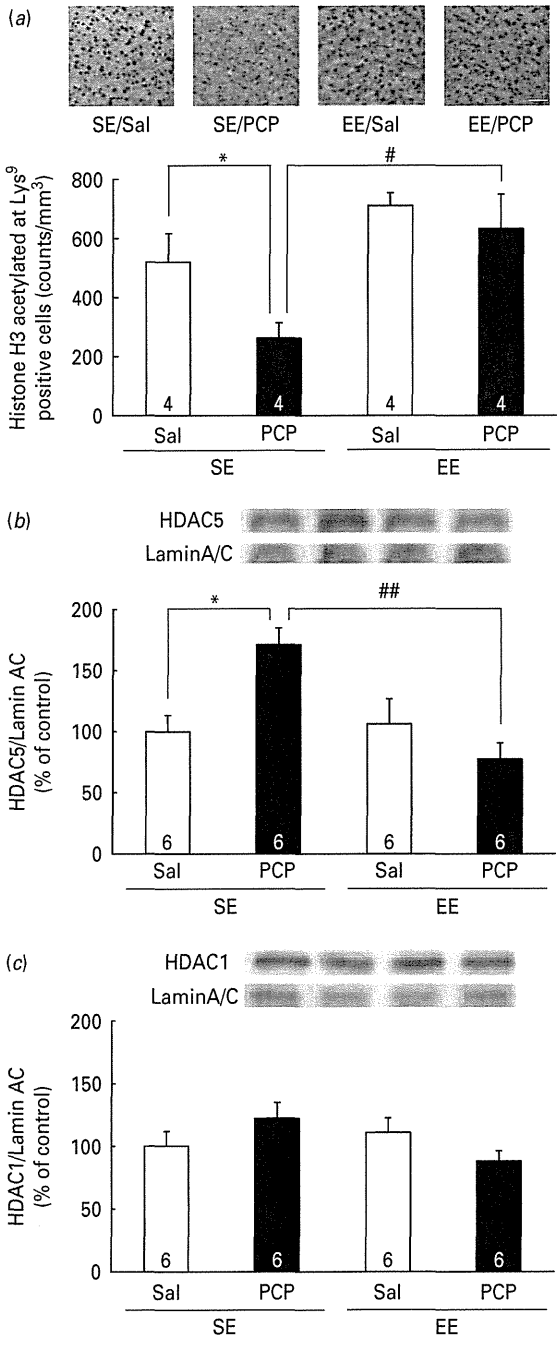

Fig. 4. Enriched environment (EE) exposure during adolescence prevented a decrease in the number of cells positive for histone H3 acetylated at Lys^9 and increase in the histone deacetylase (HDAC)5 protein expression induced by phencyclidine (PCP) in the prefrontal cortex (PFC). Mice were killed 24 h after the last PCP treatment. (*a*) Representative photomicrographs of immunostaining and quantification of the changes in the number of cells with histone H3 acetylated at Lys^9 in the PFC. Three sequential sections for the PFC were examined for counting the number of positive cells. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way analysis of variance (ANOVA) were environment: $F_{1,12}=11.59$, $p<0.01$. * $p<0.05$ compared to the results in the standard environment (SE)-exposed mice treated with saline (Sal). # $p<0.05$ compared to the results in the SE-exposed mice treated with PCP. Scale bar: 50 μm. HDAC5 (*b*) and HDAC1 (*c*) expression levels in the nuclear fraction of the PFC. The value was corrected by the level of Lamin AC. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way ANOVA were environment: $F_{1,20}=7.863$, $p<0.05$; environment × drug treatment interaction: $F_{1,20}=10.26$, $p<0.01$ for (*b*). * $p<0.05$ compared to the results in the SE-exposed mice treated with Sal. ## $p<0.01$ compared to the results of the SE-exposed mice treated with PCP.

Recently, it was reported that the levels of an 'open' chromatin marker, the acetylated Lys$^{9/14}$ of histone H3, were decreased in cultures of lymphocytes obtained from schizophrenia subjects (Gavin *et al.* 2008), whereas the levels of a 'closed' chromatin mark, the dimethylated Lys^9 of histone H3, were increased (Gavin *et al.* 2009). To investigate whether EE exposure or PCP treatment might affect chromatin modifications, we investigated the number of cells with a histone H3 acetylated at Lys^9 in the PFC (Fig. 4*a*). As shown in Fig. 4*a*, the numbers were markedly decreased in the SE-exposed mice treated repeatedly with PCP (environment: $F_{1,12}=11.59$, $p<0.05$; two-way ANOVA and $p<0.05$ by *post-hoc* comparison). In contrast, the EE-exposed mice treated with PCP showed no such decrease in the number of these cells ($p<0.05$ by *post-hoc* comparison).

