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Research report

Evaluation of object-based attention in mice

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ABSTRACT

The deficits of attention result in significant impairment in daily life, and pharmacological intervention to improve attention is the most effective treatment in clinics. However, methods which are suitable for the large scale preclinical screening of attention-improving compounds or drugs are few in the field. In this study, we have developed object-based attention task as a simple and wherever-practical method that suitable for quick drug screening in mice. Treatment with p-chlorophenylalanine (pCPA) (200 mg/kg/day, *i.p.*) for three consecutive days reduced the prefrontal cortical content of serotonin and dopamine, and increased turn-over of dopamine while decreasing turn-over of norepinephrine in the prefrontal cortex on day 7. Auditory attention and working memory, but not long-term object memory after a long (10 min) object (two objects)-exposure period, were impaired on day 7 after the same treatment paradigm with pCPA. Novel object recognition ability immediately (<10 s) after a short (3 min) object (on two objects)-exposure period was not impaired after pCPA treatment. However, novel object recognition ability immediately (<10 s) after a short (3 min), but not long (6 min), object (five objects)-exposure period was impaired after pCPA treatment. For the verification, the current task, the object-based attention task, was confirmed in an attention deficit model induced by acute phencyclidine (1 mg/kg, *i.p.*) treatment in mice. It was implied that the object-based attention task would assist the behavioral screening process of pharmacological studies on attention-improving drugs.

1. Introduction

Improving attention is one of the leading targets for new medications in neuropsychiatric disorders. However, few tests are available to assess attention in rodents in laboratory. Nevertheless, focusing on a range of behaviors that are presumed to be central to inattention, variety of animal models with distinct different neural defects have been developed to improve our understanding of the neural basis of attentional dysfunctions and to expand pharmacological interventions [1–6]. Poor attention in animal models is indicated by increased startle response for warned auditory stimuli in prepulse inhibition test and unpunctual response for visual stimuli in 5-choice serial reaction time (5-CSRT) task [7,8], while poor working/reference memory is evaluated by Y-maze and Morris water maze tests [9,10]. In these behavioral methods, especially in 5-CSRT task, detections of attentional deficits in animal models require training the subjects for long-term to enable them to acquire the behavioral skills to reach the designed criteria [8,11,12]. The long-term experimental procedures (with restriction of food or water) could negatively affect the attentional function due to the daily stress that animal receives during repeated trainings to reach a criteria before a test [13]. The requirement of learning new skills in 5-CSRT task could hardly be suitable for the studies of attentional dysfunction in neuropsychiatric disorders with impairment of learning and memory. Therefore, the time-consuming [11,14] and stress-inducing (for experimental animals) [13] drawbacks of these paradigms urge the development of new methods that could quickly evaluate attention deficits in a shorter-period, via bypassing the learning process, and without stressing the animals. Such new methods could initiate and energize the large scale pharmacological preliminary screening of attention-improving compounds or drugs for finding new candidates in the field. However, no such

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methods that rapidly evaluate the attentional function in mice have so far been reported. This study has therefore aimed to develop a behavioral method, the object-based attention task, to evaluate attentional function in a short-period in mice.

2. Methods

2.1. Animals and drug treatment

Male C57BL/6J mice (Japan SLC Inc., Shizuoka, Japan) were used. The animals were housed in plastic cages and were kept in a regulated environment (25–1 °C, 50–5% humidity), with a 12-h light/dark cycle (lights on at 08:00 AM, off at 8:00 PM). Food (CE2; Clea Japan Inc., Tokyo, Japan) and tap water were available ad libitum. Mice intraperitoneally (*i.p.*) received p-chlorophenylalanine (pCPA) (200 mg/kg/day) for three consecutive days [15] (Fig. 1) or phencyclidine (PCP) (0.3.1, and 3 mg/kg) 10 min before the tests [16,17] in physiological saline in a volume of 20 μl/10 g body weight. Control group mice *i.p.* received physiological saline. All experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University Graduate School of Pharmacy. The procedures involving animals and their care conformed to Guidelines for Proper Conduct of Animal Experiments (Science Council of Japan, 2006).

2.2. Dopamine, norepinephrine, serotonin and their metabolites

On day 4 after the cessation of 3-day pCPA treatment, the mice were anesthetized with inhalation of diethyl ether and were sacrificed by decapitation to measure the amounts of norepinephrine, dopamine, serotonin and their metabolites. Brains were rapidly removed and the prefrontal cortex was dissected out on an ice-cold plate. Each tissue sample was quickly frozen with dry ice and stored in a deep freezer at −80 °C until assayed. The amounts of dopamine (DA), norepinephrine (NE), serotonin (5-HT) and their metabolites (3,4-dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), dihydroxyphenylglycol (DHPG), and 5-hydroxyindoleacetic acid (5-HIAA) were determined using a HPLC system with an electrochemical detector (HTEC-500, Eicom Co. Ltd., Kyoto, Japan) as described previously [18]. Briefly, each frozen tissue sample was weighed, and then homogenized with an ultrasonic processor (Heat Systems Inc., New York, USA) in 350 μl of 0.2 mol/l perchloric acid containing isoproterenol (internal standard). The homogenate was placed in ice for 30 min and then centrifuged at 20,000 × *g* for 15 min at 4 °C. The supernatant was mixed with 1 mol/l sodium acetate to adjust the pH to 3 and then injected into a liquid chromatography system equipped with a reversed-phase ODS-column (Eicompak MA-5 ODS, 4.6 mm × 150 mm, Eicom). The column temperature was maintained at 25 °C and the detector potential was set at 500 mV. The mobile phase consists of 0.1 mol/l citric acid and 0.1 mol/l sodium acetate, pH 3.9, containing 14% methanol, 160 mg/l sodium-L-octanesulfonate and 5 mg/l EDTA; the flow rate was 0.5 ml/min.

2.3. Behavioral investigations

2.3.1. Prepulse inhibition (PPI) test

A standard startle chamber applicable to mice (San Diego Instruments, San Diego, CA) was used. The startle chamber consisted of a Plexiglas tube for mice (105 mm, 38 mm, 50 mm), was placed in a sound-attenuated text box, in which animals were individually placed. The tube was mounted on a plastic frame under which a piezoelectric accelerometer was mounted, which recorded and transduced the motion of the tube. Animals were randomly divided into non-stimulated and PPI groups, and subjected to the behavioral test only once. The whole experimental procedure was slightly modified from that described previously [19]. Briefly, mice were placed into the chamber in the PPI test cage, and then habituated to the experimental environment for 10 min with 65 dB of background white noise. Non-stimulated control mice were used without being subjected to PPI trials including PP4 (prepulse level 4 dB above background), PP8 (prepulse level 8 dB above background), and P16 (prepulse level 16 dB above background). The PPI test has three continuous sessions: (1) five startle trials, (2) 10 startle trials, 10 no-stimulus trials, and 40 PPI trials, and (3) five startle trials. The intertrial interval was between 10 and 20 s, and the total duration of three sessions was 17 min. The startle trial consisted of a single 120 dB white noise burst lasting 40 ms. The PPI trials consisted of a prepulse (20 ms burst of white noise with intensities of 69, 73, and 81 dB) followed, 100 ms later, by the startle stimulus (120 dB, 40 ms white noise). Each of the three prepulse trials (69, 73, and 81 dB) was presented 10 times. During the no-stimulus trial, no stimulus was presented but the movement of the animal was scored. Sixty different trials were presented pseudo-randomly, ensuring that each trial was presented 10 times and that no two consecutive trials were identical. The resulting movement of the animal in the startle chamber was measured during 100 ms after startle stimulus onset (sampling frequency 1 kHz), rectified, amplified, and fed into a computer, which calculated the maximal response over the 100-ms period. Basal startle amplitude was determined as the mean amplitude of the 10-startle trials. PPI was calculated according to the formula: 100 − [1 − (PPx/P120)]%, in which PPx was the mean of the 10 PPI trials and P120 was the basal startle amplitude.

2.3.2. Y-maze test

The Y-maze apparatus consisted of black-painted plywood. Each arm of the Y-maze was 50 cm long, 12 cm high and 4 cm wide and positioned at an equal angle. Each mouse was placed at the cross points of arms and allowed to move freely through the maze for an 8-min session. The sequence of arm entries was recorded manually. Spontaneous alternation behavior was defined as the entry into all three arms on consecutive choices in overlapping triplet sets. The percent spontaneous alternation behavior was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries − 2) × 100 as described previously [20].

2.3.3. Novel object recognition task

Inherent preference for one particular object (out of two objects) during the training session affects the novel object recognition in the test session. Therefore, to avoid object preference during the training session, two identical objects (AA or BB) are preferred. In our laboratory, with respect to this principle, we have successfully modified the object setup in training session [20–22]. We applied to two objects (A + B), which were made of the same wooden material with the similar color and smell. These two objects were different in shape but identical in size. In training session, we did not observe any preference for one particular object. In our training protocol (A + B setup), the object exposure time is 10 min, while it is 5 min for other training protocol (AA or BB setup). Other laboratories also applied the same protocol (A + B setup) successfully [23,24]. Therefore we applied the setup (A + B) for training session.

A plastic chamber (35 cm × 35 cm × 35 cm) was used in low light condition (about 40 lx) during the light phase of the light/dark cycle. The general procedure, as described elsewhere [25], consisted of three different phases: a habituation phase, an acquisition phase, and a retention phase. On the 1st day (habituation phase), mice were individually subjected to a single familiarization session of 10 min, during which they were introduced in the empty arena, in order to become familiar with the apparatus. On the 2nd day (acquisition phase) animals were subjected to a single 10-min session, during which floor-fixed two objects (A and B) were placed in a symmetric position in the central line of the arena, 10 cm from each and 8 cm from the nearest wall (each object occupies approximately 5 cm space by its size). The two objects, made of the same wooden material with the similar color and smell, were different in shape but identical in size. Mice were allowed to explore the objects in the open field. The exploration time on each object was shown (as seconds) to indicate the exploring activity of mice. On the 3rd day (retention phase), mice were allowed to explore the open field in the presence of two objects: the familiar object A and a novel object C in different shapes but in similar color and size (A and C). A recognition index (for retention session), calculated for each mouse, was expressed as the ratio (TC × 100)/(TA + TC), where TA and TC are the time spent during retention phase on object A and object C, respectively. The time spent exploring any object (nose pointing toward the object at a distance ≤1 cm, but not mounting on the object or playing with the object) was recorded (using stopwatch) by hand.

2.3.4. Object-based attention test

The apparatus is a rectangular, two-chambered opaque plexiglas boxes including exploring chamber (40 cm long, 40 cm wide, 22 cm high) and test chamber (40 cm long, 20 wide, 22 cm high). Dividing walls were made from opaque plexiglas (the dividing wall) with sliding openings which allowed access from exploring chamber into test chamber. The chambers were always bedded with fresh sawdust for every mouse after the floors of chambers were wiped with tissue paper (70% alcohol) to eliminate the odor left from the previous mouse. The general procedure consisted of three different phases: a habituation phase, an acquisition phase, and a retention phase. On the 1st phase (habituation phase), mice were individually subjected to a single familiarization session of 10 min, during which they were exposed to both empty chambers, in order to become familiar with the surroundings. On the 2nd phase (acquisition phase) animals were subjected to a single 3-min (or 6-min) session, during which floor-fixed five objects (A, B, C, D, and E) were placed separately in the center arena of the exploring chamber. Objects A, B, D, and E were placed 10 cm from each (from the inner side of one object to the inner side of another object; each object occupied approximately 5 cm space by its size) and 10 cm from the nearest wall of the exploring chamber. Object C was placed in the central point of the exploring chamber. All objects, made of the same wooden material with the similar color and smell, were different in shape but roughly identical in size. Mice were allowed to explore the objects in the open field. It has been observed that a mouse would spend equal amount time on any of the five objects during a 3-min exposure (unpublished data). Therefore, the experimenter could randomly designate any two objects (e.g., object A and object D) out of the five objects for recording of time if the recordings are done with stopwatches in two hands. As the object-exploration time, the time spent on exploring the two randomly designated objects was recorded. The exploration time on each object was shown (as seconds) to indicate the exploring activity of mice. All of the five objects, but not the mouse, were immediately withdrawn out of the exploring chamber upon the completion of the training session. On the 3rd phase (retention phase) which immediately follows the 2nd phase, an object used in training session (one of the two randomly designated objects for the recording of the exploration time in training session, e.g., object A) was placed into test chamber in parallel with its original position in the exploring chamber. A novel object F in different shape but in similar color and size was placed in the test chamber in parallel with the other one of the two randomly designated objects (e.g., object D) in

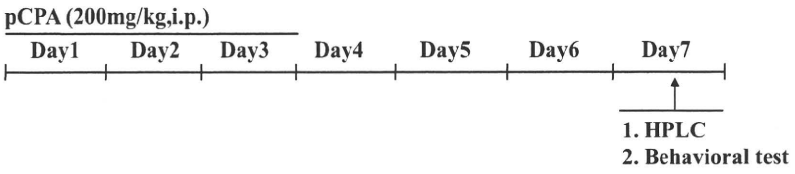

Fig. 1. Experimental schedule.

the exploring chamber (this was done before starting the training session to shorten the interval between training-retention sessions). Both objects in the test chamber were placed 7.5 cm from the dividing wall (each object approximately occupies 5 cm space by its size). Then, the mouse, which was not moved from the exploring chamber, was immediately allowed to enter the test chamber (sometimes the mouse was gently driven to the test chamber) and to explore two objects: the familiar object A and the novel object F. A recognition index (for retention session), calculated for each mouse, was expressed as the ratio (TF × 100)/(TA + TF), where TA and TF are the time spent, during retention phase, on object A and object F, respectively.

