of cells per unit length of the cell field (cells/100 μm).

5. Statistical analysis

Data were presented as means ± S.E.M and statistical analyses were done with the aid of the StatView II program for Macintosh computers (Abacus Concepts, Inc., CA, USA). Differences between means were analyzed with a one-way analysis of variance (ANOVA), and preplanned multiple comparisons were done with appropriate methods according to the data characteristics.

Ⅲ. Results

1. Effects of acute dextromethorphan on nocturnal behavior and the brain c-Fos expressions in adolescent rats

(1) Behavioral effects

Drug administration and behavior test were performed on postnatal day (PND) 28 under specific pathogen free (SPF) condition. DM at each dose (0, 10, 20, 40, 80 mg/kg) was injected introperatoneally (i.p.) in a volume of 2 ml/kg on PND 28 at 20:00 hr, one hour after the lights off. The behavioral assessment was carried out immediately after the DM injection. Animals were placed in the test room 6 hours before the test. The light in the test chamber was adjusted to 2 lux of dimness 30 min prior to the test. This dimness was just bright enough for the tester to observe the movement of rats at a distance of 0.5 meter. Rats were allowed to acclimatize to the chamber for 30 min before the DM injection, and then, immediately after the DM, ambulatory and rearing activities were recorded automatically for 150 min with 10 min intervals. Two rats a day were subjected to the behavioral test in each test chamber. Therefore, the DM dosage were randomized over the rats tested each testing day to minimize the influence

of time differences, especially because the rats used in this experiment were in the fast growing period.

The rats showed decreased cumulative locomotor activities for 150 min after the DM injections at high doses (20, 40 mg/kg). The locomotor activity of 40 mg/kg DM group was significantly impaired for the whole test time period (Fig. 2a). Ataxia occurred immediately after the DM injections at all doses and the severity persisted in a dose-dependent way for the first 60 min period after the injection (Fig. 2b). The DM rats frequently showed jerky movement and the loss of balance during rearing. The rearing decreased dose-dependently with the loss of balance (Fig. 2c). The stereotyped activity measured by head movement and sniffing did not significantly changed by all doses of DM. In addition, 40% of female rats died after DM 80 mg/kg injection.

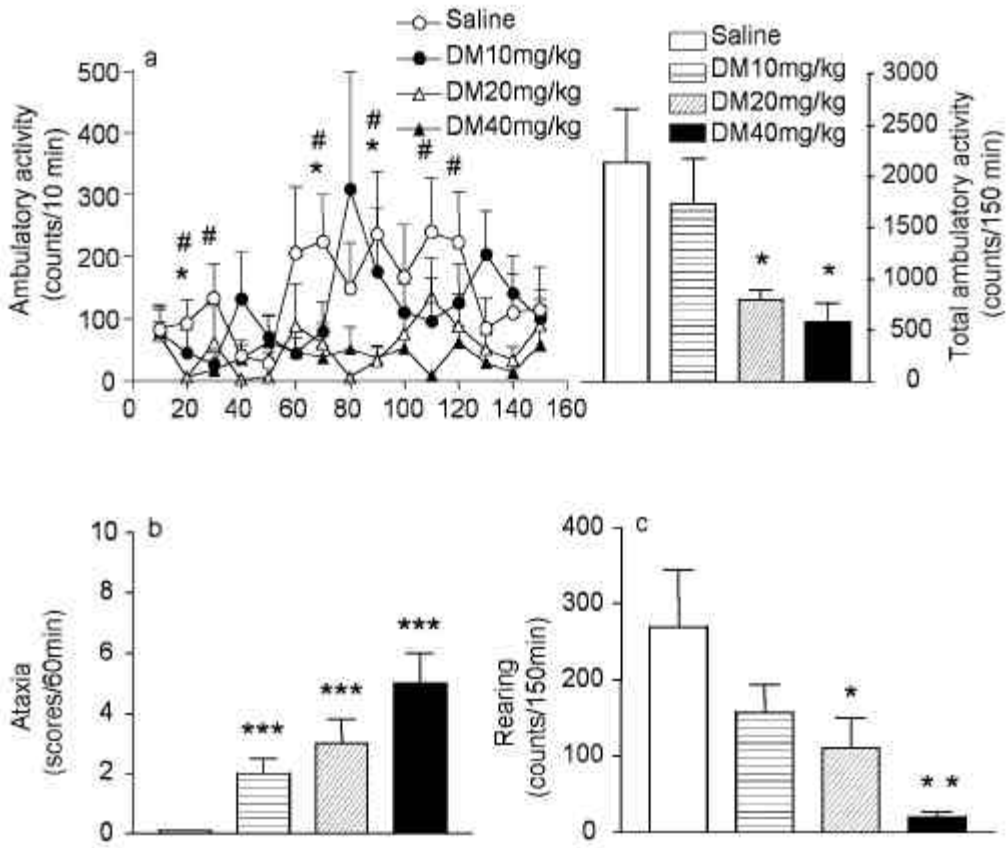

Fig. 2. Acute behavioral effects of dextromethorphan in adolescent female rats. Dextromethorphan was intraperitoneally injected into the rats 1 h after the onset of dark period with doses of 0, 10, 20, and 40 mg/kg, and the behavioral assessment was performed immediately after the injection. Ambulatory activity and rearing were measured for 150 min, and ataxia was evaluated as cumulative scores of the behavioral rating measured for 1 min at the end of every 10 min period for 60 min period. (a) ambulatory activity, (b) ataxia, (c) rearing. Numbers of animals in the saline and each dose of dextromethorphan groups were 6 (n=6), respectively. All values are mean ±S.E.M. * $p < 0.05$, degree of significance for 20 mg/kg or # $p < 0.05$, 40 mg/kg dextromethorphan group compared with the saline group in (a). * $p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, degree of significance for each dose of dextromethorphan groups compared with the saline group (b) and (c).

(2) Effects on c-Fos immunoreactivities in brain

Thirty min after the end of behavior tests, which was 3 hrs after DM administration, brains were obtained and dissected. Three consecutive sections from each brain region were collected at the levels of 1.6 and -2.56 mm from bregma,[30] respectively, and processed for c-Fos immunohistochemistry.

The numbers of c-Fos immuno-positive cells were counted using MCID image analysis system in five brain regions: the cingulated cortex (Cg), dorsal caudate putamen (Cpu), nucleus accumbens core (AcbC) and shell (AcbS), and central amygdala (CeA) (Fig. 3). The representative microscopic pictures of the brain regions of adolescent female rats show remarkable inductions of c-Fos expression by high doses of DM (Fig. 4). DM increased the numbers of c-Fos immuno-positive cells dose-dependently in all brain regions measured (Fig.5).

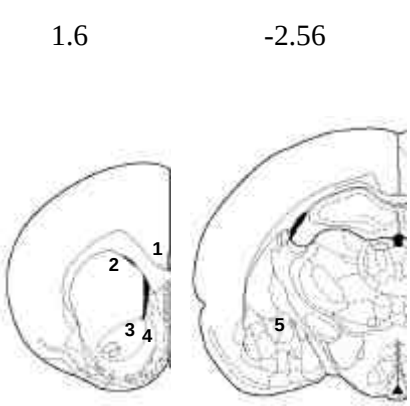

Fig. 3. Schematic diagram of rat brain region at the level of 1.6 and –2.56 mm from bregma. Numbers at the top left of each section represent the distance (in millimeters) from bregma. Numbers in the sections represent the regions analyzed as follows: 1 cingulate cortex (Cg); 2, caudate putamen (Cpu); 3, nucleus accumbens core (AcbC); 4, nucleus accumbens shell (AcbS); 5, central amygdala (CeA). Drawings were adapted from the Paxinos and Watson atlas[30].

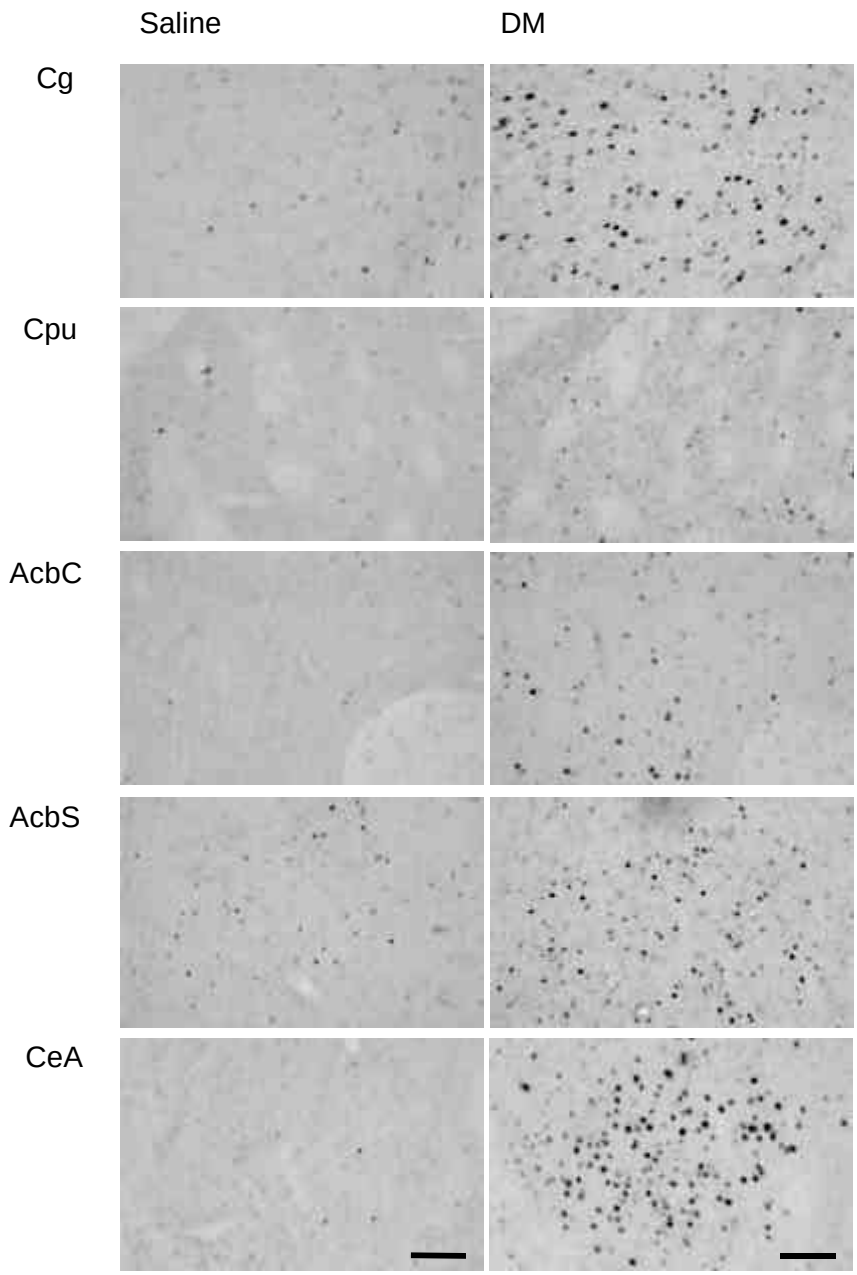

Fig. 4. c-Fos immunohistochemistry after acute treatment with dextromethorphan in 28 day-old rats. Remarkable increases in the c-Fos immunoreactivity of each brain area examined were detected 3 hours after the dextromethorphan injections. Scale bar = 100 μm. Cg, cingulate cortex; Cpu, caudate putamen; AcbC, nucleus accumbens core; AcbS, nucleus accumbens shell; CeA, central amygdala.

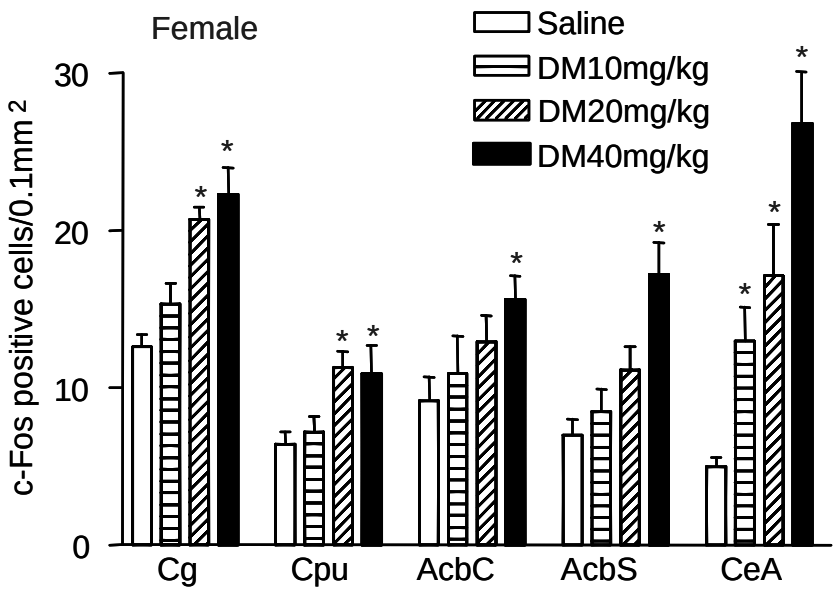

Fig. 5. c-Fos positive cells in brain regions after acute treatment with dextromethorphan in 28 day-old rats. Rats were sacrificed for the c-Fos immunohistochemistry 3 hours after the injection of dextromethorphan at different doses (0, 10, 20, 40mg/kg i.p.). Numbers of c-fos positive neurons in the examined areas significantly increased by dextromethorphan dose-dependently. CeA shows the most significant dose effect of dextromethorphan. Legends are the same as Fig. 4. * $p < 0.05$, compared with saline group, by Dunnett' s test.

