temporal psychosis in normal human subjects and to provoke schizophrenia-like symptoms (Abi-Saab *et al.* 1998). It has been suggested that the impairments of sociality and cognitive functions induced by chronic PCP treatment may be mediated by NMDA receptor dysfunction (Mouri *et al.* 2007*b*; Qiao *et al.* 2001). Indeed, repeated-dose PCP treatment has been shown to disrupt activation of CaMKII mediated by NMDA receptors, and the impairment of latent learning and emotional behaviour in PCP-treated mice has been shown to be attributable to dysfunctional NMDA–CaMKII signalling (Mouri *et al.* 2007*b*; Murai *et al.* 2007). The NMDA receptor plays a crucial role in synaptic plasticity and induction of long-term potentiation in many regions of the brain, including the PFC (Bliss & Collingridge, 1993; Zhao *et al.* 2005). Exposure to an EE was shown to enhance cortical plasticity (Duffy *et al.* 2001) and to increase NR2A and NR2B NMDA receptor subunit expression in the forebrain (Tang *et al.* 2001). The cellular mechanism of NMDA receptor-mediated enhancement of cortical plasticity was shown to be dependent on the NR2B:NR2A ratio in the PFC (Zhao *et al.* 2005). Therefore, our finding that EE exposure prevented impairments of social behaviour and recognition memory could be explained by an enhancement of the glutamatergic activities through transcriptional activation in the EE-exposed animals.

The EE-exposed mice in our study did display hypersensitivity to acute PCP treatment. However, decreased sensitivity to NMDA receptor antagonists has been reported in NR1 (Belforte *et al.* 2010) and GluR1 knockout mice (Wiedholz *et al.* 2008), which exhibit hypoglutamatergic neurotransmission. Increases in GluR1, NR2A and NR2B expressions were observed 3 h after the start of exposure to an EE, to reach substantial levels by 2 wk (Tang *et al.* 2001), indicating that glutamatergic activity is enhanced by exposure to an EE. Thus, the hypersensitivity of the locomotor-stimulant response to PCP in the EE-exposed mice could be caused by facilitation of the glutamatergic activity. Although SB treatment also potentiated locomotor activity induced by PCP treatment, the degree of increase of the locomotor activity in the EE-exposed mice (about two-fold higher compared to that in the SE-exposed mice) was higher than that in the SB-treated mice (about 1.25-fold higher compared to that in the Sal-treated mice). EE exposure affects not only transcriptional activation, such as histone acetylation, but also release of neurotransmitters (Segovia *et al.* 2009). Consistent with this notion, EE-exposed mice have been shown to exhibit elevated extracellular dopamine levels and enhanced amphetamine-induced dopamine release in the nucleus accumbens (Segovia *et al.* 2010). The enhancement of hyperlocomotion in the EE-exposed mice might also arise from the effect of EE exposure on the release of neurotransmitters, such as dopamine, in addition to its effect on histone modification.

Chromatin remodelling has been implicated in the development of several chronic psychiatric conditions, as a potential mechanism underlying long-lasting changes in gene expressions and behaviour caused by environmental stimuli (Tsankova *et al.* 2007). In schizophrenia subjects, the amount of histone H3 acetylated at Lys^9 in lymphocyte cultures is decreased (Gavin *et al.* 2008). Administration of valproic acid, which has an inhibitory effect on HDAC, produces significantly smaller increases in the amount of acetylated histone H3 in schizophrenia subjects (Sharma *et al.* 2006). These results suggest that schizophrenia is associated with 'rigid' chromatin associated with the decrease in the acetylation of Lys^9 of histone H3. We demonstrated that chronic PCP treatment decreased the amount of acetylated histone H3 in the PFC, which was blocked by EE exposure during adolescence. Our findings suggest that chronic PCP-treated animals have epigenetic abnormalities similar to those in schizophrenia subjects and that EE could prevent these epigenetic changes.

In the chromatin remodelling, HDACs repress transcription by deacetylating nucleosomal histones and other components of the transcriptional machinery. HDAC5 and histone acetylation have been suggested to have important roles in the development of psychiatric disorders and in the execution of fundamental brain functions (Renthal *et al.* 2007; Tsankova *et al.* 2006). HDAC5 decreases the levels of histone H3 acetylated at Lys^9 in the mouse brain (Tsankova *et al.* 2006). We demonstrated that HDAC5, but not HDAC1 expression, was increased in the nuclear fraction of the PFC of PCP-treated mice, which could be prevented by EE exposure during adolescence. Furthermore, administration of SB, an HDAC inhibitor, during adolescence also prevented PCP-induced behavioural abnormalities. It is suggested that these changes in HDAC5 expression may contribute to the changes in the acetylated histone H3 levels and be associated with behavioural changes.

Activation of HDAC5 is regulated through phosphorylation via neural activity-dependent mechanisms (Chawla *et al.* 2003). Activation of synaptic NMDA receptors induces translocation of HDAC5 to the cytoplasm through its phosphorylation by CaMKII, which is influenced by NMDA receptor

signalling (Renthal *et al.* 2007). Chronic PCP treatment decreases NMDA-stimulated and behaviour-associated phosphorylation of CaMKII in the PFC (Mouri *et al.* 2007*b*; Murai *et al.* 2007). The PCP-induced increase in nuclear HDAC5 expression was attributable to a disruption of NMDA receptor-mediated CaMKII activity and was prevented by EE exposure through the potentiation of glutamatergic activity.

In our study, SB treatment mimicked the effect of EE exposure during adolescence on PCP-induced behavioural and biochemical abnormalities. This result suggests that histone modification during adolescence powerfully affects PCP-induced behavioural changes. SB is a hydroxamate-based HDAC inhibitor and might affect brain function mainly through the inhibition of class I HDACs, including HDAC1, HDAC2, HDAC3 and HDAC8 (Kazantsev & Thompson, 2008), but not HDAC5. Therefore, SB treatment might prevent PCP-induced behavioural abnormalities through inhibition of HDACs except HDAC5. It remains to be clarified whether other members of the HDAC family might also have a role. Further, SB treatment in adolescence prevented not only the deacetylation of Lys^9 of histone H3, but also the increase of HDAC5 expression induced by PCP. Since suppression of the increase of HDAC5 expression induced by PCP treatment was involved in the effect of SB, the mechanism underlying this suppression by SB treatment must be clarified in the future.

In conclusion, our results suggest that EE during adolescence prevents the onset and/or development of schizophrenia through modification of the epigenetic machinery. Raine *et al.* (2003) reported that early enrichment programmes such as nutrition, education and physical exercise enrichment programmes were associated with lower levels of antisocial behaviour and schizotypal personality in adulthood in humans. Our observations support this clinical finding and suggest the involvement of epigenetic changes in the effect of enrichment of the environment, which might therefore serve as a novel prophylaxis strategy against schizophrenia.

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Statement of Interest

None.

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Prenatal NMDA Receptor Antagonism Impaired Proliferation of Neuronal Progenitor, Leading to Fewer Glutamatergic Neurons in the Prefrontal Cortex

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N-methyl-D-aspartate (NMDA) receptor is a glutamate receptor which has an important role on mammalian brain development. We have reported that prenatal treatment with phencyclidine (PCP), a NMDA receptor antagonist, induces long-lasting behavioral deficits and neurochemical changes. However, the mechanism by which the prenatal antagonism of NMDA receptor affects neurodevelopment, resulting in behavioral deficits, has remained unclear. Here, we report that prenatal NMDA receptor antagonism impaired the proliferation of neuronal progenitors, leading to a decrease in the progenitor pool in the ventricular and the subventricular zone. Furthermore, using a PCR array focused on neurogenesis and neuronal stem cells, we evaluated changes in gene expression causing the impairment of neuronal progenitor proliferation and found aberrant gene expression, such as Notch2 and Ntn1, in prenatal PCP-treated mice. Consequently, the density of glutamatergic neurons in the prefrontal cortex was decreased, probably resulting in glutamatergic hypofunction. Prenatal PCP-treated mice displayed behavioral deficits in cognitive memory and sensorimotor gating until adulthood. These findings suggest that NMDA receptors regulate the proliferation and maturation of progenitor cells for glutamatergic neuron during neurodevelopment, probably via the regulation of gene expression.
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Keywords: NMDA receptor; phencyclidine; glutamatergic neuron; neurogenesis; neuronal progenitor; schizophrenia

INTRODUCTION

A variety of amino acids and corresponding receptors, which mediate fast synaptic transmission in the central nervous system (CNS), are present before birth where they are considered to have a role in the morphogenesis of the CNS at different stages, including proliferation, migration, and differentiation. Glutamate is a major amino acid, acting on at least five types of receptors. Of the ionotropic receptors, the *N*-methyl-D-aspartate (NMDA) receptor has critical roles in neurogenesis. The expression of NMDA receptors has been described in certain cell lines and in precursor cells of the developing and adult CNS (Asahi *et al*, 1998). Functional NMDA receptors have been found in radial glia cells (López *et al*, 1997), which act as neuronal progenitors during cortical development (Heins *et al*, 2002). Glutamate has been also reported to regulate the proliferation of progenitor cells derived from the perinatal subventricular zone (SVZ) through both ionotropic and metabotropic receptors, including NMDA receptors (Brazel *et al*, 2005). Moreover, the growth of primary cultures of embryonic hippocampal progenitor cells in proliferative conditions was associated with low levels of NMDA currents (Sah *et al*, 1997).

Phencyclidine (PCP) is a noncompetitive NMDA receptor antagonist. The acute and chronic administration of PCP can induce schizophrenia-like symptoms in both humans and rodents, findings that have greatly contributed to a hypoglutamatergic hypothesis of schizophrenia. Additionally, the administration of PCP during development in rodents induces schizophrenia-like impairments in sensorimotor gating and spatial learning later in life (Wang *et al*, 2001). Previously, we reported that prenatal PCP treatment caused an impairment of cognitive memory, sensitization to PCP, and sensorimotor gating deficits (Lu *et al*, 2010, 2011). Based on the neurodevelopmental hypothesis of schizophrenia, prenatal PCP-treated animals would be a better

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pharmacological model of schizophrenia than adult PCP-treated animals. However, the mechanism by which prenatal NMDA antagonism impairs neurodevelopment, resulting in long-lasting schizophrenia-like deficits, has remained unclear.