3. Data analysis

Values are reported as the mean ± S.E.; statistical significance was considered as $p < 0.05$ and was determined with *T*-test analysis or with one-way ANOVA followed by the Bonferroni multiple comparisons test.

4. Results

4.1. pCPA treatment decreased the levels of serotonin, norepinephrine, dopamine, and their metabolites in the prefrontal cortex and hippocampus

Sub-chronic treatment with pCPA is widely accepted to deplete serotonin levels in the brain tissue of rats and mice. Brain serotonin depletion by pCPA has been reported to impair short-term memory in Y-maze test but not long-term memory in multi-trial passive avoidance task [15]. To confirm the neurochemical basis of the model, the contents of 5-HT, NE, and DA in the attention related region of the brain, the prefrontal cortex, were examined. pCPA (200 mg/kg, *i.p.*) treatment for three consecutive days resulted in a significant decrease of the content of 5-HT, 5-HIAA, DA, DOPAC, and HVA and the turn-over of 5-HT and NE, while the turn-over of DA was increased (Fig. 2A and B). Since the decreased utilization of NE (or decreased turn-over of NE), and the decreased level of 5-HT are associated with attention deficits and information-acquisition difficulties [26–36], we applied this model for the rest of the experiment.

4.2. Auditory attention and working memory, but not long-term object recognition memory, were impaired in pCPA-treated mice

To avoid stress-interference in the behavioral battery, mice were randomly selected for the different tests and were not repeatedly used. In PPI test, mice with pCPA treatment demonstrated significantly reduced PPI in comparison with their counterparts in control groups while startle amplitudes were not different between groups (Fig. 3A and B). In Y-maze test, the mice with pCPA treatment showed significantly decreased spontaneous alternation behaviors compared with the counterparts in control groups (Fig. 3C). However, the number of arm entries between groups was not different between groups (Fig. 3D). In addition, in acquisition phase of NORT, the time spent on object exploration was similar between groups (Fig. 4B, E and H). The results show the motivation, curiosity, and general behaviors are not different in both groups (Fig. 3D; Fig. 4B, E and H). During retention phase, both control and pCPA-treated

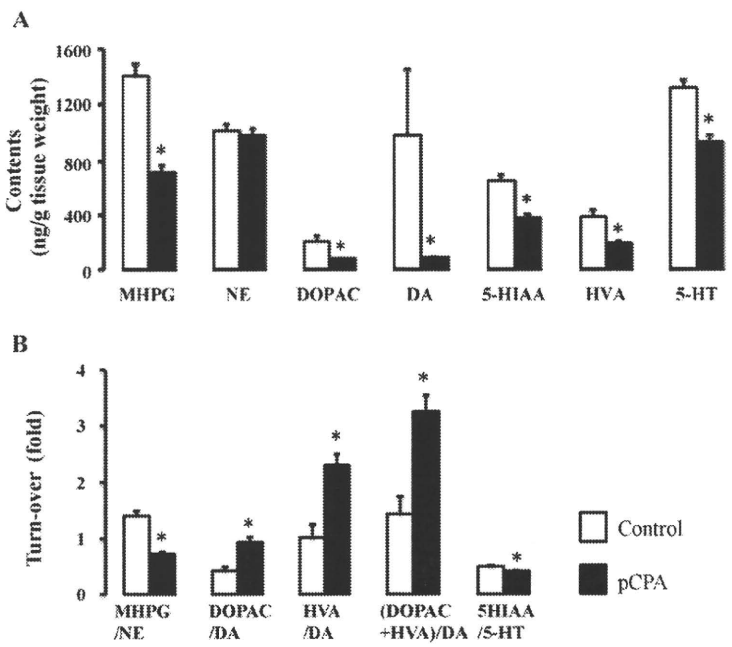

Fig. 2. The effects of pCPA treatment on the levels of serotonin, norepinephrine, and dopamine and their metabolites in the prefrontal cortex: mice were treated with the serotonin synthesis inhibitor pCPA (200 mg/kg/day, *i.p.*) for 3 consecutive days. On day 7, the prefrontal cortex was dissected out and used for HPLC analysis as described in Section 2. (A) The levels of 5-HT, NE, DA, and their corresponding metabolites are expressed in ng/g tissue weight. (B) Level of turn-over of 5-HT, NE, and DA. Values are the mean ± S.E. ($n = 9$). *$p < 0.05$ vs. control. DA: dopamine; NE: norepinephrine; 5-HT: serotonin; DOPAC: 3,4-dihydroxyphenylacetic acid; HVA: homovanillic acid; DHPG: dihydroxyphenylglycol; 5-HIAA: 5-hydroxyindoleacetic acid.

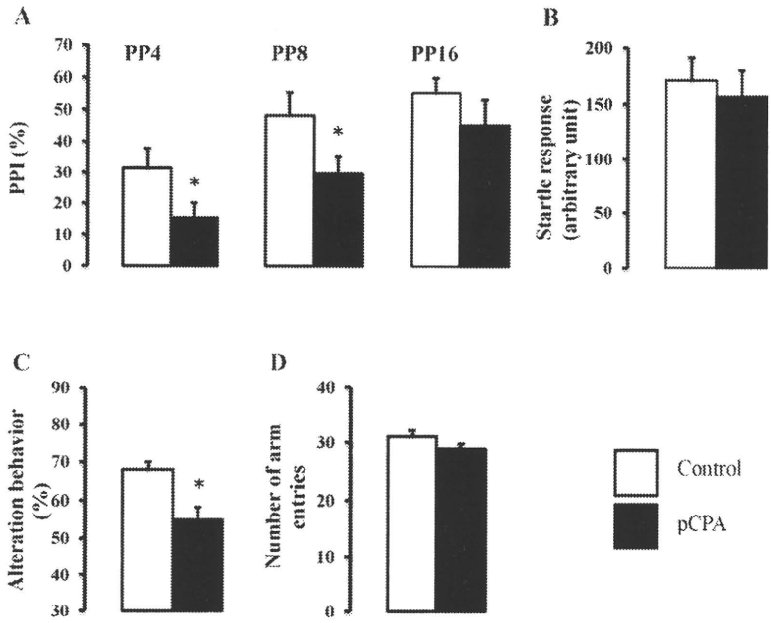

Fig. 3. Auditory attention and short term reference memory were impaired in pCPA-treated mice. Three-day treatment with pCPA (200 mg/kg/day, *i.p.*) induced significant impairment both in auditory attention in PPI test (A and B) and the immediate working memory in the Y-maze test (C and D) on day 7. Data were presented as the mean ± S.E. ($n = 10$). $^*p < 0.05$ vs. control. PP4: prepulse level 4 dB above background; PP8: prepulse level 8 dB above background; PP16: prepulse level 16 dB above background.

groups unmistakably discriminated the novel and familiar objects by showing a significantly increased exploration to the new object in an incomparable manner (Fig. 4C).

4.3. Impaired object-based attention in pCPA-treated mice

pCPA did not impair the recognition memory in NORT (Fig. 4A–C) in which exposed the mice to the object in training session for 10 min. Therefore, we shortened the time of training session for 3 min, and examined the retention in 24 h later. Unexpectedly, both control and pCPA group mice failed to discriminate the familiar object from the novel object by spending equal amount of time exploring the both in 3 min retention session (Fig. 4D–F). To examine whether the object recognition failure was due to the inability of mice to recognize the objects during the brief (3 min) object-exposure in training session or was due to the long interval (24 h) between brief training session and retention session, the interval was shortened to 10 s. With the 10 s interval, both control and pCPA group mice were able to discriminate the novel object from the familiar object by spending more time exploring the novel object (Fig. 4G–I). It was implied that 3 min exposure to the objects are enough for a mouse to pay attention to the objects to acquire the information that essential for recognizing the objects. To increase the burden of attention, the number of objects was increased to five while the time for training session still remained as 3 min. Mice were exposed to the five objects for 3 min (training session), then, after an interval of 10 s, they were exposed to two objects that include a familiar and a novel objects for 3 min (retention session) (Fig. 5A). Both pCPA and control group mice explored the two-randomly defined objects for equal amount time during the training session (Fig. 5B). In retention session, pCPA group mice still spent more time on familiar object in comparison with the novel object while control group mice spent significantly less time on familiar object (Fig. 5C). Results indicated that pCPA group mice explored the objects with a nonfocused attention during the training session that resulted in a failure to recognize the already-explored (familiar) object in retention session. To examine whether an increased object-exploring in training session could compensate the poor attention, the mice were exposed to another five objects for 6 min and tested for 3 min with a 10 s interval (Fig. 5D). The novel-object-discriminating abilities of pCPA group mice were improved up to the levels of those of control group mice (Fig. 5E and F). Then the same groups of mice were given a retention test in 24 h later by replacing the familiar and novel objects that were previously used. Both pCPA and control group of mice preferentially explored the novel object in comparison with the familiar object (the other one of the randomly recorded two objects among the five objects) (data not shown). The results implied a time-dependent improvement of object-based attentional function in mice.

4.4. Confirmation of object-based attention task methodology

To confirm the methods established above, a well-known mouse model of attention deficit was applied. PCP, an antagonist of NMDA receptor, is used at high doses for repeated treatment to establish the mouse models of schizophrenia [17,37]. However, single injection of PCP (1 mg/kg, *i.p.*) did not affect the overall activity of mice in the training trials of water-finding test but produced deficits in latent learning, which requires attention to the location of water in the single training trial, in mice [16]. Therefore, we applied PCP to establish a model of attention deficits that could be evaluated by the established method – PPI test. We confirmed that PCP (1 and 3 mg/kg) impaired PPI in mice (Fig. 6A). However, single injection of PCP (3 mg/kg, *i.p.*) increased locomotor activity and induced abnormal locomotion (wobbling) in mice soon after injection (unpublished data). Therefore, the lowest effective dose (1 mg/kg, *i.p.*) of PCP was selected in PPI test (Fig. 6A and B) to confirm the sensitivity of the new method-object-based attention task. Treatment with PCP, 10 min before the 3-min training session in object-based attention, did not affect the object exploration of mice (Fig. 6C and D); however, in test session, PCP treated mice in-discriminatively explored the familiar object and novel object (Fig. 6E). Increasing of object-exploring time to 6 min in training session, expectedly improve the novel-object-discriminating abilities of PCP group mice up to the levels of those of control group mice (Fig. 6F–H). The results provide support for the validity of the methods to evaluate attention in mice.