2. Effects of acute dextromethorphan on levels of tyrosine hydroxylase mRNA in the mesencephalon of adolescent rats

(1) Behavioral assessment

For the behavioral studies, two pups of each sex were used in a litter from 7 litters. Each littermate was distributed over the saline and the DM groups. The same rats were used for both behavioral test and brain histochemistry.

Behavioral assessment was carried immediately after the DM injection at a dose of 40 mg/kg, i.p. in a volume of 2 ml/kg on PND 28. The dose was chosen because 40 mg/kg of DM dose showed the most significant behavioral effects without mortality and 4 out of 10 rats died within 20 min after the injection of 80 mg/kg DM in our preliminary experiment done with different doses (0, 10, 20, 40 or 80 mg/kg) of DM. The behavioral test was performed between 09.00 - 13.00 hr. Rats were allowed to acclimatize to the chamber for 30 min. Then, immediately after the DM injection, the ambulatory and rearing activities were recorded automatically for 60 min at intervals of 10 min. The chambers were cleaned with 70% alcohol after each use to prevent the influence of the previously tested rat. The scores of the stereotyped behavior and ataxia were taken for 1 min at the end of every 10 min period after injection. The scores of 6 time-points were cumulated for statistical analyses.

Data were collapsed across by sexes because no sexes differences were noted. Injection with DM did not change the ambulatory activity measured for 60 min period (Fig. 6a). Ataxia occurred immediately after DM injection and continued to the time period measured (Fig. 6b). Rats frequently showed jerky movement and loss of balance. Rearing decreased by about 1/3 of the control with ataxia (Fig. 6c). Interestingly, stereotyped behaviors, measured by head movement and sniffing in the present study, were increased about 2 times of the control value

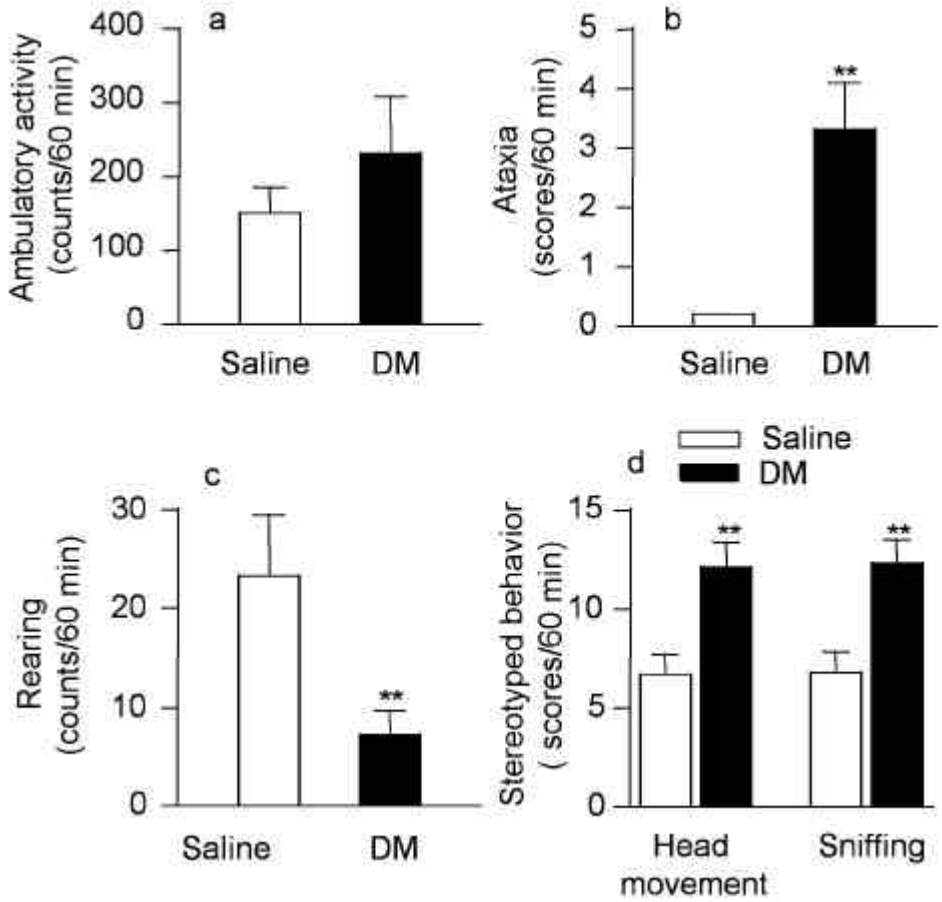

Fig. 6. Acute behavioral effects of dextromethorphan (40 mg/kg i.p.) in 28 day-old rats. Data of the ambulatory activity represent cumulative activity for 60 min immediately after injection with dextromethorphan, and data of other behaviors represent cumulative scores of behavioral rating measured 1 min at the end of every 10 min period for 60 min after injection. (a) ambulatory activity, (b) ataxia, (c) rearing, (d) head movement and sniffing. The numbers of animals in the saline and the dextromethorphan groups were 10 and 12, respectively. All values are mean ± S.E.M. ** $p < 0.01$ *vs* the saline group.

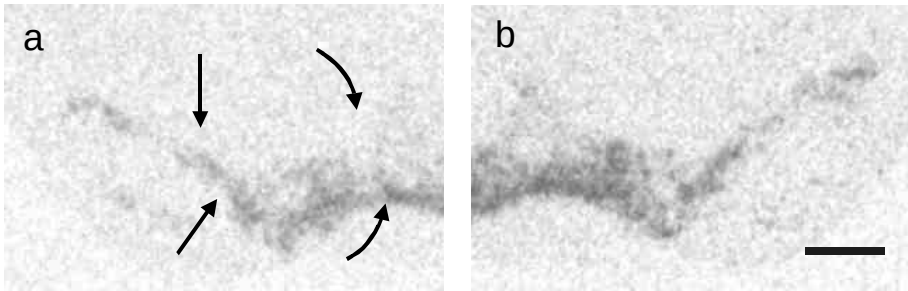

Fig. 7. Representative autoradiographies of tyrosine hydroxylase mRNA *in situ* hybridization signals in the ventral tegmental area (curved arrow) and the substantia nigra (straight arrow) of rats on postnatal day 28. Rats were sacrificed 2 hours after dextromethorphan 40 mg/kg i.p. on postnatal day 28. a, the saline group; b, the dextromethorphan group. Scale bar = 50 μm.

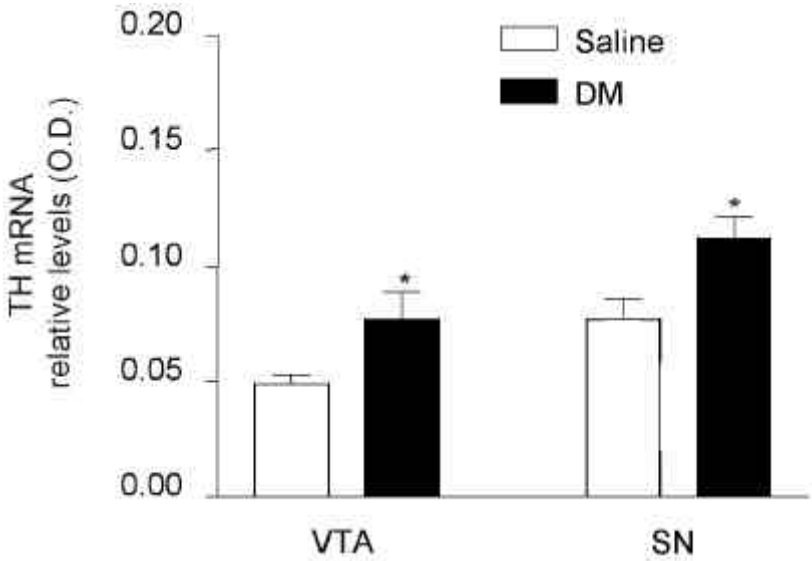

Fig. 8. Quantification of *in situ* hybridization signals in the ventral tegmental area and the substantia nigra of rats on postnatal day 28. Rats were sacrificed 2 hours after dextromethorphan (40 mg/kg i.p.) treatment. Mean optical densities of tyrosine hydroxylase mRNA expression were measured in each area by using the MCID imaging system (see the Method section for the detail). The numbers of animals in the saline and the dextromethorphan groups were 7. * $p < 0.05$ *vs* the saline group.

and the increased behaviors continued to the time period measured (Fig. 6d).

(2) TH mRNA *in situ* hybridization

One hour after the behavioral test (i.e. 2 hours after the DM or vehicle administration), the rat brains were taken and processed *in situ* hybridization. The levels of TH mRNA increased in VTA and SN 2 hours after the DM administration (Fig 7a, 7b). Relative optical densities of the autoradiographic *in situ* films showed 57.1% and 45.5% increases in the VTA and SN of DM group, respectively, compared to the control (Fig. 8).

3. Effects of chronic dextromethorphan on behavior and NMDA receptor

(1) Behavior assessment

We used pups from 10 litters for the behavioral studies. Two pups of each sex in a litter were used. In a litter, one pup was assigned to the saline-injected control group and the other was assigned to the DM-injected group. DM 40 mg/kg was injected intraperitoneally once per day for 10 days on PNDs 28-37. The same dose of DM was challenged after 7 days of drug-free period. Behavioral assessments were done just after DM injection on the 1st, 5th, 10th and the challenging day.

Results were illustrated in Fig. 9, Fig 10. Rats showed exploratory activity just after placing into the activity chamber. The exploratory activity decreased gradually, and almost no activity was noted after 30 min of acclimation period. Then, DM 40 mg/kg was injected. The saline-injected male and female rats showed almost no ambulatory activity for 150 min after injection on the 1st, 5th and 10th day of treatment. The DM-injected males showed the same pattern with the controls. However, the ambulatory activity of females showed different pattern in which increased activity was noted for 30 – 90 min period after injection. When the same

dose of DM was challenged to both the saline- and the DM- exposed groups after 7 days of drug-free period, the saline-injected males and females, and the DM-challenged males showed almost no activity during 150 min period after injection. However, activities of the females that had been exposed to DM for 10 days increased from 20 min after challenge and this increase lasted 90 min, indicating the existence of behavioral sensitization by DM. For further analyses of activities on the 10th and the challenging day, the data were log-transformed because the individual data were so variable, ranging from 133 to 9,627. The cumulative activity during 150 min after injection on the challenging day (2,681 ± 1,013) was higher than that of on the 10th day (1,965 ± 1,160) ($p < 0.01$, paired t-test using log-transformed data). In fact, 7 out of 8 rats showed higher activity on the challenging day than on the 10th day. One outlier was omitted to statistical analysis because its activity count was far higher (3,267) than the mean on the challenging day. To determine whether 10th day's activity predicted the amount of behavioral sensitization on the challenging day, Pearson's correlation coefficient between log-transformed activities of the 10th day and the challenge day was calculated, and a positive correlation between two parameters was found ($r = 0.764$, $p<0.01$) (Fig. 10).

Sniffing and head movement as stereotyped behaviors were measured for 150 min after DM injection (Fig. 11, Fig. 12). Unlike locomotor activity, these behaviors increased in both sexes during 10 days' treatment. However, sexual differences were observed in that females showed faster increase than males. On the challenging day, the sex difference became obvious and behavioral sensitization was shown only in females. Rating scores of sniffing and head movement were 1.9 times higher in female rats which had been exposed to DM than to saline. Previous exposure to DM had no effect on these behaviors on the challenging day in males.