In this study, we found that prenatal PCP treatment disturbed gene expression in neuronal progenitors and consequently impaired cell proliferation, causing the density of glutamatergic neurons to decrease in the prefrontal cortex (PFC), an area critical in schizophrenia patients, resulting in schizophrenia-like deficits until adulthood.

SUBJECTS AND METHODS

Animals

Breeder and host ICR wild-type mice were obtained from SLC Japan (Shizuoka, Japan). Noon on the day a vaginal plug was detected embryonic day (E) 0.5. E19.5 was defined as postnatal day (P) 1. After weaning on P28, pups given the same prenatal treatment were mixed by gender and randomly assigned to groups for behavioral tests on P28 and P56. Two or three litters were used in each group, and the test was repeated more than three times to reduce the influence of litters. Moreover, a balanced number of males and females were used in each experiment, because there were no significant differences between genders in this study.

PCP and 5-Bromo-2-Deoxyuridine (BrdU) Administration

PCP hydrochloride was synthesized according to the method of Maddox *et al* (1965) and checked for purity through measurements of its melting point and ultraviolet spectrum. PCP dissolved in saline (SAL) was administered (10 mg/kg/day, s.c.) to pregnant dams from E6.5 to E18.5.

To label neural progenitors cycling in S-phase in E12.5, E13.5, E14.5, or E15.5 embryos, pregnant mice were injected intraperitoneally with 50 mg/kg body weight of BrdU (Sigma) in SAL 3 h after the PCP treatment.

Novel Object Recognition Test

The novel object recognition test was carried out as described previously (Mouri *et al*, 2007) with minor modifications. The test procedure consisted of three sessions: habituation, training, and retention. Each mouse was individually habituated to the box, with 10 min of exploration in the absence of objects each day for 3 consecutive days (days 1–3) (habituation session). On day 4, each animal was allowed to explore for 10 min in the box, in which two novel objects were placed symmetrically. The time spent exploring each object was recorded (training session) The objects were different in shape and color, but similar in size. Animals were considered to be exploring an object when their heads were facing it or when they were sniffing it at a distance of <2 cm and/or touching it with their nose. After the training session, mice were immediately returned to their home cages. On day 5, the animals were placed back into the same box with one of the familiar objects used in the training session and one novel object. Animals were allowed to explore freely for 10 min and the time spent exploring each object was recorded (retention session). An exploratory preference, 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 objects, was used to assess recognition memory.

Forced Swimming Test

Each mouse was placed in a transparent glass cylinder (20 cm high, 15 cm in diameter), which contained water at 22–23 °C to a depth of 15 cm, and was forced to swim for 4 min. We measured the duration of swimming every minute with an infrared detector, SCANET MV-10 AQ (Melquest, Japan), and then calculated 'immobility time' using the score of swimming duration as follows: total time (s)–swimming time (s) = immobility time (s). Total immobility time was calculated for 3 min except the first 1 min.

Prepulse Inhibition (PPI) Test

PPI of the acoustic startle response was measured using an SR-LAB System (San Diego Instruments). The stimulus consisted of a 20-ms prepulse, a 100-ms delay, and then a 40-ms startle pulse. The intensity of the prepulse was 4, 8, or 16-dB above the 70-dB background noise. The amount of PPI was calculated as a percentage of the 120-dB acoustic startle response: 100−((startle reactivity on prepulse + startle pulse)/startle reactivity on startle pulse) × 100.

Preparation of Brain Slice and Staining

Mice were anesthetized intraperitoneally with ethyl carbamate at a dose of 1.2 g/kg and perfused transcardially with ice-cold phosphate-buffered SAL (PBS), followed by 4% paraformaldehyde (PFA) in PBS. The brains were removed, postfixed in the same fixative overnight, and then soaked in 30% (w/v) sucrose in PBS. Coronal sections 20–40 μm thick were cut with a cryostat (Micro-edge Instruments, Japan). Neonatal brains were fixed overnight in 4% PFA, cryoprotected in 30% sucrose, and cut at 8 μm. For counting the number and measuring the size of neurons, cresyl violet staining was performed and neurons with a visible nucleus and apparent outline of the entire cell were counted in a standardized area in the prelimbic region using a computer-based image analysis system, WinROOF (Mitani, Japan). Using these measurements, the upper and lower areas distinguished by the size of neurons, were calculated in a standardized area in layers II/III and IV–VI of the prelimbic region, respectively. Images were acquired with a light microscope (Biorevo BZ-9000; KEYENCE, Axioskop2 plus; Carl Zeiss). For immunohistochemistry, the brain slices were incubated with primary antibodies such as anti-phosphate-activated glutaminase (PAG) (1:500; Aviva Systems Biology), anti-NeuN (1:1000; Chemicon), anti-BrdU (1:10; from BrdU Labeling and Detection Kit II, Roche), anti-Pax6 (1:1000; Abcam), anti-proliferation cell nuclear antigen (PCNA) (1:5000; DAKO), and anti-Tbr2 (1:500; Abcam) antibody. Fluorescently conjugated secondary antibodies (Alexa 488 and 546; Molecular Probes)

were also used. Images were acquired with LSM510 meta (Carl Zeiss), and then the immuno-positive cells in the prelimbic region were counted by WinROOF.

Laser Capture Microdissection (LCM)

We collected samples of the proliferative ventricular zone (VZ) and SVZ from E13.5 mice by using LCM. Embryos were removed from dams 3 h after the administration of PCP and decapitated; their heads were frozen in liquid nitrogen and stored at −80 °C. The frozen heads were sectioned on a cryostat at a thickness of 20 μm in the coronal plane. The sections were mounted on Membrane-Slide 1.0 PEN (Carl Zeiss), dried at room temperature for 1 min, and immediately fixed in methanol for 3 min. They were counterstained using a LCM staining kit (Ambion) and viewed under a PALM-MBC MicroBeam System (PALM Microlaser Technologies GmbH, Germany). The VZ and SVZ areas in the developing cerebral cortex were identified and dissected. Immediately after the dissection, the sample was picked up on adhesive lids of 500 μl Adhesive Caps (PALM Microlaser Technologies GmbH). The cut out samples were collected from both hemispheres in 6–8 sections of one brain.

PCR Array Focused on Neurogenesis and Neural Stem Cells

RNA from the LCM samples was extracted with RNAqueous-Micro (Ambion) according to the manufacturer's instructions. Reverse transcription was carried out using an RT^2 PCR Array First Strand Kit (SA Bioscience) following the manufacturer's instructions. A total of 1 μg of RNA per sample was used. An aliquot of the diluted cDNA template was stored at −30 °C and further used in a reverse transcription-polymerase chain reaction (RT-PCR) with an ABI 7500, RT^2 Real-Time SYBR Green PCR Master Mix, and Mouse Neurogenesis and Neural Stem Cells RT^2 Profiler PCR Array (SA Bioscience), according to the manufacturer's directions. The threshold cycle (Ct) for each well was calculated using the instrument's software. To ensure that the baseline and threshold were the same across all PCR arrays run in the same analysis, internal controls provided by the manufacturer were used. Data analysis was run by the ΔΔCt method (Pfaffl, 2001).

Furthermore, this PCR array system contains a genomic DNA control, a reverse transcription control, and a positive PCR control. The genomic DNA control was to specifically detect non-transcribed genomic DNA contamination with a high level of sensitivity. The reverse transcription control tests the efficiency of the RT^2 First Strand Kit reaction with a primer set detecting the template synthesized from the kit's built-in external RNA control. The positive PCR control tests the efficiency of the polymerase chain reaction itself using a pre-dispensed artificial DNA sequence and the primer set that detects it. We confirmed that these controls were normal, and then analyzed the data obtained.

Statistical Analysis

All data were expressed as the mean ± SEM. The statistical significance of differences between two groups was determined with Student's *t*-test. The significance of differences in FST and PPI was determined using a two-way analysis of variance (ANOVA) with repeated measures, followed by Student's *t*-test. $p<0.05$ was regarded as statistically significant.

RESULTS

Prenatal PCP-Treatment Reduced Body Weight in Newborn Pups

To evaluate effects of prenatal NMDA antagonism on neurodevelopment, we administered PCP at a dose of 10 mg/kg/day to pregnant dams from E6.5 to E18.5. The body weight of PCP-treated dams decreased day by day, and significantly on E18.5 compared with SAL-treated mice (Figure 1a). Furthermore, PCP treatment also significantly reduced the body weight of newborn pups on P1 compared with SAL-treated mice without any change in the number of pups (Figures 1b and c). However, after birth, the difference decreased gradually with growth, so that there was no change on P28 and 56 (Figure 1d). These results suggest that prenatal PCP-treatment induced some developmental deficits without embryonic lethality.

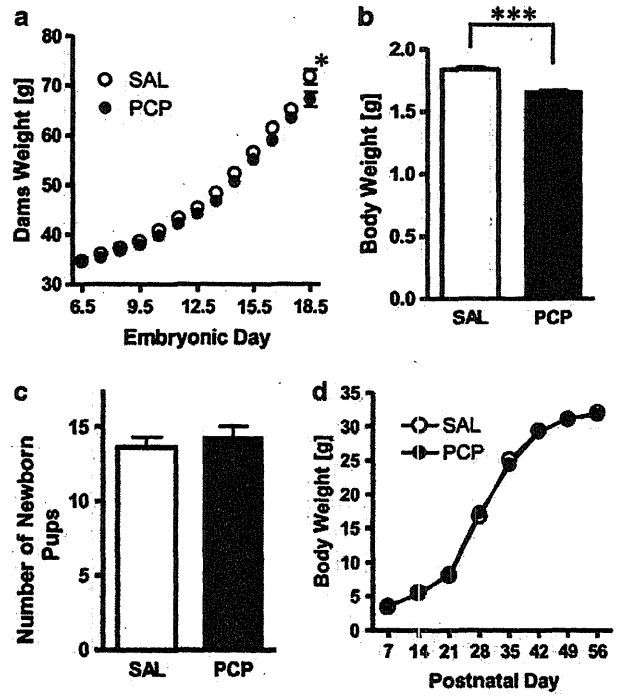

Figure 1 Prenatal PCP-treatment reduced body weight in newborn pups. Mice were administrated SAL or PCP from E6.5 to E18.5. (a) Changes in the body weight of the dams are shown (two-way ANOVA with repeated measures: $F_{Interaction(12,132)}=3.01$; $p<0.05$, $F_{Time(12,132)}=1190$; $p<0.0001$, $F_{Treatment(1,132)}=1.71$; $p>0.05$). *$p<0.05$ by Student's *t*-test ($n=6$–7). (b) The body weight on P1 ($n=50$) and (c) the number of newborn pups ($n=6$–9) are shown. ***$p<0.001$ by Student's *t*-test. (d) Changes in the body weight of the pups after birth are shown ($n=24$–28) (two-way ANOVA with repeated measures: $F_{Interaction(7,400)}=0.27$; $p>0.05$, $F_{Time(7,400)}=781$; $p<0.0001$, $F_{Treatment(1,400)}=0.01$; $p>0.05$). Values are means ± SEMs.