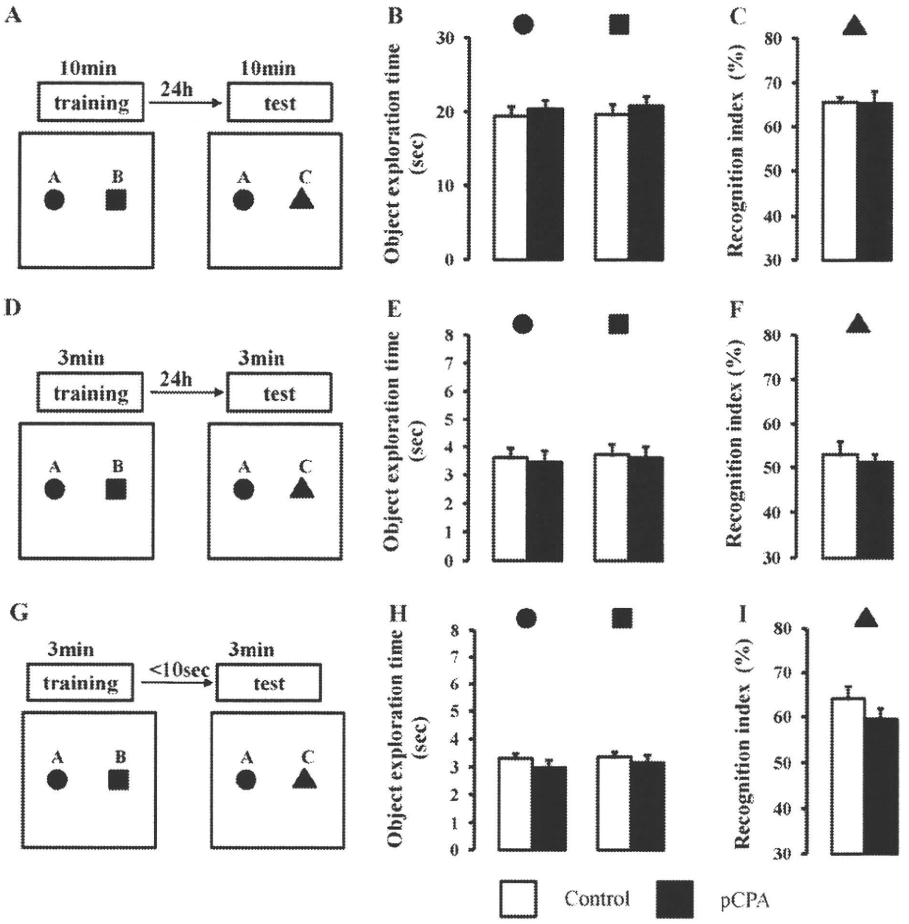

Fig. 4. Object recognition memory was not impaired in pCPA-treated mice. A NORT with two objects was performed in different schedule. Mice were treated with the serotonin synthesis inhibitor pCPA (200 mg/kg/day, *i.p.*) for 3 consecutive days. (A–C) On days 7–8, mice were subjected to training and retention sessions, respectively, of NORT. Mice were exposed to two different objects in training session for 10 min, the retention was examined in 24 h later for 10 min, both control and pCPA group mice were able to recognize the novel object (indicated by recognition index). (D–F) On days 7–8, mice were subjected to training and retention sessions, respectively, of NORT. The time of training session was 3 min, and the retention was examined in 24 h later for 3 min. Both control and pCPA group mice failed to discriminate the familiar object from the novel object by spending equal amount of time exploring the both object (indicated by recognition index) in 3 min retention session. (G–I) On day 7, mice were subjected to training and retention sessions of NORT. The time of training session was 3 min, and the retention session was examined in less than 10 s later for 3 min. Both control and pCPA group mice were able to discriminate the novel object from the familiar object by spending more time exploring the novel object as indicated by recognition index. Data were presented as the mean ± S.E. ($n = 10$). $^*p < 0.05$ vs. control.

5. Discussion

Disturbances in attentional processes are a common feature of several psychiatric disorders such as schizophrenia and attention deficit/hyperactivity disorder (ADHD) [36]. The attentional deficits, especially in young population, render the most significant impact in one's life. The ADHD affects 5–10% of children worldwide and persist through adolescence into adulthood in about half of the affected individuals [38]. In the long term, it can impair academic performance, success and social skill development. Therefore, variety animal models with distinct different neural defects have been developed to improve our understanding of the neural basis of attentional deficits (such as schizophrenia and ADHD) and to further pharmacological interventions [39–41]. Diagnosis of attentional deficits in human depends on behavioral criteria. Therefore, the animal models of the disorder focus on a range of behaviors that are presumed to be central to inattention. Consistently, animal models with attentional developmental disorders are developed with genetic approaches (e.g., DAT knock-out, SNAP-25 mutant mice) or with pharmacological approaches (e.g., anoxia, 6-hydroxydopamine) [2,4,7]. These animal models of attentional deficits show impaired reference memory depending on the task: poor exploration efficiency and working/reference in Morris water maze and in eight-arm radial maze [9,10]. In humans, attentional performance is generally assessed using the continuous performance tests (CPTs), which measures a person's selective and sustained attention to visual stimuli over a sustained period of time. Mimicking the task rules of human CPTs, the 5-choice serial reaction task (5-CSRT task) has been developed to evaluate the sustained and selective attention in rodents [42]. Since its first description by [7,43], the 5-CSRT task has been extensively used in rats and, to a limited extent, in mice for studies on attentional disorders [8,12]. The 5-CSRT task requires animals to monitor the location of a briefly presented light in one out of the five spatially arranged target areas. In this task, subjects are required to maintain 85% of free-feeding weight to drive their motivation for the reward, the food, throughout the entire training period [11]. In 5-CSRT task, the required training period for mice is longer than that for rats. The training period is approximately 2–4 months for rats [11,35] while it is approximately 3–5 months or more for mice [14,44]. The requirement of the long-term training of rodents, make the 5-CSRT task only be suitable for the studies of long-term dysfunction of attention in neuropsychiatric disorders such as schizophrenia. Apparently, in animal models of attention deficits at younger age such as attention deficit hyperactivity disorder, 5-CSRT task could not be suitable for the evaluation of attention deficits due to the

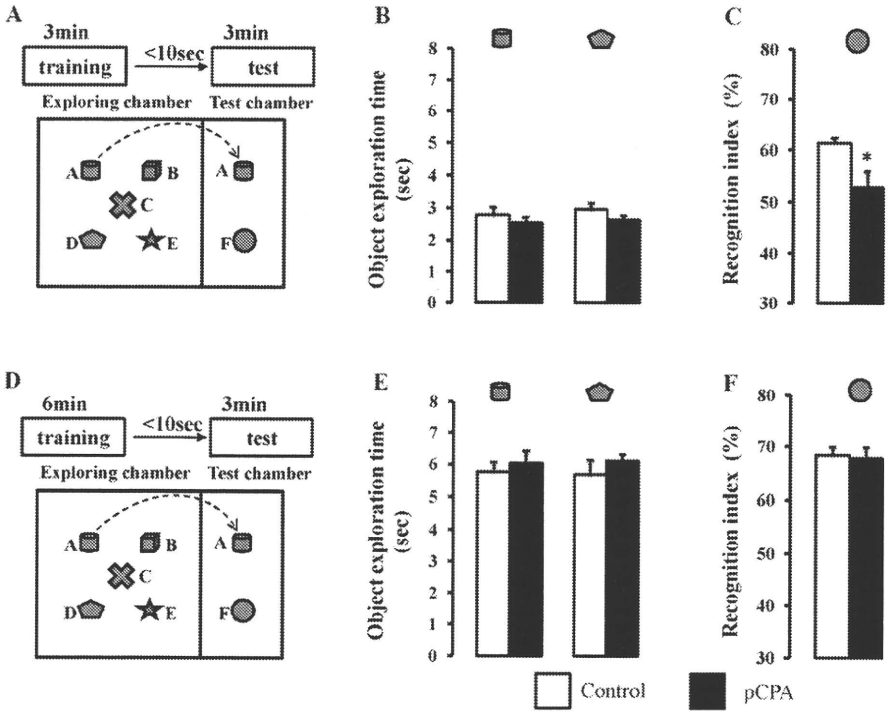

Fig. 5. Object-based attention was impaired in pCPA-treated mice. Mice were treated with the serotonin synthesis inhibitor pCPA (200 mg/kg/day, *i.p.*) for 3 consecutive days. On day 7, mice were subjected to training and retention sessions of object-based attention task. (A) Mice were exposed to five objects for 3 min (training session), then, after an interval of 10 s, they were exposed to two objects that include a familiar and a novel objects for 3 min (retention session). (B) Both pCPA and control group mice explored the two-randomly defined objects for equal amount time during the training session. (C) In retention session, pCPA group mice spent equal amount time on both novel and familiar objects while control group mice spent significantly less time on familiar object in comparison with the novel object (expressed by recognition index). (D–F) Mice were exposed to five objects for 6 min and tested for 3 min with a 10 s interval. The novel-object-discriminating abilities of pCPA group mice were improved up to the levels of those of control group mice, and no group difference in recognition index was observed. Data were presented as the mean ± S.E., $n = 10$; $^*p < 0.05$ vs. control.

long-term training. Further, the technical, spatial, and financial requirements of 5-CSRT, restrict its applicability to limited number of laboratories. In this study, we established an easy-to-use, easy-to-maintain, and easy-to-observe task, the object-based attention task. We applied pCPA-induced model of impairment of working memory in mice [15].

To confirm the neurochemical basis of the model, monoamine levels in the prefrontal cortex were examined. Not only was the level of 5-HT, but the level of DA reduced in the prefrontal cortex. This might be due to the decreased the conversion of tyrosine to DA after the treatment with pCPA [45]. The observed increase in the turn-over of DA might also contribute to the significant decrease of DA content. The increased turn-over of DA could be due to the pCPA-induced global decrease of 5-HT which generally inhibits the release of DA via 5-HT2A/C receptors [46]. It has been reported that 6-hydroxydopamine-induced lesions in the nucleus accumbens significantly decreases the frontal cortical concentrations of DA (−56%), but did not impair the discriminative accuracy of rats in 5-SCRT task [47]. It has also been reported that pCPA (500 mg/kg/day, 3 days) induced an almost total depletion of frontal cortical 5-HT and its major metabolite 5-HIAA and reduced the frontal cortical concentrations of noradrenaline (−30%) and dopamine (−42%), and induced impairment in discriminative accuracy (the indicator of attention) of rats in 5-SCRT task [35]. While the content of NE in the prefrontal was not changed by pCPA treatment, the turn-over was significantly decreased, implying a decreased release or decreased utilization of NE. However, it is not known whether the reductions of catecholamine concentrations in the prefrontal cortex seen after pCPA treatment are secondary to 5-HT depletion or due to non-specificity of pCPA treatment itself [35,45]. The decreased turn-over of both NE and 5-HT could be due to the reduced regulation (hetero-receptor regulation) by low levels of 5-HT. The modulation of higher cortical functions including attention and vigilance is associated with the NE-nergic system. Since the dysregulation of NE system is known to be the cause of attention deficits [34], and the normal tuning of all 5-HT, NE and DA in the prefrontal cortex is critical to attention [36,48,49], the concomitant abnormal changes in both NE and DA may support the impairment of working memory and attention deficits in the current model. Working memory and attention have much in common and are much dependent on the multitasking neurons in the prefrontal cortex [50–54]. A widely accepted theory regarding the function of the prefrontal cortex is that it serves as a store of short-term memory and also implements working memory. Recent studies suggest that prefrontal activity contributes more to the process of attentional selection (and selective attention) and working memory than to memory storage [54,55]. Attention requires several components including arousal and alertness, sensory processing and interpretation, selective attention, and working memory. It is well-known that NE is responsible for arousal and alertness. The decreased utilization (turn-over) of NE in the current model could undermine the prerequisites of attention, the arousal and alertness.