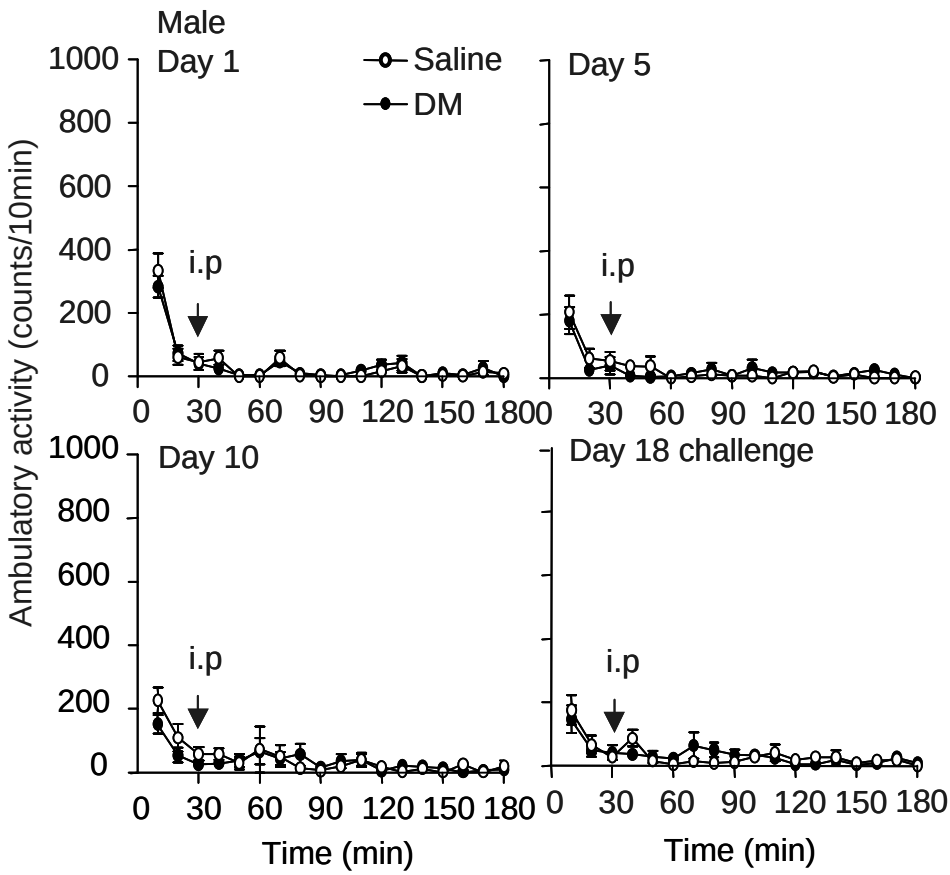

Fig. 9. Sequential effects of chronic treatment with dextromethorphan starting at postnatal day 28 on locomotor activity in male rats. Dextromethorphan was injected (i.p) daily for 10 days. The same dose of dextromethorphan was challenged on postnatal day 45 (day 18 of treatment). n=10.

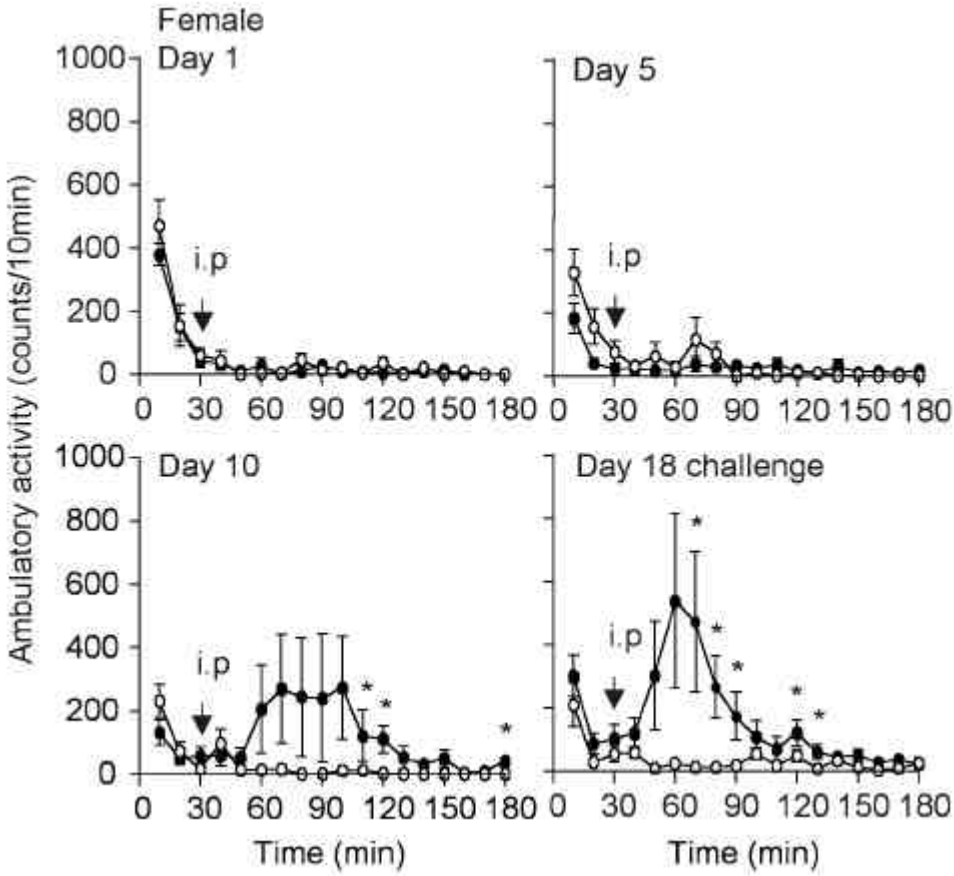

Fig. 10. Sequential effects of chronic treatment with dextromethorphan starting at postnatal day 28 on locomotor activity in female rats. Dextromethorphan was injected (i.p) daily for 10 days. The same dose of dextromethorphan was challenged on postnatal day 45 (day 18 of treatment). * $P < 0.05$ compared with control group. n=8.

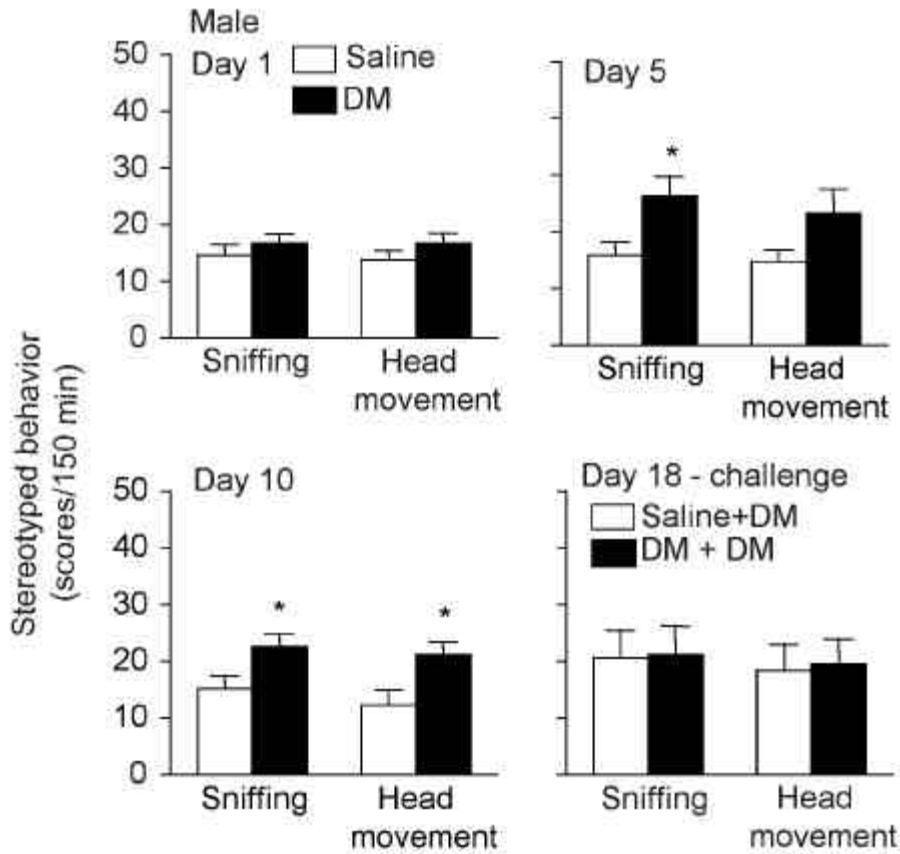

Fig. 11. Sequential effects of chronic treatment with dextromethorphan starting at postnatal day 28 on stereotyped activity in male rats. The cumulative scores of each behavior rated for 1 min of every 10 min interval during 150 min. Dextromethorphan was injected (i.p) daily for 10 days. The same dose of dextromethorphan was challenged on postnatal day 45 (day 18 of treatment). * $P < 0.05$, compared with saline group. n=10.

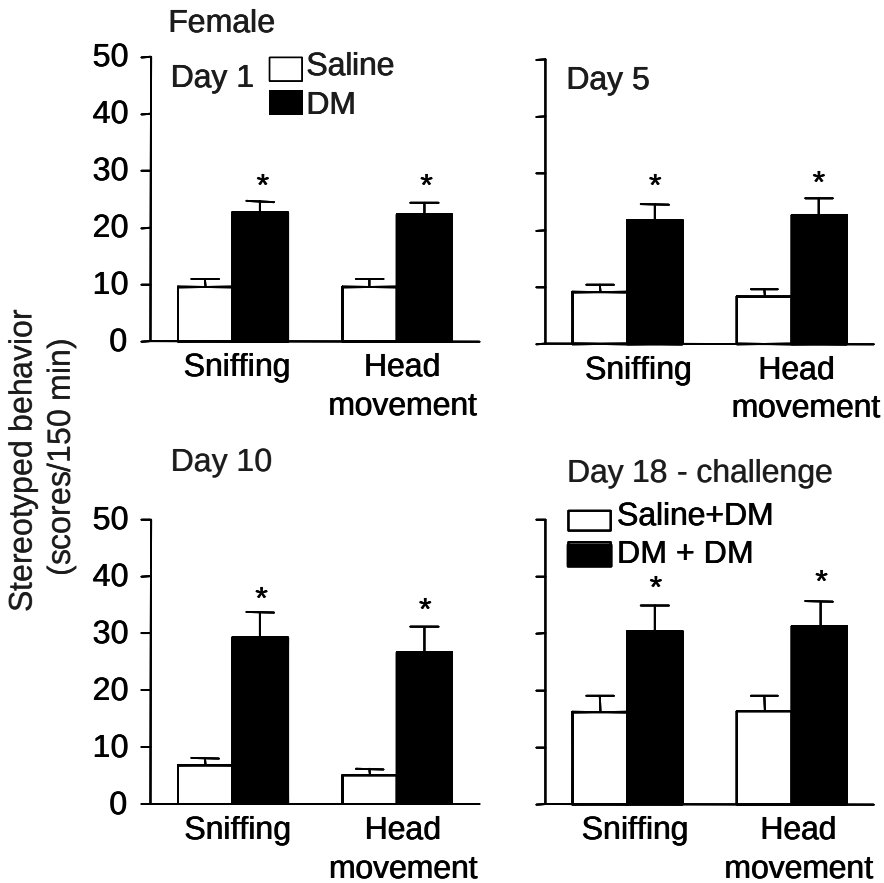

Fig. 12. Sequential effects of chronic treatment with dextromethorphan starting at postnatal day 28 on stereotyped activity in female rats. The cumulative scores of each behavior rated for 1 min of every 10 min interval during 150 min. Dextromethorphan was injected (i.p) daily for 10 days. The same dose of dextromethorphan was challenged on postnatal day 45 (day 18 of treatment). * $P < 0.05$, compared with saline group. n=8.