Prenatal PCP-Treatment Induced Behavioral Deficits in Adulthood

To investigate the effect of prenatal NMDA receptor-antagonism on behavior in adulthood, we administered PCP at a dose of 10 mg/kg/day to pregnant dams from E6.5 to E18.5, and tested the behavioral performance of their pups from P28 (Figure 2a). In novel object recognition tests on P56 to investigate the effects on recognition memory, PCP-treated mice showed changes in exploratory preference during the retention session compared with SAL-treated mice, although there was no change in exploratory time (Figures 2b and c). In the forced swimming test, the PCP-treated mice showed an increase in immobility time in comparison with SAL-treated mice, suggesting behavioral deficits like negative symptoms of schizophrenia (Figures 2d and e). These behavioral deficits were expressed from P28 (Supplementary Figure S1). Furthermore, in the test to evaluate sensory motor gating function, which is frequently impaired in schizophrenia patients, prenatal PCP treatments significantly reduced PPI compared with SAL (Figures 2f and g). These results indicated that prenatal PCP-treatments induced schizophrenia-like behavioral deficits in adulthood, similar to our previous report using PCP at a high dose of 20 mg/kg (Lu *et al*, 2010).

Prenatal PCP-Treatment Reduced the Density of Glutamatergic Neurons in PFC

To reveal the cause of these behavioral deficits, we investigated the effect of PCP-treatments on neurodevelopment in the PFC, an area critical to schizophrenia. First, we examined morphological and numerical changes in the neurons on P56 by cresyl violet staining (Figure 3a). No difference in the size of neurons in the upper (II/III) or lower (IV–VI) layer of the PFC was observed regardless of PCP treatment (Figure 3b). However, a decrease in the density of neurons was revealed in both layers of the PFC in PCP-treated mice compared with SAL-treated mice (Figure 3c).

It has been reported that hypoglutamatergic function in the PFC was found in patients with schizophrenia and an animal model treated with PCP chronically, which shows abnormal behavior (Lewis *et al*, 2003; Murai *et al*, 2007). Thus, we checked the density of glutamatergic neurons in the PFC of PCP-treated mice by immunohistochemical analysis. PAG was used as a glutamatergic neuronal marker, because it is detected only in pyramidal neurons, not in GABA-immunoreactive neurons or in nonpyramidal neurons (Kaneko and Mizuno, 1994). The result showed that number of glutamatergic neurons were reduced significantly in PCP-treated mince compared with SAL-treated mice (Figures 3d and e). These results indicated that prenatal NMDA antagonism impaired glutamatergic neurogenesis.

NMDA Receptor Antagonism Impaired Neurogenesis but not the Neural Migration

Glutamatergic neurons in the PFC are produced in the VZ and SVZ, and then migrate radially to the correct site, resulting in the formation of a layered structure with an inside-out pattern (Dehay and Kennedy, 2007). It has been

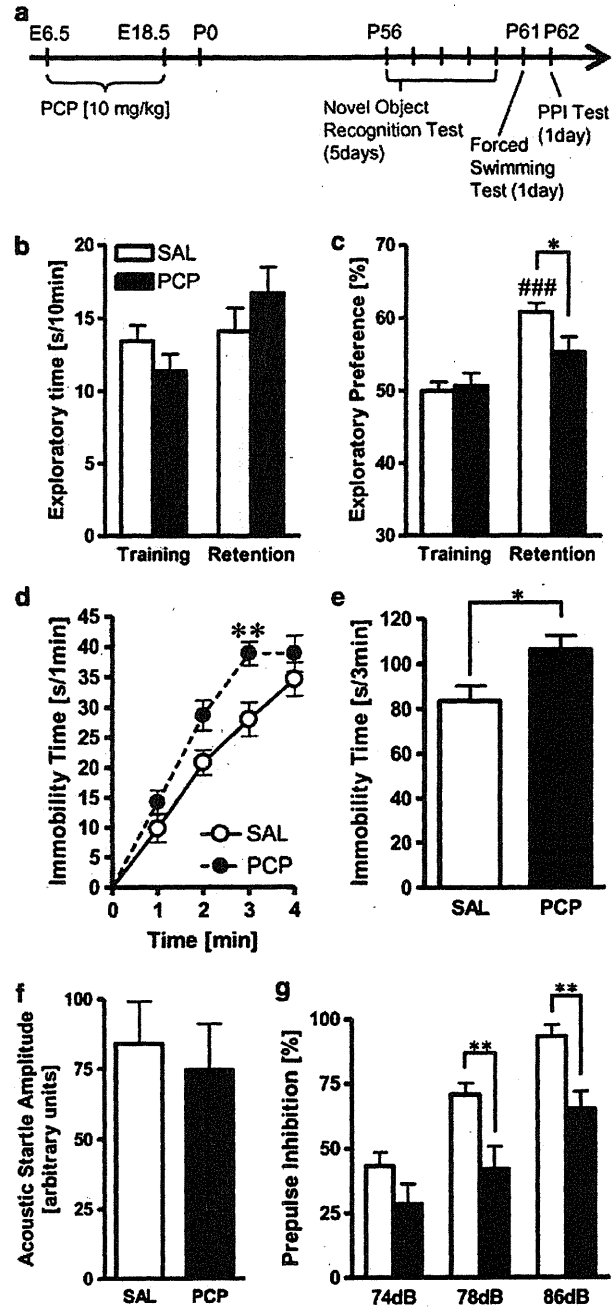

Figure 2 Prenatal PCP treatment induced behavioral deficits in adult. (a) Experimental schedule: mice were tested from P56. In the novel object recognition test, (b) exploratory time and (c) exploratory preference were determined. $^{###}p<0.001$ compared with SAL-treated mice in the training session by paired *t*-test. $^{*}p<0.05$ by Student's *t*-test. In the forced swimming test, (d) the time course (two-way ANOVA with repeated measures: $F_{Interaction(3,114)}=1.41$; $p>0.05$, $F_{Time(3,114)}=69.3$; $p<0.0001$, $F_{Treatment(1,114)}=6.97$; $p<0.05$) and (e) total scores of the immobility time were determined. $^{*}p<0.05$, $^{**}p<0.01$ by Student's *t*-test. In the PPI test, (f) acoustic startle amplitude without prepulse and (g) PPI (two-way ANOVA with repeated measures: $F_{Interaction(2,74)}=1.16$; $p>0.05$, $F_{Prepulse(2,74)}=34.4$; $p<0.0001$, $F_{Treatment(1,74)}=11.65$; $p<0.01$) were assessed. $^{**}p<0.01$ by Student's *t*-test. Values are means ± SEMs ($n=20$).

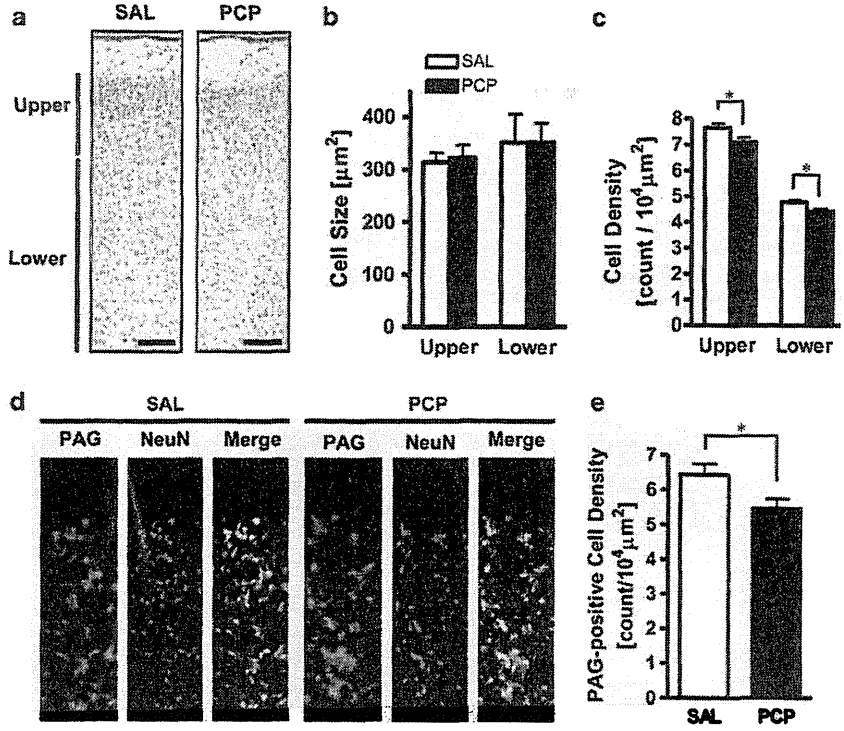

Figure 3 Prenatal PCP-treatment reduced the density of glutamatergic neurons in PFC. (a) Nissl-stained coronal sections of the PFC in SAL- and PCP-treated mice. Scale bar: 200 μm. Stereological analysis of (b) the size and (c) density of Nissl-stained cells in SAL- and PCP-treated mice ($n=6$). (d) PAG-immunostained coronal sections of the PFC in PCP- and SAL-treated mice. Stereological analysis of (e) the density of PAG-stained cells was shown. Values are means ± SEMs ($n=8$). Scale bar: 200 μm. *$p<0.05$ by Student's *t*-test.

reported that neuronal migration is activated via the NMDA receptor (Komuro and Rakic, 1993). If neural migration was impaired by PCP treatment, the density of glutamatergic neurons in the PFC might be decreased, resulting from the ectopic distribution. To verify this possibility, we performed a migration assay using BrdU, by which newborn cells at the time of injection can be labeled, because it is incorporated into the newly synthesized DNA. On E12.5, E13.5, E14.5, or E15.5, pregnant mice were injected with BrdU 3 h after receiving PCP (Figure 4a). The $BrdU^+$ cells in PCP-treated mice on P7 were distributed normally with an inside-out pattern, and there were no significant changes compared with SAL-treated mice (Figure 4b). However, the density of $BrdU^+$ cells was decreased significantly in the early developmental stage (Figure 4c). This decrease was already evident at 30 min after the BrdU administration in PCP-treated mice (Figures 4d and e). Moreover, another NMDA receptor antagonist, MK-801, induced a similar decrease in $BrdU^+$ cells. These results suggest that NMDA receptor antagonism might disturb the mitosis of neuronal progenitors.