To confirm our method to evaluate object-based attention, a different model of inattentiveness induced by PCP was applied. PCP, after chronic application, reproduces a schizophrenia-like psychosis in humans and therefore PCP-treated animals have been utilized as an animal model of schizophrenia. However, the schizophrenia-like psychosis in rats and mice are induced only after repeated treatment of higher doses (>1 mg/kg) of PCP. While repeated PCP treatment (10 mg/kg/day, *i.p.* for 14 days) in rats induces working memory impairment in a T-maze test [56], twice daily treatment with lower dose of PCP (5 mg/kg/day, *i.p.* for 5 days) did not produce significant impairments in working memory in T-maze test in rats [57]. In our laboratory, schizophrenia-like

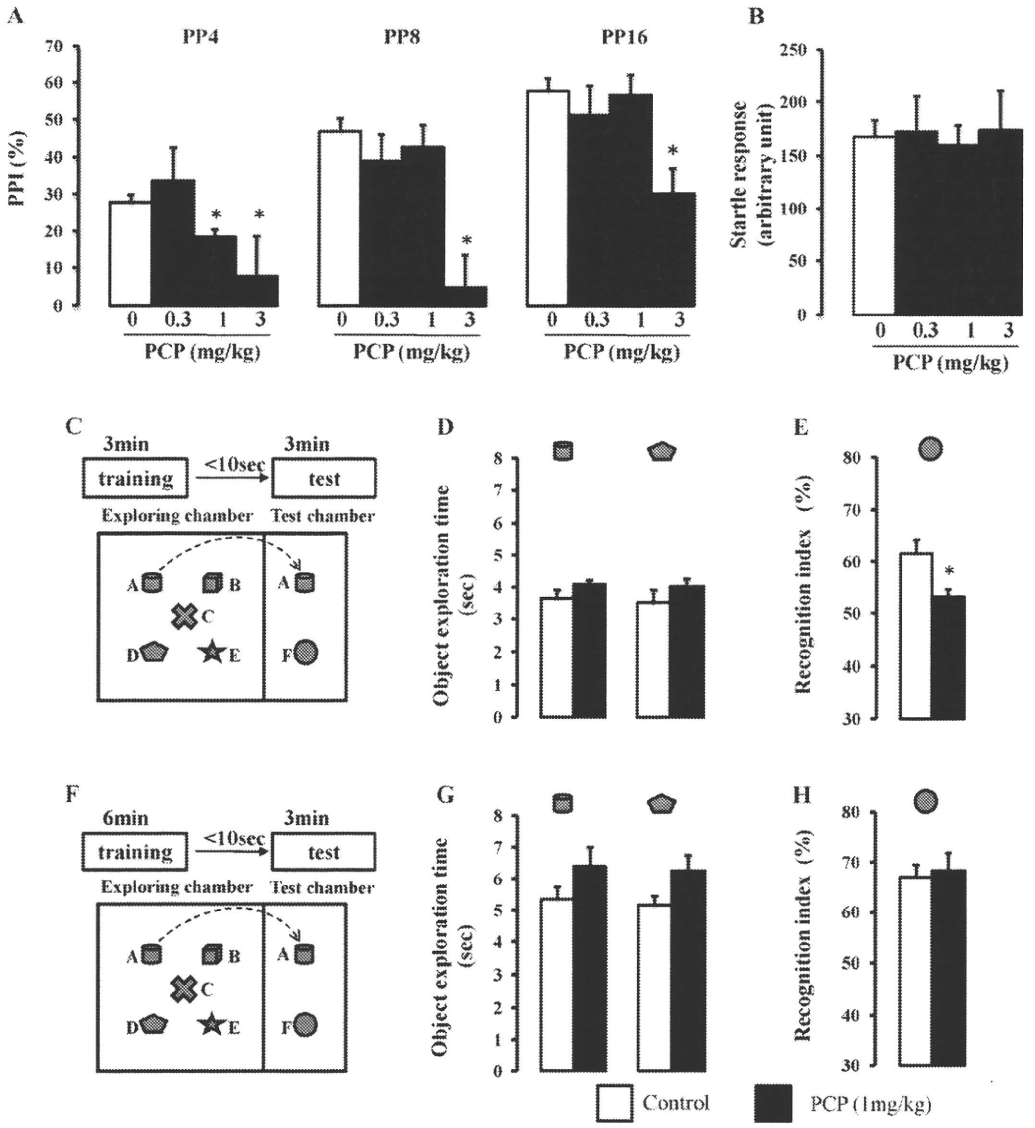

Fig. 6. Object-based attention was impaired in PCP-treated mice. (A and B) Mice received phencyclidine (PCP) (0.3, 1, and 3 mg/kg, *i.p.*) 10 min before the acclimation phase of PPI test. PCP (1 and 3 mg/kg) impaired PPI. (C and E) Treatment with PCP (1 mg/kg) was done 10 min before the 10 min habituation session of object-based attention test. PCP did not affect the object exploration of mice during 3 min training session but impaired the novel-object-recognition abilities (recognition index) compared with the control group mice. (F–H) Treatment with PCP (1 mg/kg) was done 10 min before the 10 min habituation session of object-based attention test. PCP did not affect either the object exploration of mice during 6 min training session or the novel-object-recognition abilities (recognition index) compared with the control group mice. Data were presented as the mean ± S.E., $n = 10$; $^*p < 0.05$ vs. control. PP4: prepulse level 4 dB above background; PP8: prepulse level 8 dB above background; PP16: prepulse level 16 dB above background.

behaviors were successfully induced in mice only after repeated treatment with PCP at the dose of 10 mg/kg/day for 14 days [58], but not at the dose of 3 mg/kg/day (unpublished data). It was therefore generally believed that only the repeated treatment with PCP at higher doses (e.g., 10 mg/kg) could induce schizophrenia-like psychosis in mice or rats [59]. Therefore, we applied the single injection of 1 m/kg dose of PCP for this study. PCP increases DA release and inhibits 5-HT as well as NE reuptake [37]. Acute treatment with PCP inhibits sensorimotor gating (or PPI) [17], which is an operational measure of attentional process. Acute treatment with PCP in this study induced deficits in PPI as well as in the object-based attention task, confirming the practicability of the novel method. In the object-based attention task, both pCPA and PCP induced impairment after short-term (3 min) training but not after long-term training (6 min) in mice. During the short-term exposure to objects in training session, both the control and the pCPA (PCP) group mice spent almost 3 s to explore the objects. However, pCPA (PCP) group mice were not able to differentiate the familiar object (which they explored in training session) from the novel object in test session. It was suggested that the quality of attention in pCPA (PCP) group mice was poor and 3 min exploration on the object in training session may not be enough for pCPA (PCP) group mice to be familiarized with the object they were exploring. Therefore, we examined whether an increase of exploring time (from 3 min to 6 min) in training session would help pCPA (PCP) group mice to be familiar with the object(s) and differentiate it from the novel object in the test session. During a long-term exposure to the objects in training session, both the control and the pCPA (PCP) group mice spent almost 6 s to explore the objects. In the test session, pCPA (PCP) group mice were able to differentiate the familiar object (which they explored in training session) from the novel object. It was suggested that if more time is spent on exploring the objects, more attention can be paid on the objects and this leads to the familiarity on the objects. Therefore, it was suggested that

in testing the attentional function in mice, the span of attention-time should be controlled the minimum to test the vulnerability of attentional function.

The other way to check the validity of our method may be to apply 5-CSRT task. The effect of pCPA on the attentional performance of rats in a 5-CSRT task has been reported. After training the rats for about 4 months, pCPA (500 mg/kg/day, 3 days) treatment was introduced (one rat became ill and died after the completion of the treatment). pCPA induced an almost total depletion of frontal cortical 5-HT, NE (−30%) and DA (−42%), and impairment in discriminative accuracy (the indicator of attention) as well as a decreased overall tendency to respond in rats [35]. The time-consumption on the training of C57BL/6J mice in 5-CSRT task is even greater [44]. It has been reported that C57BL/6 mice were trained for 69 sessions to enter the final parameters (in which signal duration is 1 s) in 5-CSRT task before the mice were continuously trained on the final parameters in daily sessions of 30 min duration for approximately 4 months, until mean accuracy reached 70% and no more that 50% omission errors were recorded for three consecutive days [44]. PCP, at doses up to 3 mg/kg, did not impair discriminative accuracy in C57BL/6N mice in 5-CSRT task [60]. Therefore, considering the time-consumption and the difficulties in the training of C57BL/6J mice in 5-CSRT task, simplification 5-CSRT task to 3-CSRT task is advised, and further study in using 3-CSRT is desired.

Given the unchallenging applicability and time-saving characteristics of the object-based attention task, it would assist the behavioral screening process of pharmacological studies on attention-improving drugs.

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Research report

Prenatal exposure to PCP produces behavioral deficits accompanied by the overexpression of GLAST in the prefrontal cortex of postpubertal mice

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ABSTRACT

Altered glutamatergic neurotransmission in the prefrontal cortex (PFC) has been implicated in a myriad of neuropsychiatric disorders. We previously reported that prenatal exposure to PCP produced long-lasting behavioral deficits, accompanied by the abnormal expression and dysfunction of NMDA receptors. In addition, these behavioral changes were attenuated by clozapine treatment. However, whether the prenatal exposure adversely affects pre-synaptic glutamatergic neurotransmission in postpubertal mice remains unknown. In the present study, we investigated the involvement of prefrontal glutamatergic neurotransmission in the impairment of cognitive and emotional behavior after prenatal PCP treatment (5 mg/kg/day) from E6 to E18 in mice. The PCP-treated mice showed an impairment of recognition memory in a novel object recognition test and enhancement of immobility in a forced swimming test at 8 weeks of age. Moreover, the prenatal treatment reduced the extracellular glutamate level, but increased the expression of a glial glutamate transporter (GLAST) in the PFC. The microinjection of DL-threo-β-benzyloxyaspartate (DL-TBOA, 10 nmol/site/bilaterally), a potent blocker of glutamate transporters, reversed these behavioral deficits by enhancing the prefrontal glutamatergic neurotransmission. Taken together, prenatal exposure to PCP produced impairments of long-term memory and emotional function which are associated with abnormalities of pre-synaptic glutamate transmission in the PFC of postpubertal mice. These findings suggest the prenatal inhibition of NMDA receptor function to contribute partly to the pathophysiology of neurodevelopment-related disorders, such as schizophrenia.

1. Introduction

Disruption of the brain's development at an early stage can potentially alter neural networks and may increase the risk for neuropsychiatric disorders in later life. According to the neurodevelopmental hypothesis, disruption of the developing brain predisposes the neural systems to long-lasting structural and functional abnormalities, leading to the emergence of psychopathological behavior in adulthood [3].

NMDA receptor plays a critical role in neuronal development [10]. The stimulation of NMDA receptors during development is critical for the survival, differentiation and migration of immature neurons [4,20], controls structure and plasticity [40], and establishes normal neural networks in the developing brain [12]. On the other hand, pharmacological inhibition of NMDA receptors at an early stage disturbs neural function in development [6,13,22].

The blockade of NMDA receptors with phencyclidine (PCP), a noncompetitive antagonist, produces a transient state of psychosis and schizophrenia-like deficits in normal subjects and exacerbates several symptoms in schizophrenia patients [18]. Moreover, PCP elicited a prolonged recrudescence of the acute psychotic state in patients with stable chronic schizophrenia, suggesting that a similar mechanism is compromised [21]. These observations, along with the finding of reduced glutamate levels in the cerebrospinal fluid of schizophrenic patients [19], form the basis of the glutamatergic hypofunction hypothesis of schizophrenia.

According to this hypothesis, PCP is widely used to produce abnormal behavior and biochemical changes resembling the positive symptoms, negative symptoms, and cognitive deficits of patients with schizophrenia [32,33,38]. Although a series of schizophrenia-like symptoms are observed in PCP-treated adult

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rodents, this animal model is unlikely to completely resemble the pathogenesis of schizophrenia, since at least in some cases, the pathologic abnormalities occur during development and are initiated by prenatal insults [35,39]. Therefore, it is necessary to consider the process by which the symptoms of schizophrenia develop. Based on the neurodevelopment hypothesis of psychiatric disorders, several studies have modified the classic "PCP-based animal model", with treatment using NMDA antagonists during the early development of the brain [1,12,44,49,50]. Moreover, one recent study has revealed that postnatal, but not adulthood, NMDA receptor ablation in the corticolimbic interneurons confers schizophrenia-like phenotypes in adult mice [5].