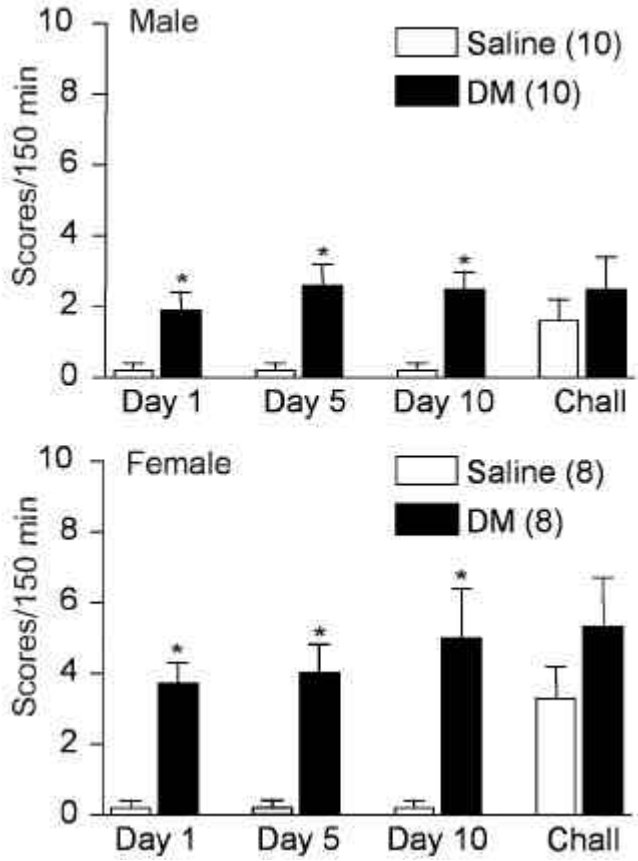

Fig. 13. Sequential effects of chronic treatment with dextromethorphan starting at postnatal day 28 on ataxia in rats. The cumulative scores of ataxia were rated for 1 min of every 10 min interval during 150 min. Dextromethorphan was injected (i.p.) daily for 10 days. The same dose of dextromethorphan was challenged on postnatal day 45 (day 18 of treatment). * $P < 0.05$, compared with saline group. Chall: challenge.

DM resulted in marked ataxia in both sexes from the 1st day of treatment. Ataxia showed peak within 30 min after injection of DM. On the challenging day, ataxia was noted at the same level of the 10th day's regardless of the experience of previous exposure to DM, and no sex differences were observed (Fig. 13).

(2) Effect of DM on NMDAR1 immunoreactivity

Brains were taken on PND 38 after 10 days injection of DM and processed NMDAR1 immunohistochemistry and Western blotting. DM had no effect on the level of NMDAR1 in the striatum, hippocampus in either sex. In males, DM had no effect on the NMDAR1 immunoreactivity in the hypothalamus (Fig. 14). In females, many stained neurons were found both in the frontal cortex and ventromedial regions of the hypothalamus in DM-treated group (Fig. 15, 16). The amount of NMDAR1 subunit immunoreactivity was up-regulated by 38.9% in the hypothalamus in DM-treated female rats by Western blotting (Fig. 16, 17).

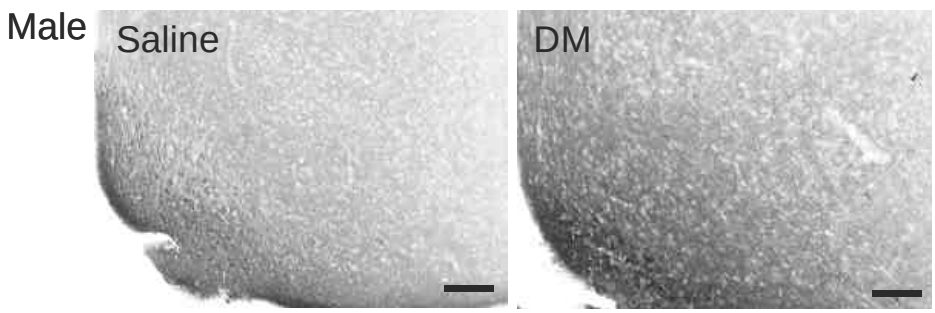

Fig. 14. Representative NMDAR1 immunoreactivity of the hypothalamus after chronic treatment with dextromethorphan in adolescent male rats. Scale bar = 100μm.

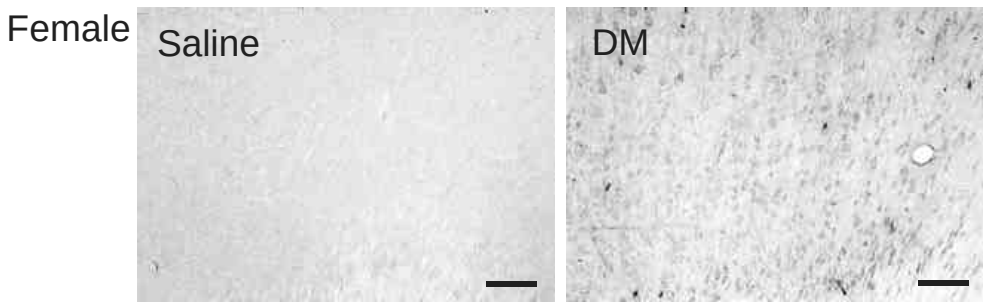

Fig. 15. Representative NMDAR1 immunoreactivity of the frontal cortex after chronic treatment with dextromethorphan in adolescent female rats. Remarkable increases in the NMDAR1 immunoreactivity of frontal cortex were detected after the 10 days dextromethorphan treatment. Scale bar = 100 μm.

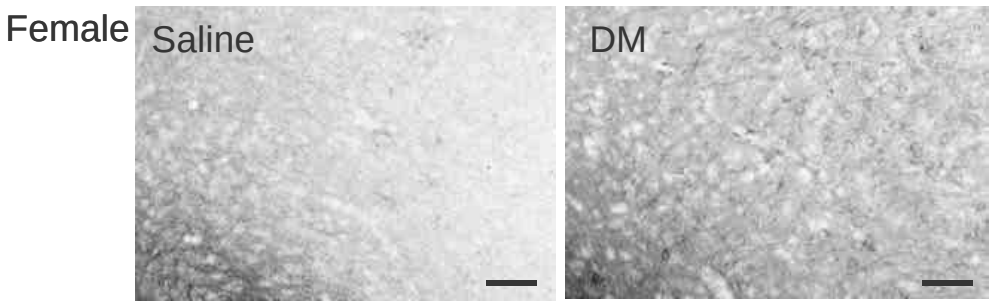

Fig. 16. Representative NMDAR1 immunoreactivity of the hypothalamus after chronic treatment with dextromethorphan in adolescent female rats. A lot of NMDAR1 positive cells were found in the hypothalamus in dextromethorphan group. Scale bar = 50 μm.

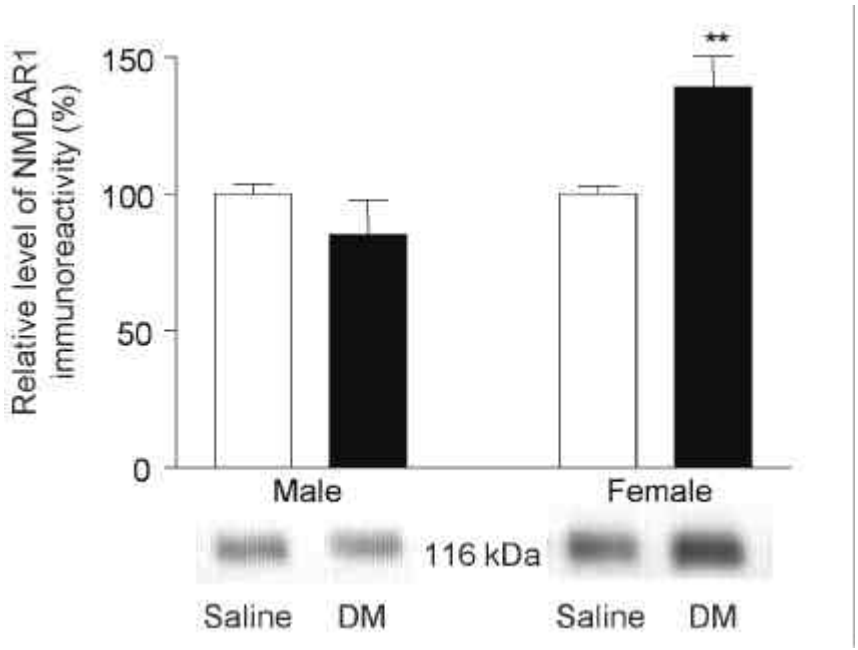

Fig. 17. Effects of chronic treatment with dextromethorphan on NMDAR1 immunoreactivity of the hypothalamus in adolescent rats. Representative result from Western blotting was presented below the graph. Mean optical densities of NMDAR1 immunoreactivity were measured by using the MCID imaging system. The numbers of animals in the saline and the dextromethorphan groups were 6. ** $P < 0.01$, compared with saline group.

4. Effects of dextromethorphan exposure at adolescent on learning and memory in late age.

(1) Autoshaping with 4 sec –delay reinforcement

Rat pups were given daily injection of saline or DM (40 mg/kg) during postnatal days 28-37. At 345 days of age, these rats were gradually deprived to and maintained at 80% of their free feeding weight. From the 360th day of age, they were fed 20 food pellets daily for 3 days in their home cages to familiarize them with the reinforcer. From the 4th day, rats were subjected to a 4 sec delay of reinforcement autoshaping for 15 daily sessions. Females learned little bit faster than males, but there was no significant difference on acquisition of extended lever touch between the saline and the DM groups in either sex. The mean numbers of extended lever touches at the first session in the male of the saline or the DM group were 5.6 ± 0.9, 3.5 ± 0.5, respectively, in the female were 4.4 ± 1.1, 4.8 ± 0.4, respectively. On the 15th session, the numbers increased to 9.3 ± 1.2, 9.5 ± 1.0 in the male of the saline or the DM group respectively, and to 11.8 ± 0.2, 10.2 ±1.1 in the female of the saline and the DM group respectively (Fig. 18, 19). It shows that rats can learn the association between the lever retraction and food pellet delivery on the repetition of the same task at this old age.

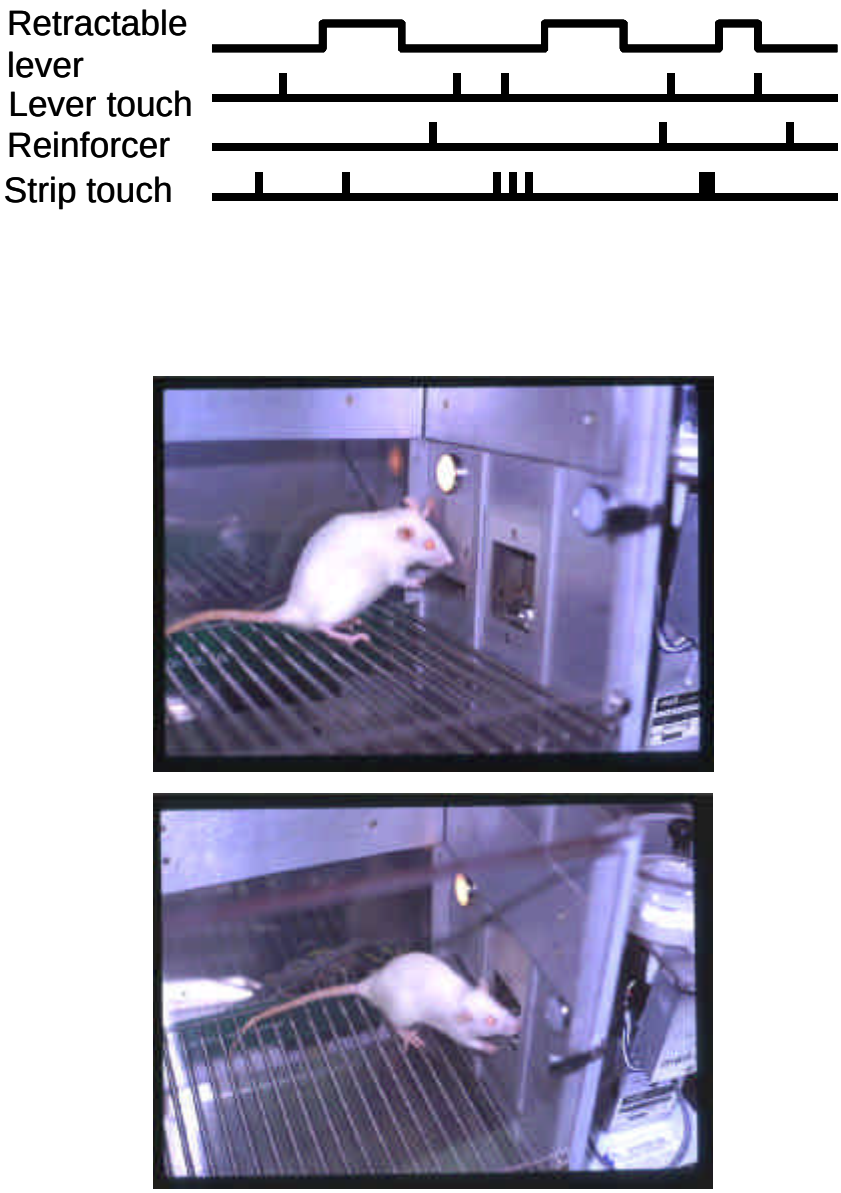

Fig. 18. The experimental schedule of the autoshaped learning test and the apparatus. Dextromethorphan (40 mg/kg) was injected (i.p.) during postnatal day 28-37 and autoshaped learning test was performed in 360 day-old rats. The lever was presented 12 times/daily Session on a random interval 45 sec schedule and retracted when the animal made a lever touch response or after 15 sec. 4 sec after lever retraction, a 40 mg pellet was delivered.