NMDA Receptor Antagonism Reduced the Density of Neuronal Progenitors in the VZ and SVZ

Three main types of cortical neuronal progenitors have been identified throughout corticogenesis: radial glial cells, short neural precursors, and basal progenitor cells (Dehay and Kennedy, 2007). Both the short neural precursors and radial glia divide at the apical surface of the VZ and expresses a transcription factor, Pax6. The basal progenitor is the other major type of neuron-producing progenitor and is located in the SVZ, and expresses the transcription factor Tbr2. The SVZ starts to form on E13.5 in the mouse and expands significantly during late corticogenesis. To investigate the effect of NMDA receptor antagonism on neurogenesis, we checked these neural progenitors in the VZ and SVZ. The results showed that the number of $Pax6^+$ neural progenitors on E13.5 was decreased in PCP-treated mice in comparison with SAL-treated mice, and $PCNA^+$, a mitotic cell marker, cells were decreased also (Figures 5a, c and d). Further, $Tbr2^+$ basal progenitors were also decreased significantly in PCP-treated mice compared with SAL-treated mice (Figures 5b and e). These results suggest that prenatal NMDA antagonism might impair the proliferation of neural progenitors, resulting in the decrease in glutamatergic neurons.

Prenatal PCP-Treatment Disrupted the Gene Expression Involved in Neurogenesis in Neural Progenitors

To uncover the changes in gene expression in neuronal progenitors causing the impairment of proliferation by PCP, we used a PCR array focusing on neurogenesis and neural stem cells. First, on E13.5, we cut out the VZ and SVZ, the areas containing neuronal progenitors, using LCM (Supplementary Figure S2). After the purification of mRNA from the tissue sections, we performed a comprehensive analysis of the expression of factors involved in neurogenesis and neuronal stem cells using the PCR array.

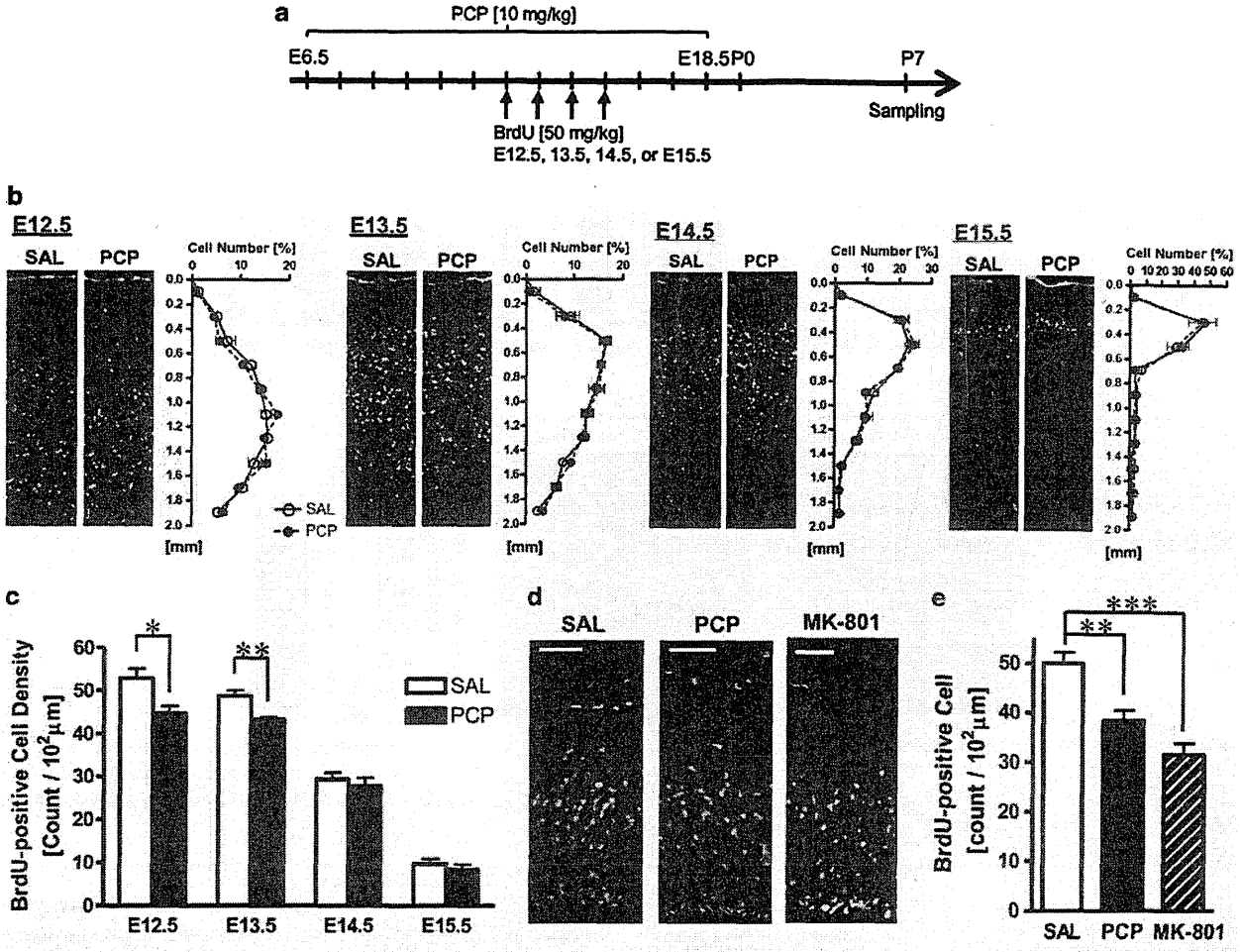

Figure 4 Prenatal PCP treatment affected the mitosis of neuronal progenitor but not neuronal migration. (a) Experimental schedule: the pregnant mice were given a single BrdU injection on E12.5, E13.5, E14.5, or E15.5, 3 h after the PCP/SAL administration to label mitotic progenitors in the embryonic brains. After the birth, the pups were fed for 7 days to examine cell migration. (b) Fluorescence images of $BrdU^+$ cells using the antibody and the percentage of $BrdU^+$ cells at each distance from the apical side are shown. (Two-way ANOVA with repeated measures: E12.5 ($F_{Interaction(9, 100)} = 1.57$; $p > 0.05$, $F_{Position(9, 100)} = 86.4$; $p < 0.0001$, $F_{Treatment(1, 100)} < 0.0001$; $p > 0.05$), E13.5 ($F_{Interaction(9, 100)} = 0.47$; $p > 0.05$, $F_{Position(9, 100)} = 55.8$; $p < 0.0001$, $F_{Treatment(1, 100)} < 0.0001$; $p > 0.05$), E14.5 ($F_{Interaction(9, 100)} = 0.45$; $p > 0.05$, $F_{Position(9, 100)} = 113$; $p < 0.0001$, $F_{Treatment(1, 100)} < 0.0001$; $p > 0.05$), E15.5 ($F_{Interaction(9, 100)} = 0.40$; $p > 0.05$, $F_{Position(9, 100)} = 68.1$; $p < 0.0001$, $F_{Treatment(1, 100)} = 0.0001$; $p > 0.05$)). (c) The number of $BrdU^+$ cells per 100 μm in the tangential direction is shown ($n = 6$). $^*p < 0.05$, $^{**}p < 0.01$ by Student's *t*-test. Furthermore, the PCP-, MK-801-, or SAL-treated pregnant mice were given a single BrdU injection 3 h before decapitation. (d) Fluorescence images of $BrdU^+$ cells in embryonic cortex are shown. Scale bar: 100 μm. (e) Stereological analysis of the number of $BrdU^+$ cells per 100 μm in the tangential direction in PCP-, MK-801-, and SAL-treated mice is shown ($n = 6$). $^{**}p < 0.01$, $^{***}p < 0.001$ by Boferroni's test (one-way ANOVA: $F_{(2, 21)} = 17.0$). Values are means ± SEMs.

Among genes whose *p*-value differed between the SAL- and PCP-treated groups by <0.05 and whose absolute value of change was larger than twofold, one gene was increased, and 16 genes decreased in expression in PCP-treated mice (Table 1). Two genes, Netrin1 (Ntn1) and Notch2, showed significant reductions in PCP-treated mice compared with SAL-treated mice. The result suggests that prenatal NMDA antagonism might disrupt the gene expression in neural progenitors, leading to the impairment of proliferation.