Our previous study confirmed that prenatal exposure to PCP (20 mg/kg) produced a cognitive deficit and hypersensitivity to PCP in terms of locomotor activity, which was associated with abnormal expression of the NMDA receptor [27]. Furthermore, these behavioral changes were attenuated by clozapine [27], an atypical antipsychotic that enhanced the function of glutamatergic transmission [9,30] and inhibited the up-regulation of glutamate transporters [29,48]. These findings suggest that the pre-synaptic glutamatergic system is involved in the behavioral deficits induced by prenatal PCP treatment. However, little attempt has been made to investigate the role of pre-synaptic glutamate transmission in this model. In this study, we evaluated the long-term effects of prenatal exposure to PCP on the prefrontal glutamatergic neurotransmission in mice.

2. Materials and methods

2.1. Animals

ICR female and male mice (8 weeks old) were obtained from Japan SLC (Shizuoka, Japan) and maintained on a 12/12 h, light/dark cycle (lights on from 08:00 to 20:00) with free access to food (CE2; Clea Japan Inc., Tokyo, Japan) and water. The mice were mated one pair per cage. Females were checked in the next morning and those with an embolus in their vaginas were considered pregnant [embryonic day 0 (E 0)]. The dams were randomly divided into saline- (SAL) and PCP-treated groups. All were housed individually till parturition. There were no maternal deaths and resorption or stillbirths caused by exposure to PCP in this study. At birth [postnatal day 0 (PD 0)], pups were culled to 8–10 per litter with a balance of males and females as possible. Maternal care behavior during feeding was monitored till weaning on PD 21. After weaning, pups given the same prenatal treatment were mixed by gender, and then randomly assigned into groups to do behavioral tests. Each behavioral test was involved 2–3 litters each time and repeated more than 3 times by using different mice to reduce the influence of litters. Moreover, a balanced number of males and females were used in each experiment, since no significant differences were observed between genders in our preliminary study [27].

The experiments with offspring were started at the age of 8 weeks and carried out in a sound-attenuated and air-conditioned room (23 ± 1 °C, 50 ± 5% humidity). The mice were habituated to the room for more than 40 min before behavioral experiments. All the behavioral tests were recorded with a digital versatile disc camera to analyze the results. The experiments were performed in accordance with the Guidelines for Animal Experiments of Meijo University Faculty of Pharmaceutical Sciences and the Guiding Principles for the Care and Use of Laboratory Animals approved by the Japanese Pharmacological Society (2008).

2.2. Drugs

PCP hydrochloride was synthesized according to the method of [28] and checked for purity. PCP was dissolved in saline. DL-threo-β-benzyloxyaspartate (DL-TBOA, Tocris, MO) was prepared as a stock solution of 100 mM in 50% dimethyl sulfoxide (DMSO) and 100 mM NaOH, and diluted with artificial cerebrospinal fluid (aCSF) before the behavioral test, or with Ringer's solution before the microdialysis analysis [34].

2.3. Drug treatment

The dams were administered SAL or PCP (5 mg/kg, s.c.) once daily at 18:00 from E 6 to E 18, the middle and late stages of pregnancy, covering the entire period of neurodevelopment in the prenatal brain from neurulation to corticogenesis [45]. The injection by s.c. was performed gently as possible to minimize potential stress-related influences on dams. The dose of PCP (5 mg/kg) was selected according to one study [44], since it was not toxic enough to affect the sensitization of dopaminergic system or tolerance of serotonergic ataxic behaviors in adults [25].

Under light anesthesia with diethyl ether, mice received an microinjection of DL-TBOA (1 or 10 nmol/μl/site; bilaterally) into the PFC [Anteroposterior (AP): +1.7 mm from Bregma; Mediolateral (ML): ±0.5 mm from Bregma; Dorsoventral (DV): +2 mm from the skull] according to the mouse brain atlas of [15], 30 min before each behavioral test [34]. DL-TBOA was injected over a period of 30 s, and the injector was left in the place for 1 min to allow diffusion. For the analysis of microdialysis, according to the effective dose observed in the behavioral tests, DL-TBOA (1 mM) was administered through the dialysis probe at a rate of 1 μl/min for a total of 30 min.

2.4. Novel object recognition test

The novel object recognition test was performed as described previously [27]. Each mouse was individually habituated to the box (L 30 × W 30 × H 35 cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects (a red painted triangular prism and a yellow painted cube) were symmetrically fixed to the floor of the box, 8 cm from the walls, and each animal was allowed to explore in the box for 10 min (day 4). An animal was considered to be exploring the object when its head was facing the object or it was touching or sniffing the object at a distance of less than 2 cm and/or touching it with the nose. The time spent exploring each object was recorded. After training, mice were immediately returned to their home cages. During the retention session, animals were placed back into the same box 24 h (day 5) after the training session, in which one of the familiar objects used during training was replaced with a novel object (a black painted golf ball). The animals were allowed to explore freely for 5 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of time spent exploring either of the two objects (training session) or the novel object (retention session) over the total amount of time spent exploring both the objects, was used to assess cognitive function.

2.5. Forced swimming test

The forced swimming test was done according to a previous report [34], with a minor modification. Mice were placed in a transparent glass cylinder (20 cm high, 15 cm in diameter), which contained water at 22 °C to a depth of 11.5 cm, and forced to swim for 6 min. The duration of swimming was measured by using a SCANET MV-10 AQ apparatus (Melquest Co. Ltd., Toyama, Japan). Immobility time was calculated as: immobility time (s) = 360 − swimming time.

2.6. Microdialysis analysis

Different mice from behavioral tests above were used for the following microdialysis analysis. Mice were anesthetized with pentobarbital-Na (50 mg/kg, i.p.) and fixed in a stereotaxic apparatus (David Kopf Instruments, CA). A dialysis probe (D-I-6-01; EICOM, Kyoto, Japan) was implanted into the PFC [AP: +1.7, ML: −0.05 from bregma, DV: −2 mm from the skull]. Twenty-four hours later, Ringer's solution (147 mM, NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) was perfused at a flow rate of 1 μl/min. Dialysate was collected every 10 min and the amount of glutamate was determined by an HPLC system (HTEC-500, EICOM) with electrochemical detection (ECD). For depolarization, 100 mM KCl-containing Ringer's solution (51 mM NaCl, 100 mM KCl, and 2.3 mM $CaCl_2$) was delivered through the dialysis probe for 30 min. Then, dialysate was collected for 90 min with Ringer's solution. For the rescue with DL-TBOA, after the collection of baseline fractions, 10 nmol of DL-TBOA dissolved in 10 μl of Ringer's solution was injected through the probe through the microinjection tube for 30 min.

2.7. Preparation of brain slice and staining

Histological procedures were performed as previously described with a minor modification [34]. Mice were anesthetized with chloral hydrate (200 mg/kg i.p.) and perfused transcardially with ice-cold phosphate-buffered saline (PBS), followed by 4% paraformaldehyde in PBS and then soaked in 10–30% (w/v) sucrose in PBS. Coronal sections 20 μm thick were cut with a cryostat (CM 1850; Leica, Germany). Cresyl violet staining was performed and the areas of brains and ventricles, and the number of neurons with a visible nucleus in the layers II/III of the prelimbic area were counted using computer-based image analysis system (WinRoof, Mitani, Japan). Images were acquired with a microscope (BZ-9000, Keyence, JP).

2.8. Western blotting analysis

Western blotting was performed as described previously with a minor modification [34]. The PFC including the cingulate and prelimbic area (Bregma +2.96 to Bregma +1.34) was rapidly dissected out, frozen, and stored at −80 °C prior to assays. The brain samples were homogenized in ice-cold buffer (50 mM Tris–HCl, pH 7.5; 150 mM NaCl; 10 mM NaF; 10 mM EDTA, 1% NP-40; 1 mM sodium orthovanadate; 10 mM sodium pyrophosphate; 0.5 mM DTT; 0.2 mM PMSF; 4 μg/ml pepstatin, 4 μg/ml aprotinin, and 4 μg/ml leupeptin). The lysate was centrifuged at 8000 × *g* for 10 min at 4 °C, and the suspension of precipitate was used.

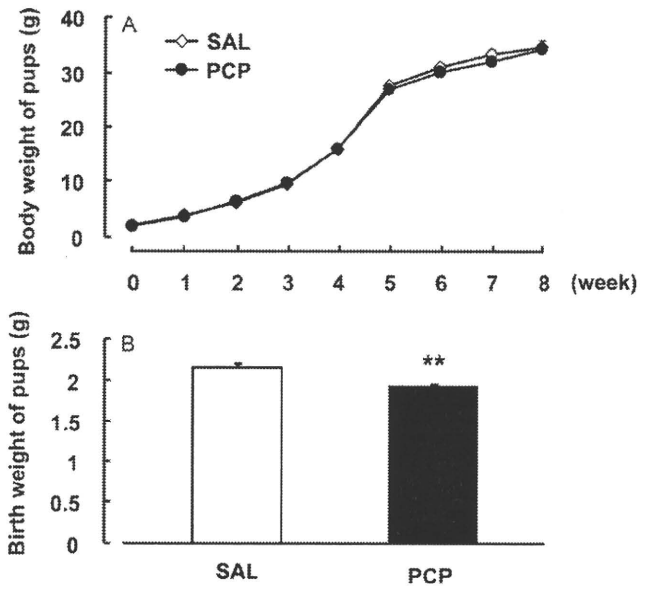

Fig. 1. Effects of prenatal PCP treatment on body weight of pups. The body weight of pups during the developing period from birth to 8 weeks old (A) (repeated one-way ANOVA with Bonferroni's test); the birth weight of pups at PD1 (B) (Student's *t*-test). $^{**}P<0.01$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M for 36–38 mice. SAL, saline; PCP, phencyclidine.

The protein concentrations were determined using a Pierce BCA Protein Assay Kit (Thermo Fishers, CA, USA). Samples were boiled at 95 °C for 5 min in sample buffer (125 mM Tris–HCl, pH 6.8, 10% 2-mercaptoethanol, 4% sodium diphosphate decahydrate, 10% sucrose and 0.0004% bromophenol blue), separated on a polyacrylamide gel, and transferred to polyvinylidene difluoride membranes (Millipore Corporation, MA, USA). The membranes were blocked with a Detector Block Kit (Kirkegaard and Perry Laboratories, MD, USA) and probed with primary anti-GLAST, anti-GLT-1 (1:1000; Upstate Biotechnology, NY, USA), and anti-GFAP (1:1000; Upstate Biotechnology) antibodies. Membranes were washed with the washing buffer (50 mM Tris–HCl, pH 7.4, 0.05% Tween 20, and 150 mM NaCl) and subsequently incubated with a secondary horseradish peroxidase-linked antibody (Kirkegaard and Perry Laboratories). Immunoreactive complexes on the membrane were detected using Western blotting detection reagents (GE Healthcare Biosciences, NJ, USA). The intensity of bands was detected by densitometry using ChemiDoc system and Quantity One Software (Bio-Rad, Hercules, CA, USA). After the GLAST, GLT-1 and GFAP proteins were detected, membranes were stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 6.7) at 50 °C for 30 min, and the expression of β-actin was detected with a primary anti-actin antibody (1:1000; Santa Cruz Biotechnology, CA, USA) as described above.

2.9. Statistical analysis

All data were expressed as the mean ± S.E.M. Significant differences between two groups were determined with Student's *t*-test. That among three groups or more was determined using a one- or two-way analysis of variance (ANOVA), or repeated one- or two way ANOVA, respectively, followed by Bonferroni's test ($P<0.05$).

3. Results

3.1. Effect of prenatal PCP treatment on body weight during development

To confirm the effects of prenatal PCP administration on development, the body weight of pups was observed throughout the development. As shown in Fig. 1, there were no significant differences between SAL- or PCP-treated offspring from birth to the age of 8 weeks old, when the behavioral tests were carried out ($F_{group\,(1,576)}=1.58$, $P>0.05$; $F_{week\,(8,576)}=2584.08$, $P<0.01$; $F_{group\times week\,(8,576)}=1.24$, $P>0.05$; repeated one-way ANOVA; Fig. 1A). These results suggested that prenatal PCP treatment did not affect the growth of body weight in pups, although there is a significant decrease in the weight at birth (SAL-treated mice, 2.15 ± 0.04 g; PCP-treated mice, 1.93 ± 0.03 g; $P<0.01$, $n=36$–38; Student's *t*-test; Fig. 1B).