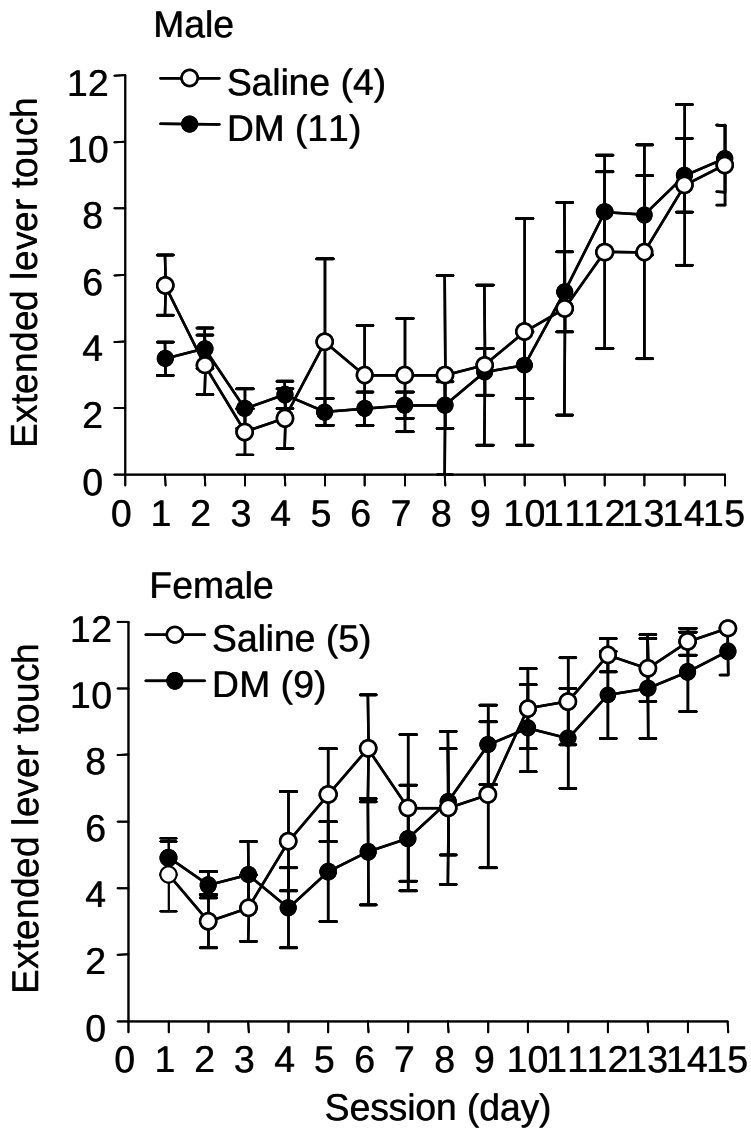

Fig. 19. Late effects of chronic treatment with dextromethorphan in adolescent on autoshaped learning test in 360 day-old rats. The lever was presented 12 times/daily session on a random interval 45 sec schedule and retracted when the animal made a lever touch response or after 15 sec.

(2) Morris water maze test

At 20 months of age, rats were subjected to the Morris water maze test. All rats showed a progressive decline in escape latency over the first nine training days (Fig 20). Four escape latencies per day were averaged and statistical analyses were performed between the saline and the DM groups in either sex. In the male, there was no significant difference on the acquisition of the submerged escape platform between the saline and the DM groups in the first nine training days. The mean escape latency was 50.0 ± 3.7 or 50.9 ± 3.5 in the saline or the DM group, respectively, on the first training day. These latencies declined to 9.5 ± 1.6 or 12.0 ± 2.2 on the 9^{th} training day, respectively. In the female, DM experienced rats spent more time to locate the escape platform in the initial training phase. The mean latency on the first training day was 49.8 ± 2.6 or 50.8 ± 5.0 in the saline or the DM group, and on the 9^{th} day, the latency of the saline or the DM group was 8.6 ± 1.5 or 18.3 ± 3, respectively. There was a significant difference between the saline and the DM group ($F(1,13) = 5.31$, $P < 0.05$).

On the first trial of the 10^{th} day, the platform was moved from the maze, and rats were allowed to swim for 60 sec to evaluate the effect of DM exposed early in life on the probe trial in late age. It was observed that DM-experienced males spent less time than the saline group in the target quadrant during the probe trial (Fig. 21, 22) ($F(1,11) = 6.76$, $P < 0.05$). However, this difference was not noted in the female.

During the reversal training phase, as the first training phase, the decline of latency was obvious as exposing the tank repeatedly in all rats tested. The retardation of acquisition of the task was found in the female but not in the male. It took longer time to find a new location of the platform at the 2^{nd} and 3^{rd} sessions in the DM-experienced female rats compared to that of

the saline group (Fig. 20, 23).

To test for existence of any sensory or motor deficit, cued trials were performed on the 16th day. The mean latency to reach the observed platform in the male of the saline or the DM group was 29.5 ± 5.2 or 26.6 ± 2.7, respectively, and in the female was 21.8 ± 2.9 or 29.7 ± 3.0, respectively. There was no significant difference between saline and the DM groups in either sex, suggesting the deficit of learning and memory was not resulted from the deficit of physical performance.

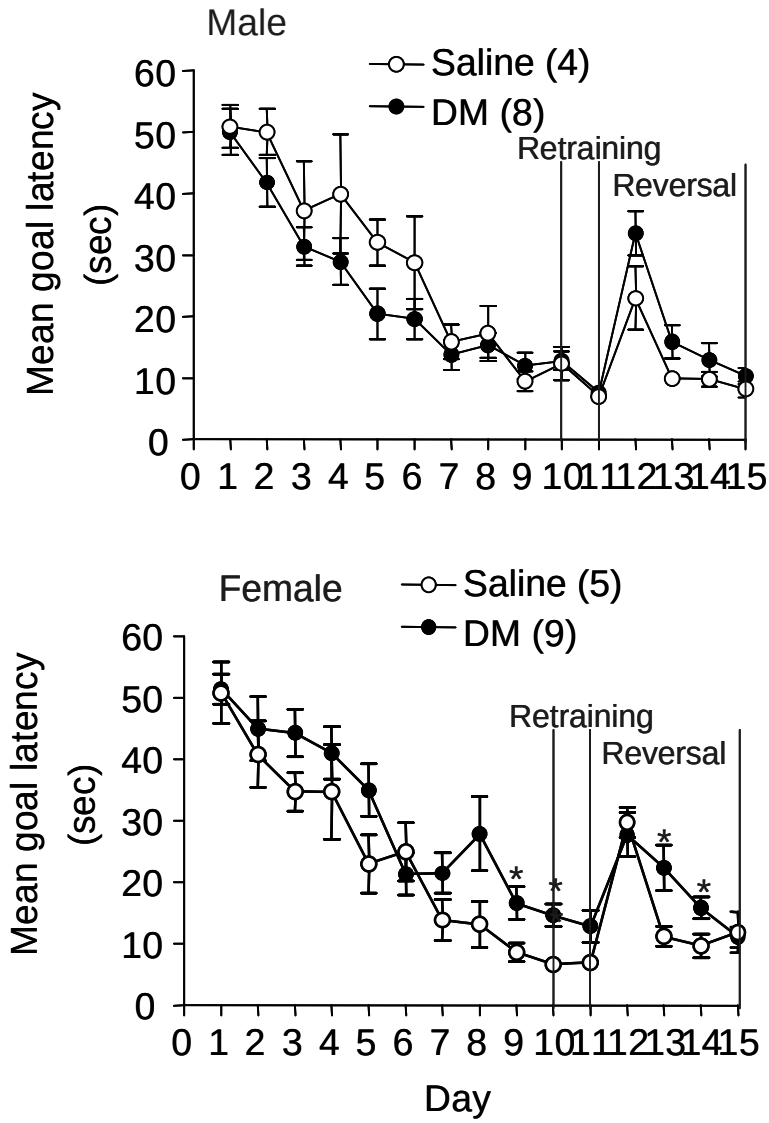

Fig. 20. Late effects of chronic treatment with dextromethorphan in adolescent on the Morris water maze test in 540 day-old rats. Dextromethorphan 40 mg/kg was injected (i.p.) to rats during postnatal days 28-37. 4 trials were given each day when the rats were 18 months old. One trial is 60 sec. * $P < 0.05$, compared with saline group.

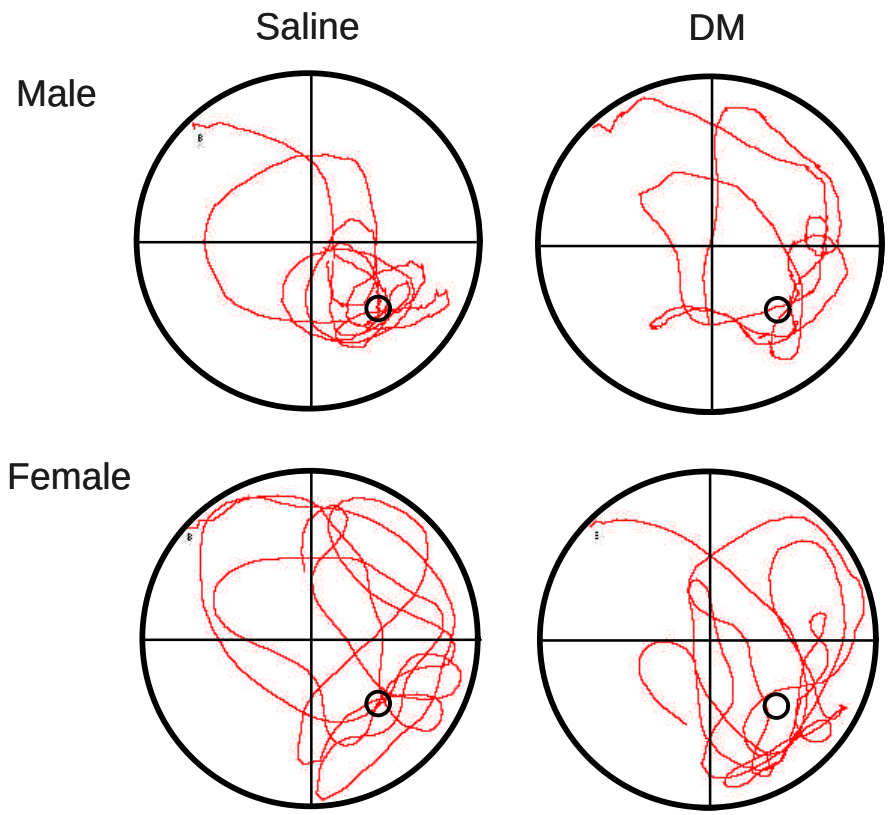

Fig. 21. Representative trace of rats in the probe trail of Morris water maze test. Rats were released from the northwest and required to swim in the pool without the escape platform for 60 sec. The time spent in each of the four quadrants was recorded.

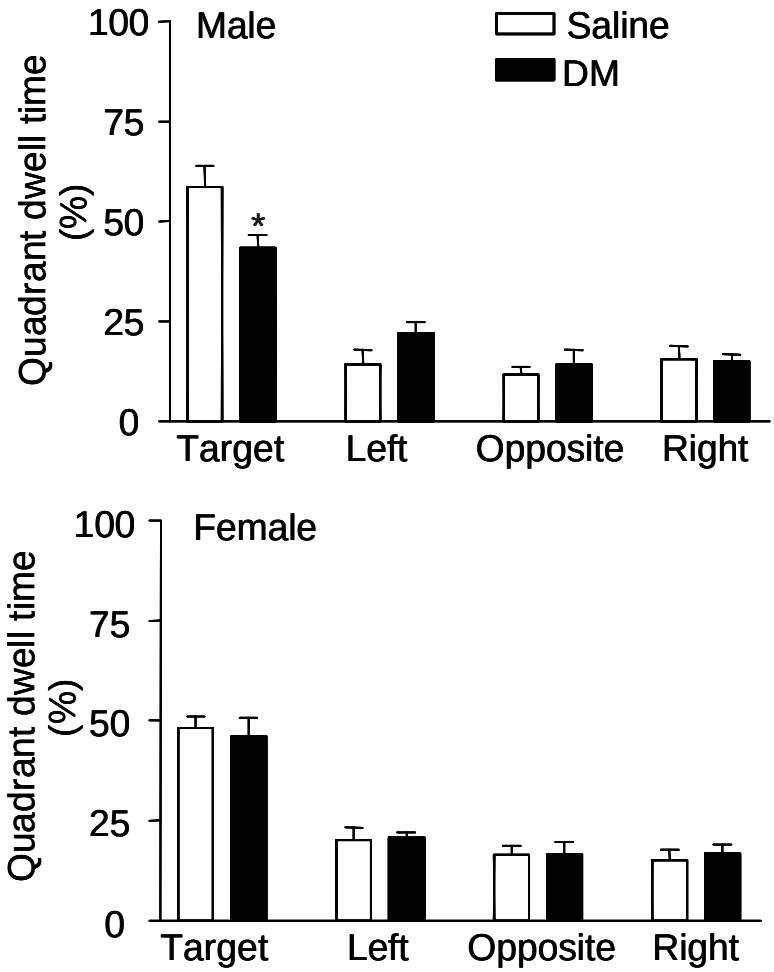

Fig. 22. Late effects of chronic treatment with dextromethorphan in adolescent on the probe trial of Morris water maze test in 553 day-old rats. Rats were released from the northwest and required to swim in the pool without the escape platform for 60 sec. The time spent in each of the four quadrants was recorded. * $P < 0.05$, compared with saline group.