DISCUSSION

In this study, mice with prenatal exposure to PCP showed schizophrenia-like behavior in adulthood, such as recognition memory deficits and PPI impairments (Figure 2). These results are consistent with our previous study with PCP (20 mg/kg) (Lu *et al*, 2010), although the dose of PCP was lower (10 mg/kg) in this experiment. These behavioral deficits are recovered by treatment with a psycotropic drug such as clozapine. By crossing the placenta (Kaufman *et al*, 1983; Nicholas *et al*, 1982), PCP affects the developing fetal brain directly. Fico and Vanderwende (1988) found that PCP was rapidly transported into the fetal brain and disappeared in 8 h after maternal exposure during pregnancy. These findings suggest that prenatal PCP exposure could block NMDA receptors on the neuronal progenitors and/or newborn cells in the fetal brain, impairing neurodevelopment. As evidence of this, we showed that prenatal PCP-treatment decreased the body weight of the dams and newborn pups (Figure 1). The decrease in weight among the dams suggests impaired neurodevelopment. However,

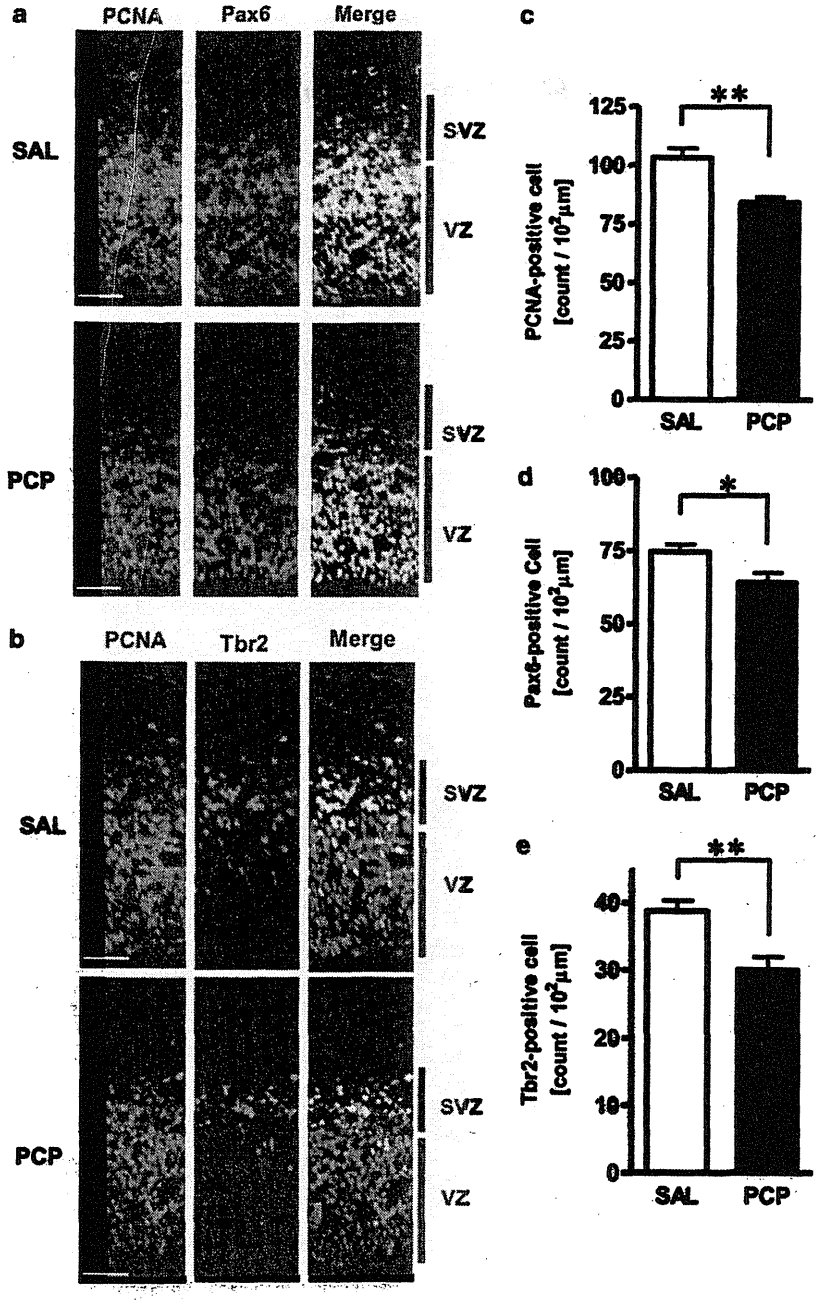

Figure 5 Prenatal PCP-treatment reduced the density of neuronal progenitors. On E13.5, mice were decapitated 6 h after the PCP/SAL injection. The pups were analyzed by immunohistochemical analysis using α-PCNA as a marker of proliferation, α-Pax6 antibody as a marker for neuronal progenitors in VZ, and α-Tbr2 antibody as a marker for basal progenitors in SVZ. Fluorescence images of PCNA-, (a) Pax6-, and (b) Tbr2-positive cells in embryonic cortex are shown. Scale bar: 100 μm. The density of (c) PCNA-, (d) Pax6-, and (e) Tbr2-positive cells per 100 μm in the tangential direction is shown. Data are means ± SEMs ($n = 6$). *$p < 0.05$, **$p < 0.01$ by Student's *t*-test.

because PCP administration was reported to reduce body weight in non-pregnant adult mice (Koseki *et al*, 2011), the decrease must result from a reduction in not only the weight of the pups but also that of the dam itself. On the other hand, the decrease in the weight of newborn pups on P1 strongly indicates that prenatal PCP treatment impaired neurodevelopment. Moreover, the difference in weight decreased with growth to the point that there was no difference on P28 and 56, suggesting that prenatal treatment with PCP has little impact on development after birth. These results are consistent with our previous study (Lu *et al*, 2010).

We found that the density, but not size, of glutamatergic neurons, was decreased in the PFC of the PCP-treated mice on P56 compared with SAL-treated mice (Figure 3). These findings might be consistent with the report that the CNS structures of NR1 knock-out mice are somewhat smaller than normal, although no region is defected obviously

Table 1 List of Gene Whose Expression in Progenitor Cells was Altered by PCP

	Gene name	Symbol	Gene number	Fold difference PCP/SAL	*t*-test *p*-value	Fold up- or down- PCP/SAL
Decrease	Disheveled 3, dsh homolog (*Drosophila*)	Dvl3	NM_007889	0.320	0.335	−3.12
	E1A-binding protein p300	Ep300	NM_177821	0.496	0.050	−2.02
	Filamin, alpha	Flna	NM_010227	0.370	0.494	−2.70
	Guanine nucleotide-binding protein, alpha o	Gnao1	NM_010308	0.148	0.294	−6.76
	Hairy/enhancer-of-split related with YRPW motif-like	Heyl	NM_013905	0.428	0.256	−2.34
	Myeloid/lymphoid or mixed-lineage leukemia 1	Mll1	NM_001081	0.394	0.241	−2.54
	Nuclear receptor coactivator 6	Ncoa6	NM_019825	0.289	0.203	−3.46
	Notch gene homolog 2 (*Drosophila*)	Notch2	NM_010928	0.546	0.047*	−1.83
	Neuronal pentraxin 1	Nptx1	NM_008730	0.440	0.333	−2.27
	Neuron-glia-CAM-related cell adhesion molecule	Nrcam	NM_176930	0.255	0.418	−3.92
	Netrin 1	Ntn1	NM_008744	0.257	0.014*	−3.89
	POU domain, class 3, transcription factor 3	Pou3f3	NM_008900	0.163	0.454	−6.14
	Pleiotrophin	Ptn	NM_008973	0.183	0.534	−5.47
	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	Sema4d	NM_013660	0.368	0.317	−2.72
	SRY-box containing gene 3	Sox3	NM_009237	0.475	0.462	−2.11
	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide	Ywhah	NM_011738	0.382	0.658	−2.62
Increase	Paired box gene 6	Pax6	NM_013627	2.666	0.573	2.67

*$p<0.05$: Student's *t*-test ($n=5$).

affected (Forrest *et al*, 1994). This reduction in the density of glutamatergic neurons would result in glutamatergic hypofunction in the PFC. In fact, we have already reported that active phosphorylated NR1, an essential subunit of the NMDA receptor, was decreased (Lu *et al*, 2010), and the basal extracellular glutamate level and high K^+-evoked glutamate release were also reduced in the PFC of PCP-treated mice (Lu *et al*, 2011). Furthermore, we have confirmed that the release of glutamate was decreased in the PFC of PCP-treated mice, and some abnormal behaviors were improved by treatment with D-serine, a co-agonist of the NMDA receptor, and a glutamate aspartate transporter inhibitor (Lu *et al*, 2010). These results suggest that a blockade of NMDA receptors by PCP might prevent glutamatergic neurodevelopment, resulting in hypoglutamatergic function in the PFC.

Although we focused on glutamatergic neurons in this study, it was reported that prenatal NMDA receptor blockade affects the development of other types of neuron such as the GABAergic neuron. For example, prenatal exposure (E15–E18) to MK-801 reduced the density of parvalbumin-positive GABA neurons in the rat medial PFC on P63 and enhanced PCP-induced hyperlocomotion (Abekawa *et al*, 2007). This finding suggests that prenatal treatment with PCP might impair the development of not only glutamatergic but also GABAergic neurons, leading to GABAergic dysfunction in the PFC. Further experiments focusing on GABAergic neurons should be performed using our model.

During the development of the mammalian cortex, neuronal progenitor cells generate neurons through asymmetric and symmetric divisions. In the early stages, $Pax6^+$-neural progenitors undergo symmetric, proliferative divisions, each of which generates two progenitor cells in the VZ (Rakic, 1995; McConnell, 1995). These divisions are followed by many asymmetric divisions, each of which generates a daughter progenitor plus a more differentiated cell such as a $Tbr2^+$-basal progenitor and neuron. The remaining neuronal progenitors in the VZ and basal progenitors in the SVZ typically undergo symmetric, differentiating divisions, each of which generates two neurons (Götz and Huttner, 2005; Hevner, 2006). These neurons migrate radially to the proper site, resulting in the formation of a layered structure with an inside-out pattern. We found that prenatal PCP treatment reduced the density of $BrdU^+$ cells in early developmental stages (Figure 4), in which mainly symmetric, proliferative divisions of neuronal progenitor were underway. Actually, we confirmed a reduction in both types of neuronal progenitor cells in the VZ and SVZ (Figure 5). Many reports have said that the NMDA receptor has an important role in the proliferation of neuronal progenitors. Glutamate stimulation via NMDA receptors significantly increased the proliferation of human neural progenitor cells derived from the fetal frontal cortex (Suzuki *et al*, 2006). Neurospheres derived from the embryonic rat brain express the NR1 and NR2B subunits of the NMDA receptor and show reductions in diameter and number (Mochizuki *et al*, 2007). Furthermore, the activation of NMDA receptors increases the proliferation of neuronal progenitors in the developing hippocampus (Joo *et al*, 2007) and striatum (Luk *et al*, 2003) as well. These findings are consistent with the present results. Further, it is easy to assume that the reduction in progenitors causes the low density of glutamatergic neurons, because these progenitors mainly produce glutamatergic neurons.