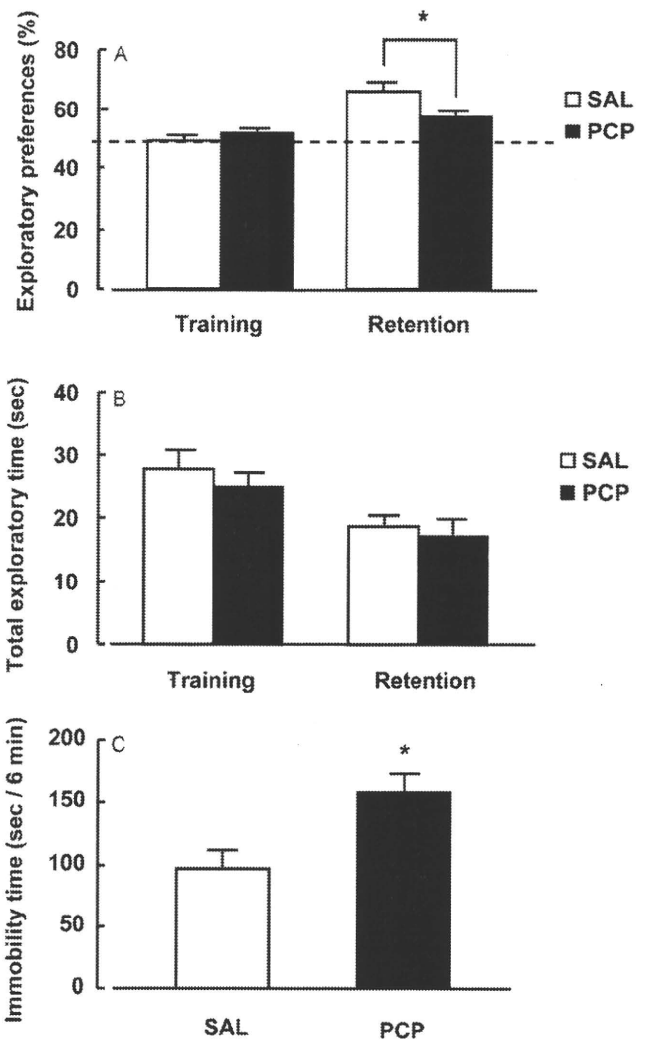

Fig. 2. Influences of prenatal PCP treatment on cognitive and emotional behavior in offspring at 8 weeks old. Exploratory preference (%) (A); and total time spent exploring the objects (s) (B) in training and retention sessions of the novel object recognition test. In the forced swimming test, immobility time (s) was assessed for 6 min (C). $^{*}P<0.05$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M. for 10–14 mice in each group (Student's *t*-test). SAL, saline; PCP, phencyclidine.

3.2. Abnormal cognitive and emotional behavior in prenatal PCP-treated mice

To investigate effects of prenatal PCP treatment on cognitive function, we performed a novel object recognition test. In the training session, the prenatal SAL- or PCP-treated mice spent almost equal amounts of time exploring either of the two objects, and there was no biased exploratory preference in each group of mice (SAL-treated mice, 49.05 ± 1.82%; PCP-treated mice, 51.76 ± 1.49%; $P>0.05$, $n=10$–14; Student's *t*-test; Fig. 2A). In addition, the total time spent on the exploration of objects in the training session did not differ between the two groups (SAL-treated mice, 27.65 ± 3.05 s; PCP-treated mice, 24.89 ± 2.38 s; $P>0.05$, $n=10$–14; Student's *t*-test; Fig. 2B). In the retention session, the PCP-treated mice showed a reduced level of exploratory preference for the novel objects compared with the SAL-treated group (SAL-treated mice, 65.95 ± 3.14%; PCP-treated mice, 57.20 ± 2.33%; $P<0.05$,

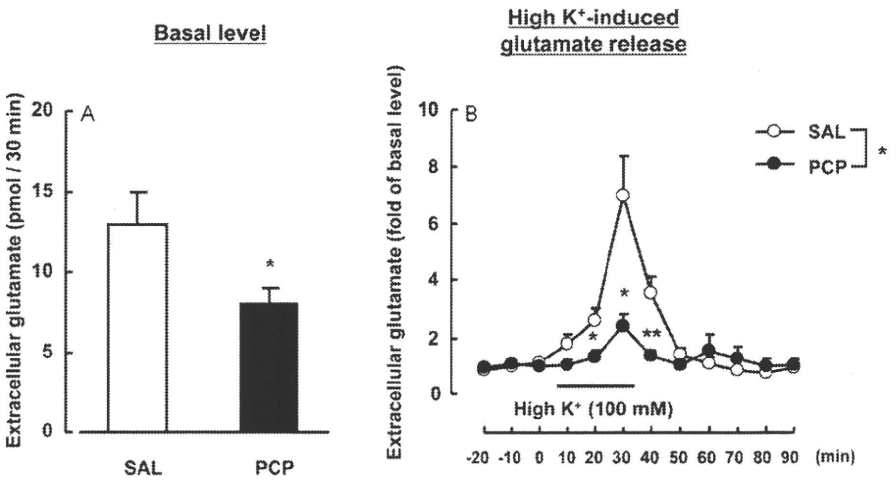

Fig. 3. Basal extracellular glutamate levels and high K^+-evoked glutamate release in the prefrontal cortex of prenatal PCP-treated mice. Basal extracellular levels of glutamate (A) and high K^+ (100 mM)-evoked glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined by microdialysis. Data are expressed as the mean ± S.E.M. for 7 mice in each group. $^*P<0.05$, $^{**}P<0.01$ compared with the prenatal SAL-treated group (Student's *t*-test or repeated one-way ANOVA with Bonferroni's test).

$n = 10–14$; Student's *t*-test; Fig. 2A). However, no significant difference was observed in the total exploration time (SAL-treated mice, 18.74 ± 1.72 s; PCP-treated mice, 17.08 ± 2.97 s; $P > 0.05$, $n = 10–14$; Student's *t*-test; Fig. 2B).

To investigate effects of prenatal exposure to PCP on emotional behavior, we performed the forced swimming test. The PCP-treated mice showed significantly prolonged immobility throughout the 6-min test, compared with the SAL-treated mice (SAL-treated mice, 96.8 ± 13.48 s; PCP-treated mice, 157.47 ± 15.69 s; $P < 0.05$, $n = 10–14$; Student's *t*-test; Fig. 2C), which implied that emotional deficits were induced by the prenatal exposure.

3.3. Reduced glutamate release in the prefrontal cortex of prenatal PCP-treated mice

To investigate whether pre-synaptic glutamatergic neurotransmission was adversely affected by prenatal exposure to PCP, we examined extracellular glutamate levels in the PFC using microdialysis. When the extracellular levels reached a steady state without any treatment, the basal release of glutamate was monitored for 30 min. The prenatal PCP-treated mice showed a dramatically reduced level of extracellular glutamate in the PFC, compared with the SAL-treated mice (SAL-treated mice, 12.97 ± 1.96 pmol; PCP-treated mice, 8.06 ± 0.95 pmol; $P < 0.05$, $n = 7$; Student's *t*-test; Fig. 3A). Next, we evaluated the glutamate release induced by high potassium (high K^+, 100 mM). The high K^+ treatment increased the release of extracellular glutamate in both the SAL- and PCP-treated mice. However, the increase was significantly lower in the PCP-treated mice ($F_{\text{treatment}(1,12)} = 20.63$, $P < 0.01$; $F_{\text{time}(4,12)} = 11.11$, $P < 0.01$; $F_{\text{treatment} \times \text{time}(4,12)} = 3.93$, $P < 0.01$; repeated one-way ANOVA for 10–50 min; Fig. 3B).

3.4. Overexpression of glutamate transporters in the prefrontal cortex of prenatal PCP-treated mice

To further examine whether the decrease in the extracellular glutamate level was due to changes to glutamate transporters, we investigated the protein expression of GLAST and GLT-1 in the PFC by Western blotting. The level of GLAST protein was significantly higher in the PCP-treated mice than SAL-treated mice (SAL-treated mice, 100 ± 10.24%; PCP-treated mice, 135.18 ± 9.98%; $P < 0.05$, $n = 6–7$; Student's *t*-test; Fig. 4A). However, we did not observe a significant difference in GLT-1 expression between the groups (SAL-treated mice, 100 ± 12.49%; PCP-treated mice, 121.98 ± 12.29%; $P > 0.05$, $n = 6–7$; Student's *t*-test; Fig. 4B). We further examined the level of GFAP expression, a marker of glial cell, by Western blotting. There was no significant difference in GFAP expression between the prenatal SAL- and PCP-treated mice (SAL-treated mice, 100 ± 5.19%; PCP-treated mice, 104.38 ± 6.72%; $P > 0.05$, $n = 6–7$; Student's *t*-test; Fig. 4C), suggesting that the increased expression of GLAST was not directly due to increasing numbers of glial cells in the PFC of mice.

3.5. The neuronal number in the prefrontal cortex of prenatal-PCP treated mice

To further investigate whether the reduced glutamate release was due to the decrease of number of neurons in adult mice, we examined morphological changes of neurons in the PFC by Cresyl violet staining. As shown in the results, there was only a tendency to decrease in the number of neurons in the layer II/III of the PFC at PD 56, but not significant difference regardless of prenatal PCP treatment (SAL-treated mice, 2247 ± 117; PCP-treated mice, 1925 ± 116; $P = 0.08$, $n = 6$; Student's *t*-test; Supplementary Fig. 1A and B). It suggested that the decrease in extracellular glutamate level might be not due to the changes of number of neurons in adult.

3.6. Effects of a potent glutamate transporter inhibitor on behavioral deficits and the impairment of glutamate release in prenatal PCP-treated mice

To further investigate whether the prenatal PCP-induced behavioral changes and glutamatergic dysfunction were associated with the overexpression of glutamate transporters, we examined the effects of a potent inhibitor of glutamate transporters, DL-TBOA. In the PCP-treated mice, DL-TBOA (10 nmol) attenuated the impairment of recognition memory in the novel recognition test. There was no biased exploratory preference ($F_{\text{group}(1,40)} = 0.17$, $P > 0.05$; $F_{\text{treatment}(2,40)} = 1.29$, $P > 0.05$; $F_{\text{group} \times \text{treatment}(2,40)} = 0.16$, $P > 0.05$; two-way ANOVA; Fig. 5A), and no difference in total exploration time between the two groups in the training session ($F_{\text{group}(1,40)} = 0.61$, $P > 0.05$; $F_{\text{treatment}(2,40)} = 2.71$, $P > 0.05$; $F_{\text{group} \times \text{treatment}(2,40)} = 0.57$, $P > 0.05$; two-way ANOVA; Fig. 5B). In the retention session, the impairment of recognition memory in the PCP-treated mice was significantly improved by the