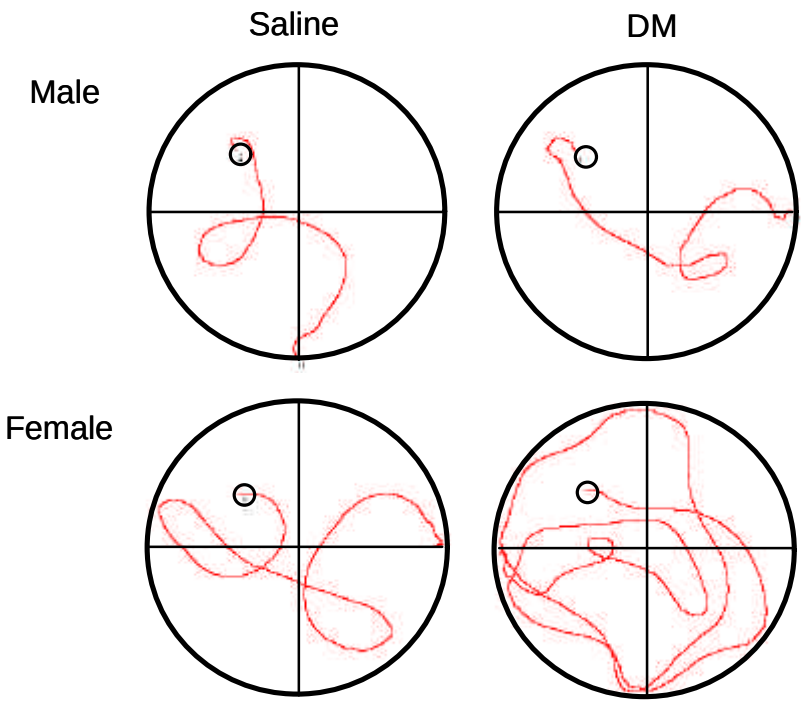

Fig. 23. Representative trace of rats in the reversal training of Morris water maze test. Platform was located on the opposite and rats were allowed swim to required a new position. 4 trials were given each day after probe trial. One trial is 60 sec.

(3) Serum corticosterone level and c-Fos immunoreactivity after cold stress

One month after the Morris water maze test, rats were subjected to the cold stress (4 °C for 2 hours). Concentrations of serum corticosterone were measured after 2 hours' exposure to the cold environment. Higher corticosterone level was noted in the DM-experienced female rats ($F(1,9) = 10.3$, $P < 0.05$), but not in males (Fig. 24). c-Fos expression in the PVN was also examined after 2 hours' cold exposure. Both groups showed many c-Fos positive cells in the PVN after the stress. However, DM-experienced male and female rats showed more c-Fos positive cells than the control (Fig. 25, 26). The mean positive cells in the male of the saline or the DM group were 254 ± 12 or 316 ± 20, respectively, in the female were 281 ± 12 or 361 ± 24, respectively.

(4) Cell density in the hippocampus

The cell densities in the CA1 and CA3 subfields of the hippocampus in 20 μm thick sections stained with cresyl violet were studied and expressed as cells/100 μm length of the cell column. Quantitative analysis indicated a significant cell loss in the CA1 and the CA3 regions in DM-experienced male rats (Fig. 27). In the DM-experienced female rats, a significant cell loss was noted in the CA3 region (Fig. 28). The numbers of cells of the CA1 and the CA3 regions in DM-experienced male rats were 83.2% and 84.1% of the control rats, respectively. That of the CA3 region in DM-experienced female rats was 86.5% of the control rats (Fig. 29).

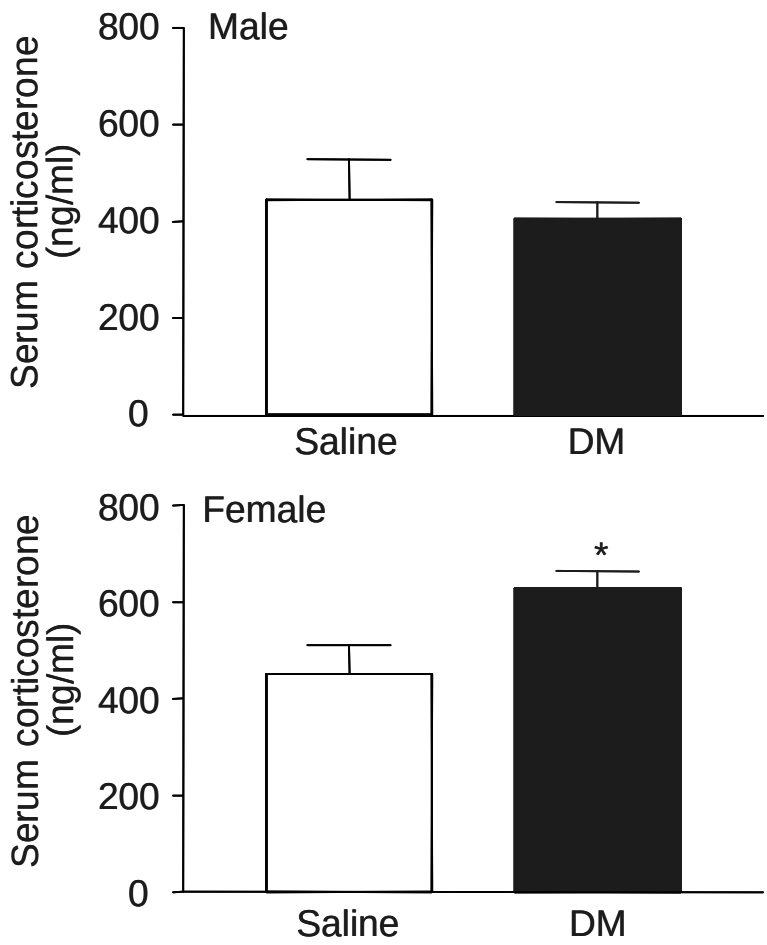

Fig. 24. Late effects of chronic treatment with dextromethorphan in adolescent on the level of serum corticosterone after cold stress in 600 day-old rats. Rats were transferred to cold room maintained at 4 °C in 20th month. Two hours later blood was taken and serum corticosterone was assayed. * $P < 0.05$, compared with saline. n=5-9.

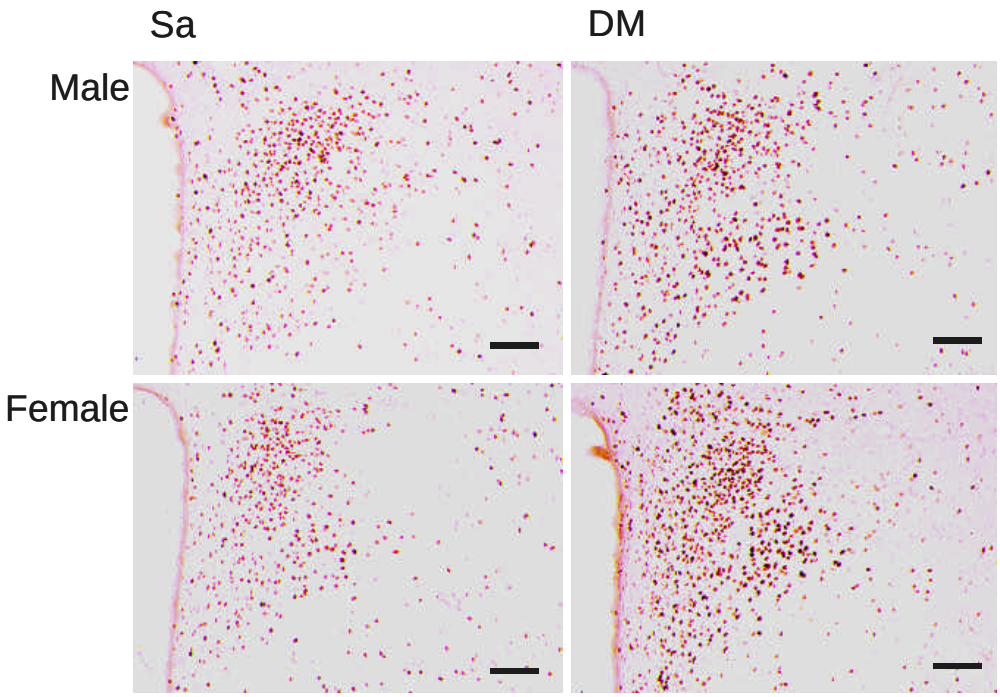

Fig. 25. Representative c-Fos immunoreactivity in the paraventricular nucleus of 600 day-old rats after cold stress. Remarkable increases in the c-Fos immunoreactivity of each brain area examined were detected 2 hours after the cold stress. Scale bar = 100 μm.

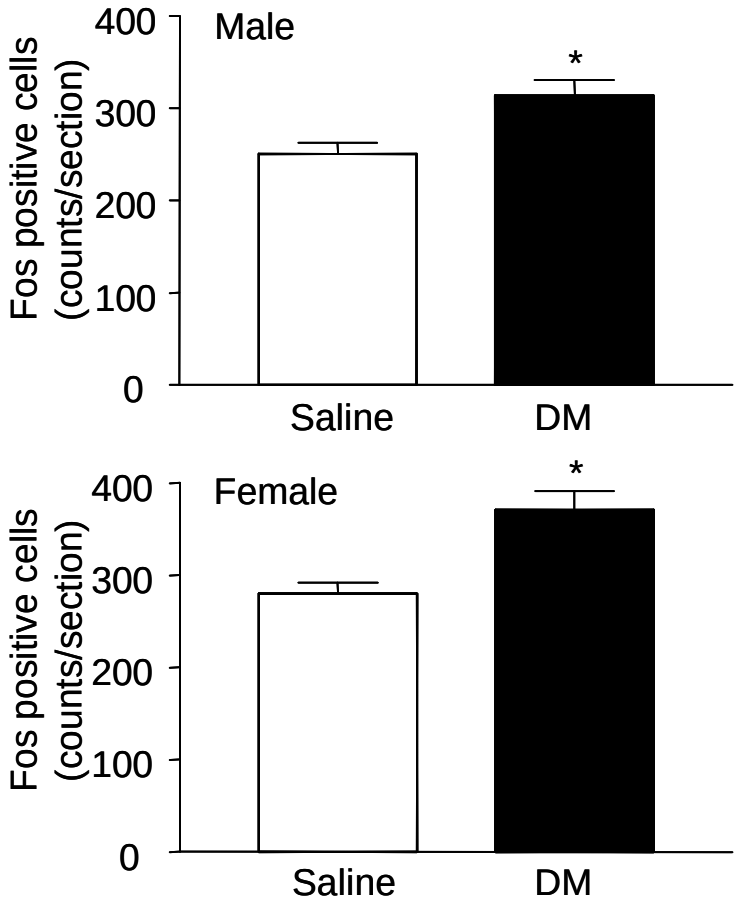

Fig. 26. Late effects of chronic treatment with dextromethorphan in adolescent on c-Fos immunoreactivity after cold stress in 600 day-old rats. Rats were transferred to cold room maintained at 4 °C. Two hours later brain was taken. Numbers of c-Fos positive cells in the paraventricular nucleus area were significantly increased by cold stress. * $P < 0.05$, compared with saline. n=5-9.