Previous reports have shown that the stimulation of NMDA receptors promotes the radial migration of neurons

(Komuro and Rakic, 1993). However, in this study, prenatal PCP treatment did not affect the migration. One explanation is that the half-life of PCP in brain tissue is very short: the concentration of PCP in rat fetal brain peaked 30 min after its injection into pregnant dams, and the half-life of PCP on E15 and E18 in brain tissue was 126 and 27 min, respectively (Fico and Vanderwende, 1988). The neuronal migration might be impaired temporarily by PCP, but soon return to normal as the PCP in fetal brain disappears. However, prenatal PCP treatment might have a critical impact on neuronal progenitors because of repeated exposure.

We showed that prenatal PCP-treatment disrupted the gene expression of Notch2 and Ntn1, in neural progenitors (Table 1). The Notch pathway is involved in a wide array of cell fate decisions during development (Louvi and Artavanis-Tsakonas, 2006). Mammalian Notch proteins appear to have an important role in preventing cell differentiation in a variety of cell lineages. Notably, in the developing cerebellar cortex, Notch2 signaling in granule neuron precursors has been reported to inhibit differentiation into neurons and maintain precursor proliferation (Solecki *et al*, 2001). This suggests that decreased Notch2 expression in progenitor cells of PCP-treated mice might prevent the proliferation of neuronal progenitors in early developmental stages, leading to the low density. Further, Ntn1 is a diffusible protein, which provides informational cues for several cellular functions, including cell adhesion, migration, axon guidance, proliferation, differentiation, and cell survival (Cirulli and Yebra, 2007). A recent report has demonstrated that one Ntn1 receptor, Neogenin, is expressed in neuronal progenitors including radial glia during neurogenesis in the forebrain. The Neogenin-positive progenitor displays higher proliferative and neurogenic potential than its negative counterpart (Fitzgerald *et al*, 2007), suggesting that Ntn1 signaling might activate neurogenesis. As well as Notch2, the decreased Ntn1 expression might bring about the impairment of proliferation. Furthermore, both Notch2 and Ntn1 signaling has been reported to be involved in apoptotic cell death. Apoptosis is enhanced in both Notch2 and Ntn1 mutant fetal brain (Hamada *et al*, 1999; Llambi *et al*, 2001). Interestingly, it also occurred in the fetal brains of prenatal PCP-treated rats (Ikonomidou *et al*, 1999). Apoptosis via the decrease in Notch2 and Ntn1 might be one of the causes of the low density of neuronal progenitors.

In this study, prenatal PCP treatment affected the expression of only two genes in the neuronal progenitors, although it induced very severe behavioral deficits in adulthood. The expression of more genes is likely to be changed by the treatment. However, the PCR array system used in this study can evaluate the expression of only 84 genes. Moreover, genes having important functions other than in neurogenesis were not checked. To evaluate more exhaustively the effect of NMDA receptor antagonism on gene expression, PCR arrays and micro arrays with a broader range would be required.

In conclusion, as a mechanism by which a prenatal NMDA receptor blockade causes long-lasting behavioral changes, we demonstrated that prenatal NMDA receptor antagonism impaired the proliferation of neuronal progenitors through aberrant gene expression, and consequently decreased the density of glutamatergic neurons in the PFC, resulting in glutamatergic hypofunction. These findings lead us to speculate that NMDA receptors regulate the proliferation and maturation of progenitor cells in the VZ during neurodevelopment via the regulation of gene expression.

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DISCLOSURE

The authors declare no conflict of interest.

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ORIGINAL INVESTIGATION

Dissociable role of tumor necrosis factor alpha gene deletion in methamphetamine self-administration and cue-induced relapsing behavior in mice

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Abstract
Rationale During the development of addiction, addictive drugs induce transient and long-lasting changes in the brain including expression of endogenous molecules and alteration of morphological structure. Of the altered endogenous molecules, some facilitate but others slow the development of drug addiction. Previously, we have reported that tumor necrosis factor alpha (TNF-α) is a critical molecule among endogenous anti-addictive modulators using animal models of drug-conditioned place preference and drug discrimination.
Objectives Does targeted deletion of the TNF-α gene in mice affect methamphetamine (METH) self-administration, motivation to self-administer METH, cue-induced reinstatement of METH-seeking behavior, and food reinforcement or seeking behavior?
Methods Both METH self-administration and reinstatement of drug-seeking behavior and food self-delivery and food-seeking behavior were measured in TNF-α (−/−) and wild-type mice.
Results There were an upward shift of dose responses to METH self-administration under a fixed ratio schedule of reinforcement and higher breaking points under a progressive ratio schedule of reinforcement in TNF-α knockout (TNF-α (−/−)) mice as compared with wild-type mice. There was no significant difference in cue-induced reinstatement of METH-seeking behavior, food-maintained operant behavior, motivation to natural food, and cue-induced food-seeking behavior between TNF-α (−/−) and wild-type mice.
Conclusion TNF-α affects METH self-administration and motivation to self-administer METH but contributes to neither METH-associated cue-induced relapsing behavior nor food reward and food-seeking behavior. TNF-α may be explored for use as a diagnostic biomarker for the early stage of drug addiction.

Keywords TNF-α · Methamphetamine · Self-administration · Motivation · Cue-induced reinstatement · Diagnostic biomarker · Mice

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Introduction

Proinflammatory cytokine tumor necrosis factor alpha (TNF-α) is produced by macrophages and circulating monocytes. TNF-α has been implicated in brain function directly or indirectly through stimulation of vagal afferents (Maier and Watkins 1998). In one study, the level of TNF-α expressed in the brain (locus coeruleus) of human opiate addicts was higher than that of control subjects (Dyuizen

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and Lamash 2009). Yet reports of the level of circulating TNF-α in human alcoholics, opiate addicts, and cocaine or marijuana abusers are not consistent. Some reports show that the level increases (Gonzalez-Quintela et al. 2008; Peng et al. 1999; Irwin et al. 2009), and others show that the level decreases or does not change (Baldwin et al. 1997; Irwin et al. 2007; Sacerdote et al. 2008; Li et al. 2009; Franchi et al. 2010). In animals, repeated administration of psychostimulants or opiates induced TNF-α production in the brain or immune system (Friedman and Eisenstein 2004; Nakajima et al. 2004; Niwa et al. 2007a; Kubera et al. 2008). TNF-α knockout (TNF-α (−/−)) mice are more sensitive to methamphetamine (METH)- or morphine-conditioned place preference than wild-type animals (Nakajima et al. 2004; Niwa et al. 2007a, b). Exogenous TNF-α treatment attenuates METH- or morphine-induced reward, METH-associated discriminative stimulus effects, and dopaminergic neurotoxicity (Nakajima et al. 2004; Niwa et al. 2007a, b). We hypothesized that as an endogenous modulator, TNF-α activates the plasmalemmal dopamine transporter and vesicular monoamine transporter-2 and inhibits addictive drug-induced dopamine release as neuroprotection in drug reward and neurotoxicity (Nakajima et al. 2004; Yamada 2008).

To investigate the neurological mechanisms of drug addiction and screen potential therapeutics against addiction, several animal behavioral models have been created. Among them are locomotor sensitization, conditioned place preference, drug discrimination, drug self-administration, and reinstatement of drug-seeking behavior. The models are designed to mimic the physiological or behavioral effects induced by various drugs of abuse (Niwa et al. 2008). Drug self-administration and reinstatement of drug-seeking behavior which mimic some clinical symptoms of human addicts are the gold standard for studying the development of drug addiction (Deroche-Gamonet et al. 2004; Vanderschuren and Everitt 2004; O'Brien and Gardner 2005; Epstein et al. 2006; Panlilio and Goldberg 2007; Niwa et al. 2008). Using our well-established mouse models of drug self-administration and reinstatement, we determined whether targeted deletion of TNF-α gene in mice affects METH self-administration, motivation to self-administer METH, or cue-induced reinstatement of METH-seeking behavior.

Materials and methods

Subjects and drug

Male C57BL/6-TNF-α (−/−) and wild-type (C57BL/6) mice, 8 weeks old, weighed 20–25 g at the beginning of the experiments. TNF-α (−/−) mice, derived from the TT2 ES cell line (established from C57BL/6×CBA/JNCrj Fi blastocyte), were backcrossed to C57BL/6 for more than eight generations (Taniguchi et al. 1997). Homozygous TNF-α (−/−) mice were obtained by interbreeding of heterozygotes and confirmed by Southern blot analysis for the TNF-α allele. The wild-type littermates were used as control for homozygous TNF-α (−/−) mice. All animals were kept in a regulated environment (23±0.5°C, 50±0.5% humidity) with a 12-h light/dark cycle (lights on at 9:00 A.M.). Both water and food were available ad libitum throughout the experiments unless otherwise noted. All procedures followed the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Nagoya University Animal Care and Use Committee. METH hydrochloride (Dainippon Pharmaceutical Ltd., Osaka, Japan) was dissolved in sterile saline and self-administered by the mice at 0.1 mg/kg/infusion over 5 s (infusion volume, 2.1 μl) (Yan et al. 2006, 2007).

Food-reinforced operant behavior and reinstatement of food-seeking behavior

Food-reinforced operant behavior and motivation

Food-reinforced behavior and motivation were tested in standard mouse operant conditioning chambers as described previously (Yan et al. 2006, 2007). Briefly, the chamber was equipped with two nose-poke sensors (ENV-313 M, Med Associates, Georgia, VT) in two holes, two cue-lamps in and above each hole, and a food pellet dispenser (ENV-203-20, Med Associates) connected to a rectangular opening (2.25 cm×2.25 cm) between the two holes. The bottom of the opening was 5 mm above the chamber floor and equidistant from the holes. A house light was located at the top of the chamber opposite the holes. During the tests for food-reinforced operant behavior and motivation, one hole was defined as active, and the other, as inactive. Nose-pokes in the active hole delivered a single food pellet (dustless precision pellets 20 mg, A Holton Industries Co., Frenchtown, NJ) to the opening by the dispenser (ENV302M, Med Associates) and inactivated the cue-lamp and hole-lamp for 5 s followed by a 5-s timeout period. Nose-pokes in the active or inactive hole during the timeout period had no consequences but were recorded by the software MED-PC for Windows (Med Associates).