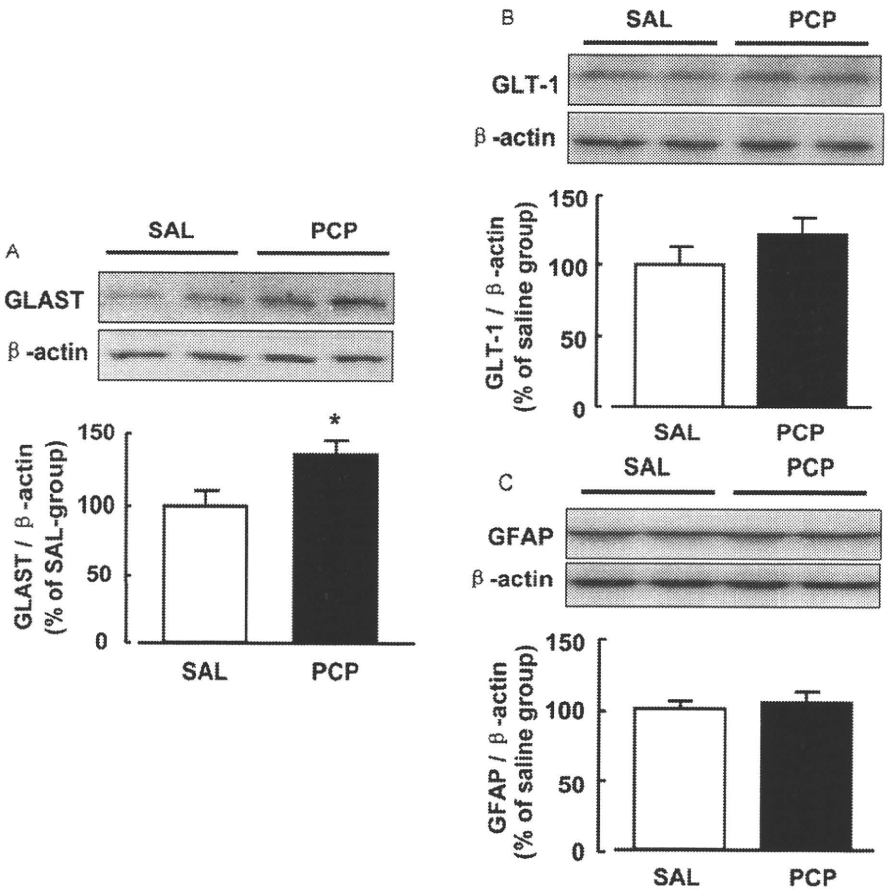

Fig. 4. Changes in the expression of glutamate transporters in the prefrontal cortex of prenatal PCP-treated mice. Representative Western blots band for the expression of GLAST, GLT-1 and GFAP. The amount of protein (30 μg/well) loaded was normalized to that of β-actin. Results are represented as the level of GLAST (A), and GLT-1 (B), as well as GFAP (C) in the prefrontal cortex. *$P < 0.05$ compared with the prenatal SAL-treated group. Data are expressed as the mean ± S.E.M. for 6–7 mice in each group (Student's *t*-test). SAL, saline; PCP, phencyclidine.

higher dose of DL-TBOA (10 nmol) ($F_{group\,(1,40)} = 24.66$, $P < 0.01$; $F_{treatment\,(2,40)} = 3.96$, $P < 0.05$; $F_{group \times treatment\,(2,40)} = 6.51$, two-way ANOVA; $P < 0.01$, Fig. 5C). However, there were no significant differences in total exploration time in the retention session among each group ($F_{group\,(1,40)} = 0.14$, $P > 0.05$; $F_{treatment\,(2,40)} = 0.02$, $P > 0.05$; $F_{group \times treatment\,(2,40)} = 1.27$ $P > 0.05$; two-way ANOVA; Fig. 5D). These results suggested that DL-TBOA did not affect motivation or curiosity, but ameliorated the impairment of recognition memory in the prenatal PCP-treated mice.

Next, we evaluated the effects of DL-TBOA on the prolonged immobility time in the forced swimming test in the PCP-treated mice. DL-TBOA (10 nmol) significantly reversed the prolonged immobility induced by prenatal PCP exposure in the forced swimming test ($F_{group\,(1,40)} = 18.03$, $P < 0.01$; $F_{treatment\,(2,40)} = 7.42$, $P < 0.01$; $F_{group \times treatment\,(2,40)} = 3.10$, $P = 0.06$; two-way ANOVA; Fig. 5E), but did not change immobility time in the SAL-treated mice. These results indicated DL-TBOA to be effective in correcting emotional abnormalities induced by prenatal exposure to PCP without affecting motility.

Furthermore, we continued to evaluate the effects of DL-TBOA on the reduced extracellular glutamate level and impairment of K^+-induced glutamate release induced by prenatal PCP treatment. After the basal levels of glutamate reached a steady state, DL-TBOA was given through the probe for dialysis and the basal release of glutamate was monitored for 90 min. DL-TBOA tended to normalize the level of extracellular glutamate in the prenatal PCP-treated mice ($F_{(2,18)} = 2.60$, $P = 0.10$; one-way ANOVA; Fig. 6A). Moreover, it clearly improved the high K^+ (100 mM)-induced glutamate release reduced by prenatal exposure to PCP ($F_{group\,(2,18)} = 9.09$, $P < 0.01$; $F_{time\,(4,18)} = 22.45$, $P < 0.01$; $F_{group \times time\,(8,18)} = 2.43$, $P < 0.05$; repeated two-way ANOVA for 10–50 min; Fig. 6B). These results indicated that DL-TBOA reversed the reduction in glutamatergic neurotransmission observed in the prenatal PCP-treated mice.

4. Discussion

The blockade of NMDA receptors by PCP in the developing brain has been found to impair learning and memory. For instance, prenatal exposure to PCP disrupts passive avoidance and pole-climbing avoidance responses [36], and impairs performance in the eight-arm maze and Morris water maze in adult rats [2,51]. In the present study, prenatal PCP treatment produced an impairment of memory in the novel object recognition test, consistently suggested a cognitive deficit in this model. Furthermore, the prenatal PCP-treated mice showed a prolonged immobility in the forced swimming test, which are frequently observed in PCP animal models displaying schizophrenia-like negative symptom [38]. Taken together, these results indicate that the blockade of NMDA receptors by PCP in the prenatal period triggers cognitive and emotional abnormalities in postpubertal mice.

Glutamate neurotransmission plays a critical role in synaptic activity and plasticity throughout the brain, including cognition-, emotion- and reward-related circuits [31]. In schizophrenic patients, evidence of abnormal glutamatergic transmission has been found, such as disturbances of cortical glutamate release

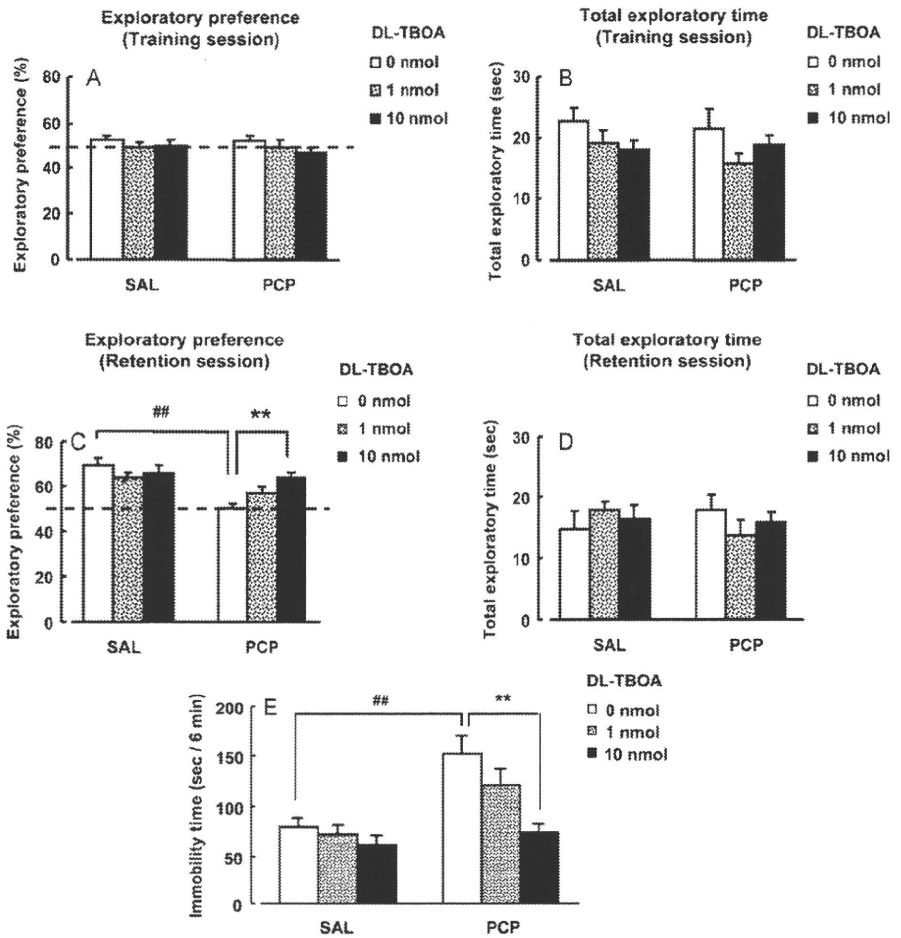

Fig. 5. Effects of DL-TBOA on the behavior in the prenatal PCP-treated mice. DL-TBOA (1 and 10 nmol) was administered by microinjection directly into the prefrontal cortex 30 min before each behavioral test. Exploratory preference (%) in the training session (A) and retention session (C). Total exploration time (s) in the training session (B) and retention session (D) of the novel object recognition test. Immobility time (s) was assessed for 6 min in the forced swimming test (E). Data are expressed as the mean ± S.E.M. for 7–8 mice in each group. $^{\#\#}P<0.01$ compared with the prenatal SAL-treated group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the prenatal PCP-treated group (two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

[7,8,14,24], lower glutamate levels in the PFC [46], and decreased levels of glutamate in cerebrospinal fluid [19], as well as reduced glutamatergic tone in the cortex area [16]. In PCP-treated adult mice, a decrease in spontaneous extracellular glutamate release [34] and in the level of phosphorylated-NR1 [33,34], but an increase in levels of GLAST expression has been observed in the PFC [34]. In the present study, we found a decrease in both the extracellular glutamate concentration and high K^+-induced release of glutamate in the PFC of the PCP-treated mice compared with the SAL-treated mice, suggesting that prenatal exposure to PCP produced a pre-

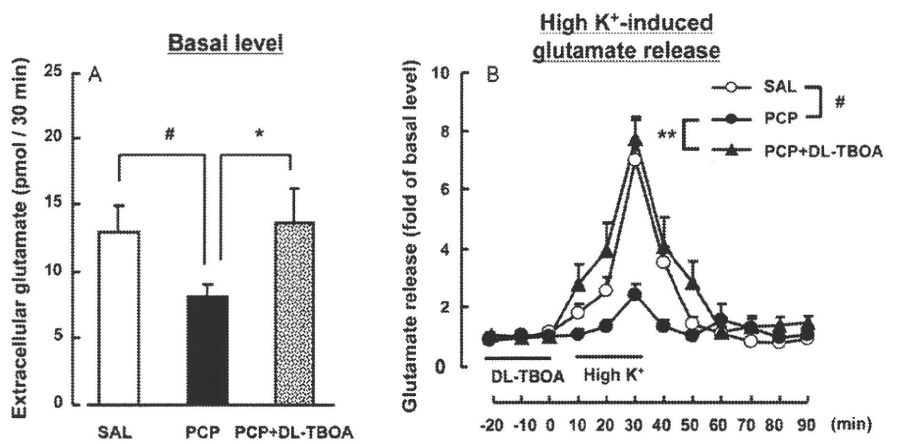

Fig. 6. Effects of DL-TBOA on the reduction of glutamate release in the prenatal PCP-treated mice. DL-TBOA (1 mM) was administered through a micodialysis tube into the prefrontal cortex of mice for 30 min (μl/min). After the administration, basal glutamate release (A) and K^+-evoked (100 mM) glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined. Data are expressed as the mean ± S.E.M. for 7 mice in each group. $^{\#}P<0.05$ compared with the prenatal SAL-treated group; $^{*}P<0.05$, $^{**}P<0.01$ compared with the prenatal PCP-treated group (one-way ANOVA or repeated two-way ANOVA with Bonferroni's test). SAL, saline; PCP, phencyclidine.

synaptic hypofunctional glutamatergic neurotransmission. Given that glutamate neurotransmission plays a critical role in synaptic plasticity and long-term potentiation [31], it is possible that the hypofunctional glutamatergic neurotransmission was associated with these behavioral deficits induced by prenatal PCP treatment.