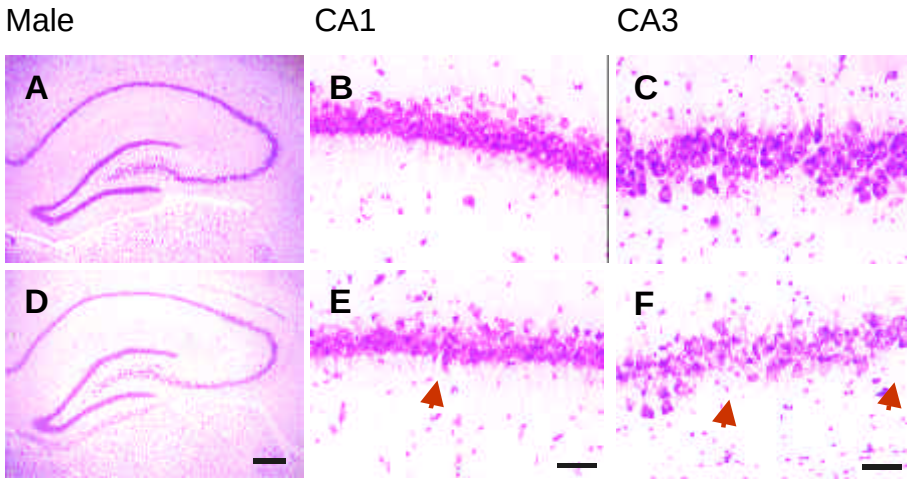

Fig. 27. Representative photomicrographs of the dorsal hippocampus of 600 day-old male rats in groups of the control (A) and the dextromethorphan exposed during adolescent (D). Dextromethorphan group showed a decrease in the number of pyramidal cell bodies in the CA1 (E) and CA3 (F) fields compared to control group (B, C). Scale bar in (A and D) = 400 µm, Scale bar (in B, C, E, F)=100 µm, respectively.

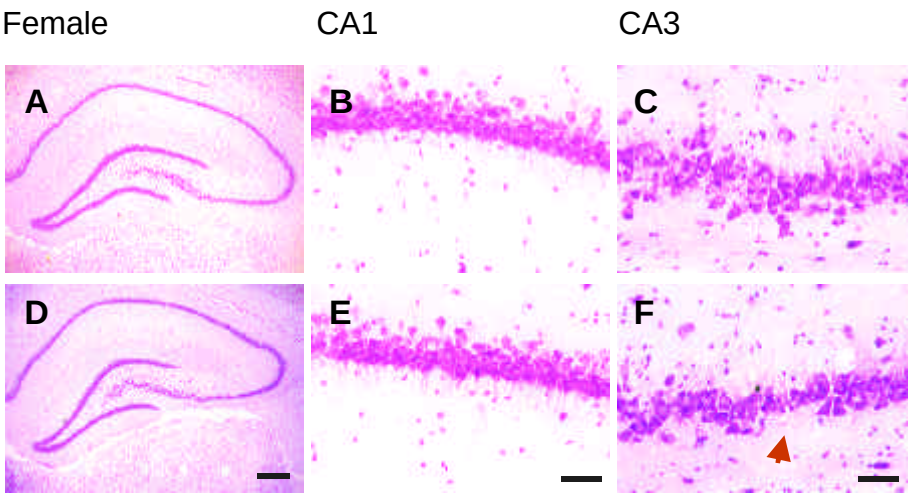

Fig. 28. Representative photomicrographs of the dorsal hippocampus of 600 day-old female rats in groups of the control (A) and the dextromethorphan exposed during adolescent (D). Dextromethorphan group showed a decrease in the number of pyramidal cell bodies in the CA3 (F) field compared to control group (C). Scale bar in (A and D) = 400 µm, Scale bar (in B, C, E, F)=100 µm, respectively.

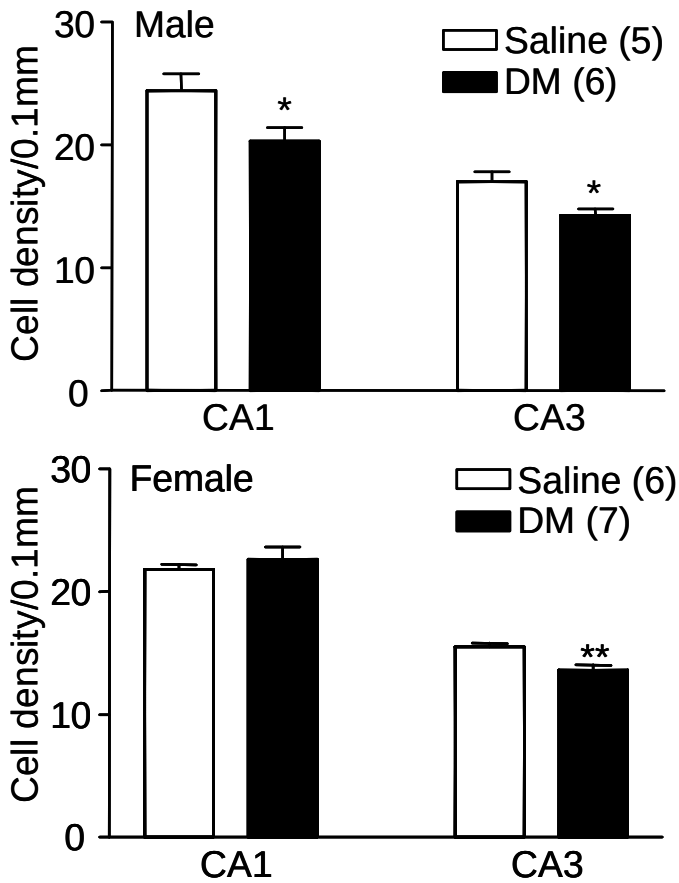

Fig. 29. Late effects of chronic treatment with dextromethorphan in adolescent on neuron densities in CA1 and CA3 pyramidal cell fields of the hippocampus in 600 day-old rats. Dextromethorphan (40 mg/kg) was injected (i.p.) during postnatal day 28-37. * $P < 0.05$, ** $P < 0.01$, compared with saline group.

Ⅳ. Discussion

1. Effects of acute dextromethorphan on nocturnal behavior and the brain c-Fos expressions in adolescent rats

The results showed that acute dextromethorphan at high doses decreases the nocturnal locomotor activity of rats in a dose-dependent manner. Numbers of previously published reports examining daytime behavior have shown inconsistency in its results about the effect of dextromethorphan on locomotor activity. Whereas Ginski and Witkin[31] reported that dextromethorphan had no effect on the daytime locomotor activity at all, Wu et al.[8] reported that acute dextromethorphan at a high dose (60 mg/kg) increased the locomotor activity 60 min after intraperitoneal injection, but not after subcutaneous injection. Wu et al. suggested that the increased locomotor activity following intraperitoneal dextromethorphan, but not subcutaneous injection, implies that the major metabolite of dextromethorphan, dextrophan, which produces phencyclidine-like behavioral effects, may be in charge of the effects on the locomotor activity. Nevertheless, it is less likely that the effect of acute dextromethorphan in increasing the locomotor activity is mediated by its metabolite, dextrophan, because the structure of dextrophan glucuronide exhibits a hydrophilic nature which may provide limited access to the brain.[32] In the present experiment, no change was found in daytime locomotor activity by dextromethorphan. Meanwhile, it was noticed that the rats showed minimal baseline activity during daytime, no doubt because they are nocturnal animals. With being suspicious of the daytime results examined by others while rats show very low baseline activity, the rats' locomotor activity at nighttime was examined while their life cycle was in active phase. It was found that a high dose of dextromethorphan immediately decreased nocturnal locomotor

activity. In consistency with the decreased ambulation, rearing also decreased by dextromethorphan administration in a dose-dependent manner. Acute dextromethorphan at the doses of 5-80 mg/kg has been reported to cause modest muscle relaxation in mice,[33] which might be attributed to the effects of this drug in decreasing both locomotor activity and rearing.

It was discerned that all the previous reports about the effects of dextromethorphan on locomotor activity were consisted of daytime studies using both male and female adult rats. On the contrary, in this study the behavioral tests during nighttime were performed and only adolescent female rats were used. It was believed that these different experimental conditions might have produced different results, because it was previously found that not only the nocturnal ambulatory activity of rats was far higher than their daytime activity, but also female rats exhibited significantly greater behavioral activities than males at adolescence.[13]

This study demonstrated that significant inductions of c-Fos expression by dextromethorphan were detected in the nucleus accumbens, both core and shell, and in the caudate putamen. The striatum is considered to be the main brain region involved in the alteration of stereotyped behavior induced by dopaminergic agonists,[34] and actually, a high dose of dextromethorphan acutely increased the gene expression of tyrosine hydroxylase, the rate limiting enzyme of dopamine biosynthesis, in the midbrain dopaminergic neurons (discussed in the next chapter). In the present report, the dextromethorphan induced c-Fos expressions in the striatum may partly be a secondary effect of the dextromethorphan- induced activation of the midbrain dopamine neurons innervated to the striatum. Taken together, dextromethorphan at high doses appear to activate the nigro-striatal dopaminergic pathway and this may partly contribute to an increase in the stereotyped behavior. However, dextromethorphan did not significantly change the stereotyped behavior of female rats during nighttime in the present

study. It is speculated that the effect of dextromethorphan in increasing the stereotyped behavior, if it did, might be hidden by the increased baseline activity during nighttime.

It was also found that dextromethorphan dose-dependently induced c-Fos expressions in the cingulate cortex and the central amygdala. C-Fos induction by dextromethorphan in the cingulate cortex may reflect a cytotoxic effect of dextromethorphan as a NMDA antagonist, because it has been reported that the cingulate cortex is a predominant site of the cytotoxic effect by various NMDA antagonists, such as phencyclidine, MK-801, and even dextrophan, the major metabolite of dextromethorphan.[35,36] The nucleus of central amygdala appears to be implicated in the reward pathway. There are bi-directional innervations between the ventral tegmental area and the central amygdala. Dopaminergic neurons in the ventral tegmental area project to the central amygdala and the efferent fibers from the central amygdala project to the ventral tegmental area, all of which constitute a part of the mesolimbic reward circuit.[37]The dose-dependent induction of c-Fos expression by dextromethorphan in the central amygdala suggests that this area might be targeted by high doses of dextromethorphan for the emotional seeking behavior to this drug of abuse.

The mechanism of c-Fos induction by dextromethorphan is not known. It is just speculated that the c-Fos inductions by dextromethorphan may be mediated, at least partly, by the sigma sites, because the sigma ligands, such as phencyclidine and SKF10047 (N-allylnormetazone), induce c-Fos expressions in the brain regions[38,39,40] and because the major metabolite of dextromethorphan, dextrophan, binds to the phencyclidine site.[41] Although the effect of dextrophan on locomotor activity has been reported with opposite findings from present study,[41,42] the behavioral effects of dextromethorphan in this result occurred too quickly to expect its metabolite action.

The pattern of c-Fos induction by acute dextromethorphan might have not been directly related to its behavioral effects in this study. However, it should be noticed that the reward pathway appeared to be activated by a single injection of dextromethorphan, that the behavioral alterations occurred concomitantly.

2. Effects of acute dextromethorphan on levels of tyrosine hydroxylase mRNA in the mesencephalon of adolescent rats

The results demonstrated that the single injection of DM at a dose of 40 mg/kg increased mRNA levels of TH in the VTA and the SN. This is the first report that DM acutely changes the gene expressions of TH, the rate-limiting enzyme of dopamine biosynthesis, in the dopaminergic neurons of mesencephalon. This finding suggests that DM at a high dose may activate mesolimbic and nigrostriatal dopaminergic systems, even with a single injection. One possible mechanism of DM-induced expressions of TH could be explained by the effect of NMDA antagonism on the dopamine release in the midbrain dopamine neurons.[43]It was also reported that the activation of NMDA receptors exerted an inhibitory effect on dopamine release via the GABA system in the mesencephlon where dopamine neurons receive plenty of GABAergic inputs,[44] and MK-801, another NMDA antagonist, also increased TH mRNA in the midbrain.[45,46]Therefore, it is likely that the NMDA antagonistic property of DM disinhibited the suppressed dopamine release in the physiological condition, and the resulting depletion of the dopamine storage induced the gene expression of TH. However, it should be noticed that dopaminergic neurons in the reward pathway appeared to be activated by a single injection of DM, that the behavioral alterations occurred concomitantly, and that, therefore, repeated

injections of DM, as commonly occurring with drugs of abuse, may generate more provocative effects on the brain function and behavior. In this regard, it was found that behavioral sensitization had occurred with multiple injections of DM at a high dose (next chapter).

Acute DM increased stereotyped behavior. The elevated TH mRNA level in the SN may contribute to the increase in stereotyped behavior, because the nigrostriatal pathway is responsible for the expression of stereotyped behavior.[47]It can be also postulated that the elevated TH mRNA level in the VTA, which may activate the mesolimbic pathway, may result in the increase in locomotor activity. Similarly, the stimulatory effects of MK-801, a non-competitive NMDA antagonist, on locomotor activity was mediated by the mesolimbic dopaminergic system.[48]However, a significant increase in locomotor activity was not detected in daytime study. It is speculated that the increasing tendency of locomotor activity might be covered by the severe ataxia occurred with DM at this high dose.