Naive TNF-α (−/−) and wild-type mice (n=7 for each genotype) were deprived of food for 20 h (water remained available ad libitum throughout the experiments). The next day, both genotypes were able to nose-poke for food pellets in the chambers as described above. After each session of nose-poking for food pellets, the mice were returned to their home cages and given unlimited amounts of food for 2 h. The daily sessions of food-reinforced nose-poking by TNF-α (−/−) and wild-type mice were initially performed under a fixed ratio

(FR) 1 schedule for 3 h. Once the mice showed stable nose-poking for food pellets (deviations of less than 15% of the mean of active responses in three consecutive training sessions), the reinforcement schedule was changed to an FR2 until the same criterion was achieved. The same groups of mice were then subjected to nose-poking for food pellets under a progressive ratio (PR) schedule. The number of active nose-pokes required to obtain a single food pellet escalated according to the following series: 1, 2, 4, 6, 9, 12, 15, 20, 25, 32, 40, 50, 62, 77, 95, 118, 145,178, 219, 268, 328, 402, 492, 603, 737, etc. (Roberts and Bennett 1993). Each session under the PR schedule lasted for 5 h or until the mice failed to respond within 1 h. The "breaking point," expressed as the total number of food pellets earned by animals, reflected the intensity of motivation for earning food pellets. Within two to four daily sessions, TNF-α (−/−) and wild-type mice demonstrated stable active nose-poke responses for food pellets (deviations of less than 15% of the mean of the total active responses in two consecutive sessions).

Extinction and reinstatement of food-seeking behavior

During this phase, both food and water were available ad libitum in the home cages. After the test for motivation to take food pellets under the PR schedule, the same groups of TNF-α (−/−) and wild-type mice were then subjected to seven daily 3-h sessions of extinction. Throughout the extinction session, the house light was on. The food-associated cue- and hole-lamps and the system that delivered food pellets were turned off. Nose-pokes into the previously active hole neither delivered food pellets nor reinstated food-associated cues (cue- and hole-lamps). Once the mice met the criterion of extinction (fewer than 15 active responses or 25% of active responses in the stable phase of self-administration in two consecutive sessions), the mice were placed in the chambers for 3 h under the same conditions as those for extinction training. The number of nose-pokes in active or inactive holes was counted as baseline (no-cue data). On the next day, the mice were then subjected to the test for food-associated cue-induced reinstatement in a 3-h session. The test was performed under the same conditions as the food-reinforced operant behavioral test under the FR2 schedule, except that there was no delivery of food pellets after nose-pokes into a previously active hole. Nose-pokes in the previously active or inactive hole were counted as active and inactive, respectively.

Surgery and apparatus for METH self-administration

Catheterization

New groups of naive TNF-α (−/−) and wild-type mice were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). Indwelling catheters were made of micro-silicone tubing (inner diameter, 0.50 mm; outer diameter, 0.7 mm; IMG, Imamura Co., Ltd., Tokyo, Japan) and polyethylene tubing (inner diameter, 0.50 mm; outer diameter, 0.8 mm). Incisions were made on the skin of the head and ventral neck, and the right jugular vein was externalized. The end of the catheter was inserted into the jugular vein via a small incision and was secured to the vein and surrounding tissue with silk sutures. The exit port of the catheter passed subcutaneously to the top of the skull where it was attached to a modified 24-gauge cannula, which was secured to the mouse's skull with quick self-curing acrylic resin (Shofu Inc., Tokyo, Japan). To extend catheter patency, the catheters were flushed immediately after surgery, and in the morning and evening of the following days, with 0.03 ml of an antibiotic solution of cefmetazole sodium (20.0 mg/ml; Sankyo Co., Ltd., Tokyo, Japan) dissolved in heparinized saline (70 unit/ml; Leo Pharmaceutical Products Ltd., Tokyo, Japan). The patency of the catheter was usually confirmed once a week before behavior tests by infusion of a pentobarbital sodium solution (6.0 mg/ml, 0.15 ml/mouse) into the jugular vein. If the mice could not be knocked down within 5 s, the corresponding data were excluded from the statistical analysis.

Apparatus for METH self-administration

METH self-administration was conducted in the standard mouse chambers located within ventilated sound attenuation cubicles as described previously (Yan et al. 2006, 2007). Briefly, the chambers were equipped with nose-poke sensors in two holes located on one side of the chamber 1.0 cm above the floor, cue- and hole-lamps located above and in each hole, and a red house light located on the top of the chamber opposite the holes. During self-administration, one hole was defined as active, and the other, as inactive. Nose-pokes in the active hole activated the infusion pump (PHM-100, Med Associates) and inactivated the cue-lamp and hole-lamp. Nose-pokes in the inactive or active holes during the timeout period had no programmed consequences but were recorded. The components of the infusion line were connected to each other from the injector to the exit port of the mouse's catheter by joint FEP tubing (inner diameter, 0.25 mm; outer diameter, 0.55 mm; Eicom Co., Ltd., Japan), which was encased in steel spring leashes (Instech, Plymouth Meeting, PA). Swivels were suspended above the chamber. For each chamber, one pump/syringe set was located inside the cubicle.

METH self-administration under a fixed ratio or progressive ratio of reinforcement schedule

Acquisition of METH self-administration

After recovery from catheterization, the TNF-α (−/−) (n=21) and wild-type mice (n=20) were initially subjected to METH

self-administration under an FR1 schedule at a dose of 0.1 mg/kg/infusion. Within four to six sessions (days), most mice discriminated active from inactive nose-poke responses under the FR1 schedule of reinforcement. Once the mean of active nose-pokes was more than 60% of the total nose-pokes (active plus inactive) and the mice received no fewer than ten infusions of METH over two consecutive sessions, the METH reinforcement schedule was changed to FR2. Under the FR2 schedule, METH self-administration behavior stabilized gradually (deviations of less than 15% of the mean of active responses in three consecutive training sessions).

Dose responses for METH self-administration under an FR2 schedule

After drug self-administration behavior stabilized, TNF-α (−/−) and wild-type mice were subjected to METH self-administration under the FR2 reinforcement schedule in the dose range of 0.003–0.1 mg/kg/infusion from higher to lower dose. At each dose, the animals were subjected to two to four daily 3-h sessions of METH self-administration until active nose-poke responses were stable (deviations of less than 15% of the mean of the total active responses in two consecutive sessions).

Motivation for METH self-administration under a PR schedule

After testing for the dose–response curve, the TNF-α (−/−) and wild-type mice received additional two daily 3-h sessions of METH self-administration at a dose of 0.1 mg/kg/infusion. Then, they were subjected to METH self-administration under the PR schedule at a dose of 0.1 mg/kg/infusion. The "breaking point," or the final ratio (the number of active nose-pokes needed to earn the last infusion of METH), reflected the intensity of motivation. Each session lasted for 5 h or until the mice failed to respond within 1 h. Each mouse was subjected to two to five sessions of METH self-administration under the PR schedule, and active nose-pokes for METH infusion stabilized in both genotypes of animals within two to five sessions (as described in the section of dose response). Five TNF-α (−/−) and four wild-type mice were excluded from the final data analyses of acquisition, dose–response curve, and breaking points because of catheter patency or health problems.

Extinction and cue-induced reinstatement

After testing for motivation for METH self-administration, TNF-α (−/−) and wild-type mice were subjected to six to ten daily 3-h sessions of extinction training. Throughout the extinction session, the house light was on. The METH-associated cue- and hole-lamps and the pump for METH infusion were turned off. Therefore, nose-pokes into the previously active hole did not start an infusion of METH nor METH-associated cues (cue- and hole-lamps or pump noise for METH infusion). Once the extinction criterion was met (fewer than 15 active responses or 25% of active responses in the stable phase of self-administration in two consecutive sessions), the TNF-α (−/−) and wild-type mice were first placed in the chambers for 3 h under the same conditions as during extinction training. The number of nose-pokes in active or inactive holes was counted as baseline (no-cue data). On the next day, both genotypes of mice were then subjected to a daily 3-h session to test for cue-induced reinstatement of METH-seeking behavior. The cue-induced reinstatement tests were conducted under the same conditions as the METH self-administration tests under the FR2 schedule, except that METH was unavailable. Nose-pokes in the previously active or inactive hole were counted as active or inactive, respectively. Three TNF-α (−/−) and two wild-type mice were excluded from the final data analyses of cue-induced reinstatement because of health problems.

Data analysis

All data are expressed as the mean±SEM. A three-way analysis of variance (ANOVA) with repeated measures was performed for the difference in nose-poke responses between the two genotypes of mice during food self-delivery, food extinction, METH self-administration, dose–response curve, and METH extinction, followed by Fisher's least significant difference (LSD) post hoc test. A two-way ANOVA with repeated measures was performed for the difference in nose-poke responses between the two genotypes of mice during cue-induced food-seeking behavior and cue-induced drug-relapsing behavior, followed by the Bonferroni/Dunn post hoc test. The Mann–Whitney test was used to analyze the breaking points under the PR schedule; the Student's *t*-test was used to analyze the total intake of METH during drug self-administration. In all cases, a significant difference was set at $P<0.05$.

Results

No difference in food-reinforced operant behavior and motivation to obtain food pellets between TNF-α (−/−) and wild-type mice

Naive TNF-α (−/−) and wild-type mice were trained to make nose-pokes for food pellets under the FR or PR schedule in daily 3-h sessions. A three-way repeated measure ANOVA analysis with genotype, nose-poke, and day (session) as the

main factors showed a significant effect of nose-poke [$F_{(1, 24)}$= 1276.01, P<0.001], day [$F_{(9, 216)}$=70.12, P<0.001], but no effect of genotype [$F_{(1, 24)}$=0.79, P=0.38]. There was a significant nose-poke×day interaction [$F_{(9, 216)}$=92.31, P<0.001], but there were no significant interactions with genotype (all had P>0.71). An LSD post hoc analysis revealed that after day one, both TNF-α (−/−) and wild-type mice could discriminate active from inactive nose-poke responses for food pellets (P<0.001), but there was no significant difference between groups in either active or inactive nose-poke responses for food pellets under the FR schedules (Fig. 1a). There also was no significant difference in the number of training sessions needed to stabilize operant behavior between TNF-α (−/−) and wild-type mice. TNF-α (−/−) and wild-type mice had similar breaking points for food pellets under the PR schedule (Fig. 1b, Mann–Whitney test, P>0.05). Figure 1c represents typical curves of cumulative active nose-poke responses for food pellets under the PR schedule in one of TNF-α (−/−) or wild-type mice. These findings suggest that targeted deletion of the TNF-α gene does not affect food-reinforced operant performance or motivation.