Glutamate transporters, GLAST and GLT-1, are considered to regulate glutamate transmission by rapidly clearing glutamate from extracellular fluid [11]. It is suggested that an increase in GLAST expression contributes to a decrease in extracellular glutamate release, because the regulation of extracellular glutamate levels by membrane transporters is important for terminating synaptic transmission in the brain [43]. Furthermore, increased numbers of binding sites and protein expression of glutamate transporters have been observed in the postmortem brain of schizophrenia patients [9,11,19,37,42]. Additionally, antipsychotics such as clozapine inhibites the increase in glutamate transporters [29,48]. In this study, we observed the overexpression of GLAST protein in the prenatal PCP-treated mice, although no significant change in GLT-1 expression. GLAST is produced during embryogenesis in rodents, while GLT-1 is expressed in the forebrain postnatally [47]. Therefore, prenatal exposure to PCP might lead to different patterns of GLAST and GLT-1 expression. Furthermore, we found that DL-TBOA, a potent glutamate transporter blocker, attenuated the cognitive and emotional deficits by normalizing the extracellular release of glutamate. These results suggested that the elevated expression of GLAST protein was, at least in part, responsible for the dysfunctional glutamate transmission associated with these behavioral changes in the prenatal PCP-treated mice.

GLAST proteins are expressed in glial cells of the adult brain and spinal cord [23,41]. However, in this study, we failed to detect a significant change in the expression of GFAP, a marker of glial cells. Thus, it is unlikely that the increase in GLAST expression is due to the activation of glial cells. It was reported that Ca^{2+} influx inhibits GLAST expression in astrocytes [26]. Since PCP inhibited the influx of Ca^{2+} by blocking the NMDA receptor, one possibility is that the disruption of Ca^{2+} influx into astrocytes is associated with the up-regulation of GLAST expression. Furthermore, any factors which enhance the gene transcription of GLAST or disrupt the protein's degradation might also contribute to the up-regulated expression. The precise mechanism remains to be elucidated.

The NMDA receptors are thought to control the differentiation and migration of immature neurons [4,20]. Many neurons undergo a stage when they are critically dependent on stimulation by glutamate through the NMDA receptors, and sustained deprivation of this input by NMDA receptor antagonists during development activates apoptosis [17]. Therefore, we could not exclude the possibility that a loss of glutamatergic neurons contributes to the glutamatergic hypofunction, although here, we did not observe any significant decrease in the total number of neurons in adult mice. Thus, any neurodevelopmental disturbances caused by prenatal exposure to PCP in development are potentially implicated in these behavioral and biochemical changes.

5. Conclusion

The present findings indicate that prenatal exposure to PCP leads to cognitive impairment and emotional dysfunction, which are accompanied by a disruption to pre-synaptic glutamate neurotransmission through the enhanced expression of glutamate transporters in the PFC. Since the abnormal glutamatergic release and the altered expression of glutamate transporters are involved in the pathophysiology of schizophrenia, this study provides further insights into how psychiatric illnesses develop.

Conflicts of interest

The authors state no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbr.2011.01.035.

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Irradiation in Adulthood as a New Model of Schizophrenia

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Abstract

Background: Epidemiological studies suggest that radiation exposure may be a potential risk factor for schizophrenia in adult humans. Here, we investigated whether adult irradiation in rats caused behavioral abnormalities relevant to schizophrenia.

Methodology/Principal Findings: A total dose of 15-Gy irradiation in six fractionations during 3 weeks was exposed to the forebrain including the subventricular zone (SVZ) and subgranular zone (SGZ) with male rats in the prone position. Behavioral, immunohistochemical, and neurochemical studies were performed three months after fractionated ionizing irradiation. Three months after fractionated ionizing irradiation, the total numbers of BrdU-positive cells in both the SVZ and SGZ zones of irradiated rats were significantly lower than those of control (sham-irradiated) rats. Hyperactivity after administration of the dopaminergic agonist methamphetamine, but not the N-methyl-D-aspartate (NMDA) receptor antagonist dizocilpine, was significantly enhanced in the irradiated rats although spontaneous locomotion in the irradiated rats was significantly lower than that of controls. Behavioral abnormalities including auditory sensory gating deficits, social interaction deficits, and working memory deficits were observed in the irradiated rats.

Conclusion/Significance: The present study suggests that irradiation in adulthood caused behavioral abnormalities relevant to schizophrenia, and that reduction of adult neurogenesis by irradiation may be associated with schizophrenia-like behaviors in rats.

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Introduction

Schizophrenia is a heterogeneous and multifactorial disease with complex interactions between genetic liability and environmental factors. A number of epidemiological studies have proposed perinatal events with potential harmful neurodevelopmental impacts as major environmental risk factors [1–4], but few studies have revealed risk factors in adulthood. Interestingly, some epidemiological studies suggest that exposure to ionizing radiation may be a risk factor for schizophrenia in adult humans [5]. First, a higher prevalence (6%) for schizophrenia was reported in the atomic bomb survivors in Nagasaki, Japan [6]. Second, four years after the Chernobyl accident in 1986, the incidence of schizophrenia in the exclusion zone was significantly higher than that in the general population (5.4 per 10,000 in the exclusion zone versus 1.1 per 10,000 in the Ukraine in 1990) [7]. Third, the incidence for schizophrenia was shown to be high in people living in the region of the Semipalatinsk nuclear weapon testing area in Kazakhstan: 29% of all registered mental patients residing in the area were suffering from schizophrenia and among those, 42.3% were born before the first nuclear test explosions [5]. Furthermore, the incidence for schizophrenia has also been shown to be high in rural areas in India that have high natural background radiation [5]. Taken together, the findings suggest that ionizing radiation may be an environmental trigger that can actualize a predisposition to schizophrenia or indeed cause schizophrenia-like disorders [5].

In both pediatric and adult patients, cranial radiation therapy causes debilitating cognitive deficits that are poorly understood [8]. However, accumulating evidence suggests that radiation-induced cognitive deficits in animals may be associated with a decrease in hippocampal proliferation and a decrease in adult

neurogenesis [9–15]. Interestingly, Reif et al. [16] reported a reduction in the proliferation of hippocampal neural stem cells in the postmortem brains of schizophrenic patients. Therefore, it is likely that adult neurogenesis plays an important role in the pathophysiology of psychiatric diseases including schizophrenia [17]. Given the role of neurogenesis in radiation-induced cognitive deficits, we hypothesized that reduction of adult neurogenesis by irradiation may be implicated in the pathophysiology of schizophrenia in adulthood. The present study was, therefore, undertaken to examine whether irradiation in adult rats causes behavioral abnormalities relevant to schizophrenia.

Methods

Animals

Adult male Sprague-Dawley rats (Japan SLC, Hamamatsu, Japan), aged 8 weeks and weighing 280–300 g, were housed in groups of three animals per cage under standard conditions (22±0.5°C, 12:12 light-dark cycle, lights on at 7:00 AM). All procedures were approved by the Guide for Animal Experimentation of the Hamamatsu University School of Medicine and Chiba University Graduate School of Medicine. Irradiated group and control group were 148 and 144 rats, respectively. All analyses were performed three months after the last irradiation. Six rats from both groups were used for neurotransmitter quantification, and eight rats were used for cell counting. Nocturnal activity, methamphetamine-treated response, and dizocilpine-treated response were measured using 17, 17 and 18 pairs of rats, respectively. Thirty-five irradiated rats and 33 control rats were used for cognitive function tests of social interaction (6 rats each), eight-arm radial maze (17 irradiated and 15 control rats), and Morris water maze (12 rats each). Prepulse inhibition (PPI) test was analysed in 23 irradiated and 21 control rats. Twelve rats for each group were used for analysis of clozapine effect on PPI deficits.

Fractionated ionizing irradiation

The irradiation was done with a Stabilipan 2 (Siemens) therapeutic unit (150 kV and 20 mA). A total dose of 15-Gy irradiation in six fractionations during 3 weeks was exposed to the forebrain including the subventricular zone (SVZ) and subgranular zone (SGZ) with rats in the prone position. The other parts of the head and whole body were protected by a lead shield. Sham-irradiation controls underwent the same procedures as the experimental animals, but did not receive irradiation.

Immunohistochemistry and stereological analysis

Twenty-four hours after intraperitoneal injection of BrdU (100 mg/kg; Sigma-Aldrich Japan Inc., Tokyo, Japan), brains were fixed with 4% paraformaldehyde. They were coronally sectioned at 30 μm, and eight-section series were collected. Serial sections were stained with mouse monoclonal anti-BrdU antibody (0.6 μg/mL; Becton Dickinson Immunocytometry Systems, CA, USA) and biotinylated horse-anti-mouse IgG (1:160; Vector Lab. Inc., CA, USA). The signal was visualized using an ABC kit (Vector Lab. Inc., CA, USA) and 3, 3′-diaminobenzidine (Sigma-Aldrich Japan Inc., Tokyo, Japan). Other series were stained with Cresyl Violet for counting granule cells. The numbers of BrdU-labeled nuclei in the SVZ and SGZ of the dentate gyrus, and granule cells in the dentate gyrus were evaluated with Stereo Investigator (version 6, MicroBrightField Japan, Inc., Chiba, Japan). The SVZ estimates were made from two sections each anterior and posterior to the decussation of the corpus callosum (Bregma 1.60 mm). SGZ and granule cell layer estimates were made from an 8-section series between the top and end of the hippocampus. The volumes of each portion were estimated using Cavalieri's principle [18].

Measurement of dopamine, DOPAC and amino acids

Dopamine and its major metabolite DOPAC in rat brain sample were measured by high-performance liquid chromatography (HPLC) coupled with electrochemical detection (Eicom Co., Ltd., Kyoto, Japan) as reported previously [19]. Amino acids (glutamine, glycine, glutamate, D-serine, L-serine) in rat brain samples were measured by column-switching HPLC (Shimadzu Co., Ltd., Kyoto, Japan) as reported previously [20].

Psychostimulant-induced hyperlocomotion

Spontaneous nocturnal locomotor activity was measured for six hours in the middle of dark phase (21:00–3:00). Abnormalities in dopaminergic neurotransmission were tested by hyperlocomotion induced by the psychostimulant methamphetamine. Locomotor activity was monitored under an infrared ray passive sensor system (SCANET-SV20, Melquest Ltd., Toyama, Japan). After a 30-minute acclimation period, rats were intraperitonealy (i.p.) injected with methamphetamine (2.0 mg/kg, Dainippon Pharmaceuticals Ltd, Osaka, Japan) or dizocilpine ((+)-MK-801; 0.03 mg/kg, Sigma-Aldrich, St Louis, MO), and horizontal locomotor activities were measured for 2 hours.

Social interaction

Social interaction was tested in a wooden arena (90×90×30 cm high) placed in a dimly lit room. Each rat was tested for 10 min with a weight-matched partner that had a similar treatment condition but was from a different home cage. Social interaction was assessed by the time spent interacting, including sniffing, following, crawling over or under, grooming, and aggressive behaviors.

Eight-arm radial maze

Spatial working memory was analyzed with an automated eight-arm radial maze system in a manner similar to that described previously [21]. Rats were placed on the central platform and allowed to get all eight pellets within 10 min. The rats went through 1 trial per day. When a rat could take seven pellets within 1 error for five consecutive days, the rat was administrated 10 daily sessions for working memory assessment. A 30-sec delay was initiated after four pellets had been taken by confining the rats in the center with a shutter. After opening the shutter, the rat was allowed to get the remaining 4 pellets. The number of revisits to arms from which pellets had already been taken was used as the working memory error. Data acquisition and control of shutter were performed using Image RM 2.00 (O'Hara & Co., Ltd. Tokyo, Japan), modified NIH Image program (available at http://rsb.info.nih.gov/nih-image/).

Morris water maze

Spatial reference memory was assessed using the Morris water maze (180 cm in diameter circular pool). A submerged translucent platform was fixed in the center of a quadrant (north). Training sessions consisted of placing the rat into the water maze at one of three randomly chosen start positions (south, east, west) and allowing it to swim to the platform for 60 sec. On the next day, after rats were trained for 5 days with four trials per day, the platform was moved to the opposite quadrant (south). A probe trial was carried out after four trials identical to the training sessions. The platform was removed and rats were allowed to swim freely for 60 sec. The time spent in the quadrant where the platform has