This study firstly reports that a high dose of DM increased levels of TH mRNA in the VTA and the SN of adolescent rats, and also the behavioral alterations related to drug abuse were detected. It is concluded that the activation of midbrain dopaminergic neurons by a high dose of DM may correlate with its behavioral effect, and a caution should be taken in the use of DM, especially at high doses because of the abuse potential of DM.

3. Effects of chronic dextromethorphan on behavior and NMDA receptor

Repeated exposure to drugs of abuse results in a progressive augmentation of behavioral responses and this response persists even after long-term withdrawal. This phenomenon,

termed behavioral sensitization, underlies certain aspects of human drug addiction.[17] The present study firstly demonstrated that repeated exposure to DM at a dose of 40mg/kg elicited a behavioral sensitization. This phenomenon of DM suggested that DM has a drug addiction property.

The dopaminergic reward system has been recognized playing a critical role in drug addiction.[49]The previous study has demonstrated that DM activates the mesolimbic dopaminergic reward pathway. On the other hand, it has been reported that MK-801 prevents the sensitization to stimulants but produces sensitization to itself, suggesting that glutamatergic system also involved in inducing behavioral sensitization.[50,51] It is possible that DM-induced behavioral sensitization is mediated by changed dopaminergic and/or glutamatergic systems. The repetitive administration of DM may change the dopaminergic and glutamatergic interconnections in many brain regions such as VTA, nucleus accumbens and amygdala.[52,53] It has now been thought that dopaminergic, glutamatergic, GABAergic pathways are involved in the induction of behavioral sensitization, and different neural substrates mediate behavioral sensitization caused by different drugs.[51] For example, morphine acts at the μ-opioid receptors, amphetamine acts at monoamine transporters, and MK-801 acts on the NMDA receptor.

This hypothesis that dopaminergic and glutamatergic systems are involved in the induction of DM sensitization can be supported by its activating effect on dopaminergic system and its NMDA antagonistic property. DM significantly increased the c-Fos expression in the dopaminergic terminal regions and increased TH mRNA expression in the VTA and SN. Similar to the case of DM, it has been reported that chronic treatment with NMDA antagonist such as MK-801 or PCP resulted in a modest behavioral sensitization,[54,55] while acute treatment

with PCP showed no effects.[25] Furthermore, NMDA receptor antagonists such as PCP, ketamine are known to interfere with the function of dopamine uptake sites,[56] and ketamine increased dopamine release in prefrontal cortex.[57] DM may also have the same effect, but it has not been demonstrated.

The neurochemical results showed that the up-regulation of NMDA receptor might be, at least partly, involved in the sensitization to the effect of DM. The susceptibility of the brain to NMDA toxicity changes during development. MK-801 induces rapid and persistent up-regulation of NMDA receptors in 7 days old rats[58] and produces morphological changes in cortical neurons of the young adult female guinea pig.[59] A continuous hyperstimulation of the neurons in the retrosplenial cortex would occur after blockade of NMDA receptors.[59] Surprisingly, a significant up-regulation of NMDAR1 was detected in the frontal cortex, retrosplenial cortex and hypothalamus in female rats after 10 days treatment with DM in the present study. Since recent studies in nonhuman primates have shown that the connectivity of the prefrontal cortex is substantially refined during adolescence, suggesting that these development changes may be critical for the appearance of the features of schizophrenia.[23] Thus developmental events occurring during this period may play more serious role than during adult. In addition, the developmental studies of the thalamic projections to prefrontal cortex may attribute to some relationship between abnormalities in the thalamus and the frontal cortex in schizophrenia. Based on this information, it was suggested that up-regulation of NMDA receptor in frontal cortex may be, at least, involved in the behavioral sensitization effect of DM.

The importance of gender differences in brain and behavior was conceived to highlight for research community.[60] Present study showed that female rats showed more sensitive to DM. It has been reported that female rats are more sensitive to NMDA antagonist.[61]The gender

difference may be related to estrogen effect and gender-specific brain structure. Ovariectomy attenuated striatal dopamine release induced by amphetamine, and estrogen treatment to ovariectomized rats increased dopamine release induced by amphetamine.[62,63] However, estrogen does not produce the same effects in the castrectomized male rats.[62] Interestingly, the ovariectomy attenuated DM behavioral sensitization. However, the level of locomotor activity was still higher than that of male (data not shown). These results suggest that both estrogen and gender-specific structure of the female rats may responsible to behavioral sensitization elicited by DM.

In summary, 10 days' treatment with DM at a dose of 40 mg/kg elicits a behavioral sensitization in adolescent female rats. The activated dopaminergic reward pathway and up-regulated NMDA receptors may contribute to the induction of behavioral sensitization by DM. The estrogen and gender-specific structure of the female may also play a role in the induction of behavioral sensitization.

4. Effects of DM exposure at adolescent period on learning and memory in late age

The present study showed that daily exposure to DM at 40 mg/kg for 10 days at adolescent period impairs learning performance in water maze in aged rats. The difference in performance was not attributable to differential swimming abilities or sensor deficit, since the initial swimming speed was no difference, and a cued trial (visible platform) on the 16th day after training showed no difference. Although acute DM has motor relax effect,[11,64] the effect seems not permanently exist. Therefore, these spatial memory deficits in DM-experienced rats may be related to the hippocampal damage seen in these animals. Previous reports showed that spatial memory deficit was induced by lesioning of the dorsal hippocampus.[65] The mechanism of brain

damage may be related to sudden withdrawal of DM. It has been reported that 6 months of alcohol consumption followed by 4-6 weeks of withdrawal impairs spatial memory, while receiving alcohol 20-28 weeks without withdrawal showed no impairment in the radial maze performance of rats.[66] Memory impairment induce by NMDA antagonists may be related to the fact that repeated treatment with NMDA antagonists resulted in up-regulation of NMDAR in some brain regions including hippocampus.[67,68] In the chronic treatment study, significant up-regulations of the NMDA receptor were found in the frontal cortex and hypothalamus in the female. Withdrawn from the treatment of DM, may result in a large calcium influx to neurons, which may produce neuronal damage.[69] Therefore, it is postulated that chronic treatment with DM up-regulates NMDA receptors and sudden withdrawal elicits a large calcium influx through the up-regulated NDMA receptors, which is responsible for the neuronal damage. On the other hand, high doses of NMDA antagonists result in neuronal apoptosis through another mechanisms.[25,26,70] Involvement of GABA system has been suggested.[71] Neuronal damage can be resulted from over excitation of excitatory pyramidal neurons that normally receive a lot of GABA inhibition. NMDA antagonists disinhibit the function of GABAergic neuron which has NMDA receptors.

Another important consideration is that the altered hypothalamic-pituitary-adrenal axis may play an important role in the cell loss of hippocampus and impairment of learning in water maze. Present data showed that a significant c-Fos expression induced by a cold stress in the PVN of the DM-experienced male and female rats. An elevated concentration of serum corticosterone was found in the DM-experienced female rats after 2 hours of cold stress. It could be suggested that altered HPA function may at least partly contribute to the impairment in the water maze test because there have been many reports demonstrating the relationship

between HPA and hippocampal functioning.[18,72] It has been reported that maintaining high corticosterone level induced by chronic stress may result in cell loss specifically in the hippocampus.[73] Similarly, treatment with a high dose of corticosterone may impair learning in water maze test,[74] and resulted in decreased numbers of apical dendritic branch points and total apical dendritic length in CA3 pyramidal cells in rats, and resulted in even a loss of CA3 pyramidal cells.[75]

Sex differences were noted in the Morris water maze test. Males showed decreased spatial memory at the probe trial, while females showed retardation of acquisition of the task in the initial learning phase as well as the reversal training phase. Sex-dependent patterns of cell loss, found in the present study, may be responsible for this sexual dichotomy. The exact mechanism of this sexual dichotomy is not known yet. However estrogen may have a role for this phenomenon because the neuroregeneration in the hippocampus of females may better than males.[76]

Unlike Morris water maze test performed 18 months of age, no differences were found in the autoshaped learning test performed 12 months of age. The age may be not enough to express behavioral differences because subtle neuronal damage was expressed into altered behavior only in aged life.[18] Active compensatory mechanisms of brain can mask a subtle neuronal damage during young age.[77]

The results indicate that treatment with a high dose of DM at adolescent period produce significant cognitive deficits in aged life in rats. And it has also been reported that the experience of repeated high doses of DM may result in long-term memory deficiency in humans.[78] Therefore DM should be reconsidered as an agent prescribed under control.

V. Conclusion

DM was administered acutely or chronically in adolescent rats, and alterations in behavior and neurochemistry were assessed during adolescent or aged periods. The following results were obtained:

1. Acute DM decreased nocturnal behavior dose-dependently in adolescent rats.

2. Acute DM increased c-Fos expressions in the dopaminergic reward pathway and increased tyrosine hydroxylase mRNA expression in mesencephalon in adolescent rats.

3. Repetitive DM induced behavioral sensitization in locomotor activity and stereotyped behavior, and these phenomena were obvious in female.

4. Repetitive DM increased NMDAR1 immunoreactivity in the hypothalamus and this was obvious in female.

5. Ten days exposure to DM during adolescent period results in impaired learning and memory and stress responses with cell loss in the CA1 and CA3 field of hippocampus in male and CA3 in female at old age.

These data indicate that DM has an abuse liability with behavioral sensitization. Furthermore, exposure to DM at adolescent period impairs spatial learning with accelerated cell loss in the hippocampus in aged life. The results suggest that altered NMDA receptor regulation and stress axis contribute to the consequences of DM exposure.

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국문요약

흰쥐에서 청소년기에 대량의 dextromethorphan노출에 의한 성장후 행동 및 신경화학적 변동

Dextromethorphan은 1950년부터 비의존성 진해제로 널리 사용되어왔다. 그러나, 청소년층 사이에서 dextromethorphan 의 대량투여에 의한 환각작용이 인지되었고, 최근에는 한국 청소년층에서도 남용되고 있는 약물로서 그 폐해가 심각하게 대두되고 있다. 또한 dextromethorphan은 NMDA 수용체 봉쇄작용이 있는 것으로 알려져 있기 때문에, 뇌기능이 왕성한 청소년 시기의 dextromethorphan 남용은 나이든 시기 뇌신경계 및 행동에 영구적인 장애를 초래할 가능성이 내재되어 있다.

따라서 본 연구에서는 청소년기 dextromethorphan 노출에 의한 행동 및 뇌 신경계 변동을 동물실험을 통하여 급성영향, 만성영향 및 장기간 효과로 구분하여 Sprague-Dawley 흰쥐에서 검색하였다.

본 연구의 결과는 다음과 같다.

1. Dextromethorphan을 청소년기에 투여한 후 행동학적 급성영향을 살펴본 바 야간 활동도가 약물 용량 의존적으로 감소되었다.
2. Dextromethorphan을 청소년기에 투여한 후 급성 신경화학적 변동을 살펴본 바 도파민성 보상회로상의 c-Fos 발현이 증가되어 있었고, 중뇌영역에서 tyrosine hydroxylase의 mRNA발현 또한 증가되어 있었다.
3. Dextromethorphan을 청소년기에 반복적으로 투여한 경우 활동도와 반복 행동에

있어서 행동학적 민감화 (behavioral sensitization) 가 유도됨을 알 수 있었고, 이는 암컷 흰쥐에서 뚜렷하였다.

4. 시상하부 NMDA R1 수용체의 면역활성도가 dextromethorphan을 청소년기에 반복적으로 투여한 경우 암컷 흰쥐에서 증가되어 있었다.
5. Dextromethorphan을 생후 28일부터 37일까지 10일 동안 투여받은 흰쥐는 나이든 시기인 18개월 연령에서 기억과 학습능력 그리고 스트레스반응이 변동되어 있었다.

본 연구결과를 종합해 보면 dextromethorphan은 행동민감화를 수반하는 남용성 약물로서의 특징을 갖고 있으며, 청소년 시기의 dextromethorphan 경험만으로도 나이든 시기에서도 행동변이가 초래됨을 알 수 있었다. 이것은 dextromethorphan의 경험에 의해 유도된 NMDA수용체 활성변동과 이와 연관된 스트레스반응계의 변동이 관련되어 있을 것으로 사료된다.

핵심되는말: dextromethorphan, 약물남용, 행동학적 민감화, 기억과 학습, tyrosine hydroxylase, NMDA 수용체, c-Fos, autoshaping, Morris water maze