No difference in extinction and cue-induced food-seeking behavior between TNF-α (−/−) and wild-type mice

To further evaluate the potential influence of targeted deletion of the TNF-α gene on cue-induced food-seeking behavior, the same groups of TNF-α (−/−) and wild-type mice were then subjected to extinction training. A three-way repeated measure ANOVA analysis with genotype, nose-poke, and day (session) as the main factors showed a significant effect of nose-poke [$F_{(1, 24)}$=50.03, P<0.001], day [$F_{(9, 144)}$=23.87, P< 0.001], but no effect of genotype [$F_{(1, 24)}$=158.97, P= 0.77]. There was a significant nose-poke×day interaction [$F_{(6, 144)}$=11.31, P<0.001], but there were no significant interactions with genotype (all had P>0.054). An LSD post hoc analysis revealed significant differences between active and inactive nose-pokes during extinction training (Ext 1–5) in wild-type mice (P<0.001 on Ext 1–2 and 4, P<0.01 on Ext 3, and P<0.05 on Ext 5) and in TNF-α (−/−) mice (P<0.001 on Ext 1 and P<0.05 on Ext 4–5). As shown in Fig. 2a, both TNF-α (−/−) and wild-type mice reduced their active nose-pokes, and there was no significant difference during extinctions six to seven. Both TNF-α (−/−) and wild-type mice met the extinction criterion after the same number of daily 3-h sessions of training. On the next day, food-associated cue-induced reinstatement was tested in both genotypes of mice. Food-associated cues reliably triggered reinstatement of food-seeking behavior in both TNF-α (−/−) and wild-type mice (Fig. 2b; active–inactive nose-poke $F_{(1,48)}$= 10.47, P<0.001; no-cue–cue $F_{(1,48)}$=16.86, P<0.001; interaction $F_{(3,48)}$=4.63, P<0.01). There also was no significant difference in cue-induced reinstatement behavior between TNF-α (−/−) and wild-type mice (Fig. 2b; $F_{(1,24)}$=3.61, P>0.05). Our results suggest that the effect of TNF-α gene deletion in cue-induced food-seeking behavior is minimal.

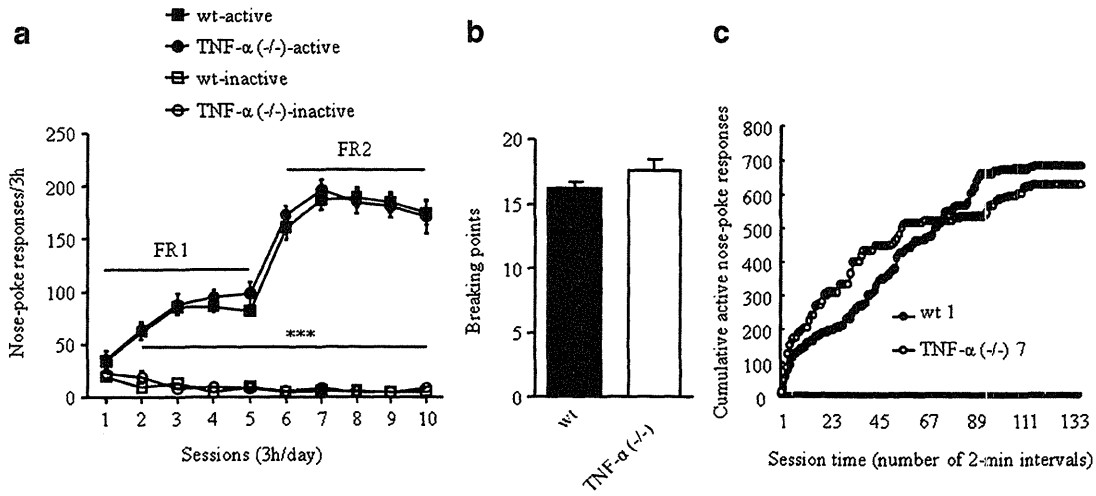

Fig. 1 Food-reinforced operant behavior and natural motivation in TNF-α (−/−) and wild-type mice. **a** Active and inactive nose-poke responses for food self-delivery under the FR2 schedule. ***P<0.001 versus inactive nose-poke responses in the same genotype. **b** Breaking points (total food pellets earned) for food reinforcement under the PR schedule. **c** Typical accumulative active nose-poke responses for food pellets under the PR schedule in the two genotypes of animals. *wt 1* wild-type mouse no. 1; *TNF-α (−/−) 7* TNF-α (−/−) mouse no. 7. Data are presented as the mean±SEM. N=7 for each genotype

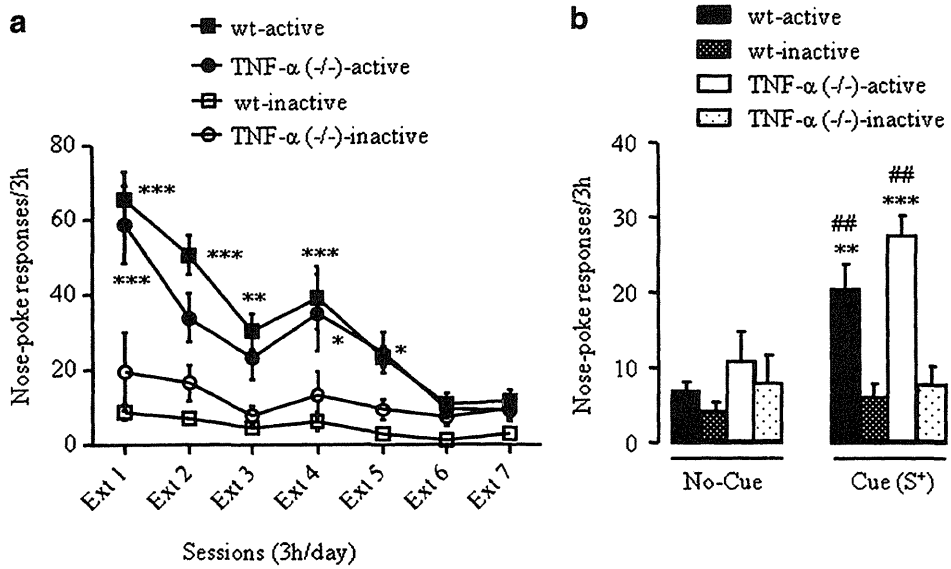

Fig. 2 Extinction and cue-induced food-seeking behavior in TNF-α (−/−) and wild-type mice. **a** Active or inactive nose-poke responses during extinction training. *P<0.05, **P<0.01, ***P<0.001 versus inactive nose-poke responses in the same genotype. **b** Active or inactive nose-poke responses during the test for food-associated cue-induced reinstatement. **P<0.01, ***P<0.001 versus inactive nose-poke responses in the same genotype. $^{\#\#}P$<0.01 versus no-cue in the same genotype (active–inactive nose-poke $F_{(1,48)}$=10.47, P<0.001; no-cue–cue $F_{(1,48)}$=16.86, P<0.001; interaction $F_{(3,48)}$=4.63, P<0.01). Data are presented as the mean±SEM. N=7 for each genotype

Upward shift dose responses to METH self-administration and motivation to self-administer METH solution in TNF-α (−/−) mice

There was no significant difference in the number of training sessions needed to stabilize METH self-administration between the two genotypes of mice (data not shown). A three-way repeated measure ANOVA analysis with genotype, nose-poke, and day (session) as the main factors showed a significant effect of nose-poke [$F_{(1,\ 56)}$=249.56, P<0.001], day [$F_{(10,\ 560)}$=85.64, P<0.001], but no effect of genotype [$F_{(1,\ 56)}$=0.28, P=0.60]. There was a significant nose-poke× day interaction [$F_{(10,\ 560)}$=67.68, P<0.001], but there were no significant interactions with genotype (all had P>0.16). An LSD post hoc analysis revealed that after day five, both TNF-α (−/−) and wild-type mice could discriminate active from inactive nose-poke responses for METH-taking at a dose of 0.1 mg/kg/infusion (P<0.001), but there was no significant difference between groups in either active or inactive nose-poke responses for METH self-administration under the FR2 schedule (Fig. 3a). Either TNF-α (−/−) or wild-type mice could discriminate active from inactive nose-poke responses after day five (Fig. 3a). There was no significant difference in total intake of METH accumulated for 11 daily 3-h sessions of METH self-administration training between TNF-α (−/−) and wild-type mice (Fig. 3b; 32.0±1.8 and 34.4±2.6 mg/kg, respectively; Student's t-test, P>0.05). A three-way repeated measure ANOVA analysis with genotype, nose-poke, and METH dose as the main factors showed a significant effect of nose-poke [$F_{(1,\ 55)}$=300.32, P<0.001], METH dose [$F_{(4,\ 220)}$=36.49, P<0.001], and genotype [$F_{(1,\ 55)}$=3.48, P<0.05]. There was a significant nose-poke×METH dose interaction [$F_{(4,\ 220)}$=18.63, P<0.001], nose-poke×genotype interaction [$F_{(1,\ 55)}$=5.06, P<0.05], genotype×nose-poke× METH dose interaction [$F_{(4,\ 220)}$=2.48, P<0.05], but no genotype×METH dose interaction [$F_{(4,\ 220)}$=1.94, P=0.11]. In the dose range of 0.01–0.03 mg/kg/infusion, the dose response to METH self-administration in TNF-α (−/−) mice showed an upward shift compared with the responses in the wild-type mice (Fig. 3c). An LSD post hoc analysis revealed that TNF-α (−/−) mice had more active nose-poke responses for METH-taking than the wild-type mice at a dose of 0.01 mg/kg/infusion (P<0.01) or 0.03 mg/kg/infusion (P<0.05). There was no significant difference in active nose-poke responses to self-administer METH at a dose of 0.003 mg/kg/infusion between TNF-α (−/−) and wild-type mice. When saline was substituted for METH, no significant difference was observed in self-administration behavior between the two genotypes of mice (Fig. 3c).

The same groups of TNF-α (−/−) and wild-type mice were then subjected to METH (0.1 mg/kg/infusion) self-administration under the PR schedule. The breaking point increased significantly in TNF-α (−/−) mice compared with that in wild-type mice (Fig. 4a; Mann–Whitney test, P<0.01). The representative curves for METH self-administration under the PR schedule in the two genotypes of mice appear in Fig. 4b. Deletion of the TNF-α gene gave the mice a greater motivation to self-administer METH.