Furthermore, to investigate whether the protein expression levels of HDACs might be altered by EE exposure or PCP treatment, we performed Western blotting to examine the changes in the expression level of HDAC5 and HDAC1 in the PFC of the mice (Fig. 4*b*, *c*). The HDAC5 level increased with PCP treatment in the SE-exposed mice (environment: $F_{1,20}=7.863$, $p<0.05$; environment × drug treatment interaction: $F_{1,20}=10.26$, $p<0.01$; two-way ANOVA and $p<0.05$ by *post-hoc* comparison), whereas no such increase in the HDAC5 protein expression induced by PCP was observed in the EE-exposed mice (Fig. 4*b*). There were no differences in the HDAC1 protein expression levels among the four groups (Fig. 4*c*).

Inhibition of HDACs during adolescence prevented PCP-impaired sociality and objective recognition memory

To investigate whether the inhibition of HDACs during adolescence prevents PCP-induced abnormal

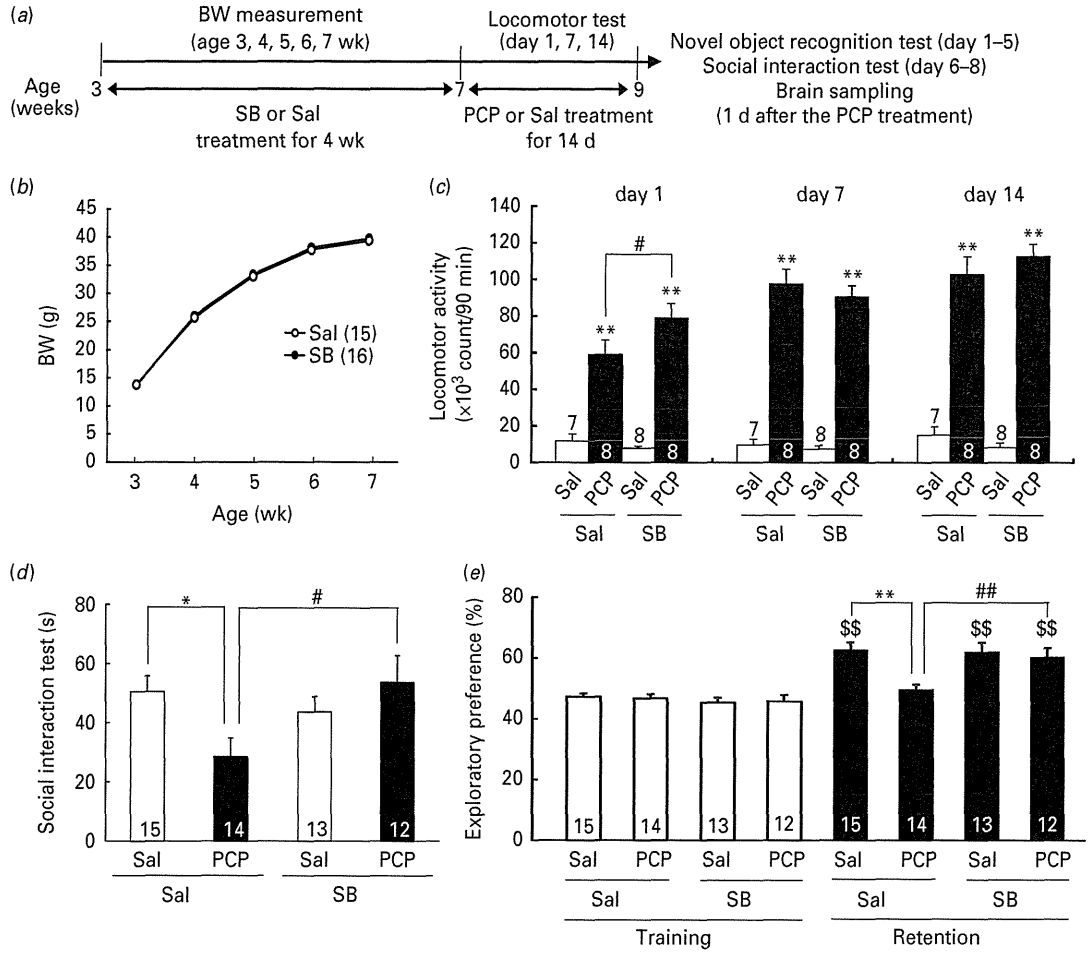

Fig. 5. Phencyclidine (PCP)-induced hyperlocomotion and impairments of sociality and recognition memory in the sodium butyrate (SB)-treated mice. (*a*) Experimental design. Mice were treated with SB (1 g/kg.d i.p.) or saline (Sal) for 4 wk from age 3 wk. After the SB treatment period, the mice were treated with PCP (10 mg/kg.d s.c.) or Sal for 2 wk. Social interaction and novel object recognition tests were conducted after the PCP treatment period. (*b*) Body weight (BW) during the SB treatment period. The number of mice is indicated in parentheses. (*c*) Locomotor changes during the PCP treatment period. Values indicate the means ± S.E. The number of mice is indicated on or within the columns. Results of two-way repeated-measures analysis of variance (ANOVA) were group: $F_{3,54}=88.34$, $p<0.01$; day: $F_{2,54}=35.08$, $p<0.01$; group × day interaction: $F_{6,54}=5.948$, $p<0.01$. ** $p<0.01$ compared to the results in the Sal-treated mice in the Sal group on the same day. # $p<0.05$ compared to the results in the Sal-treated mice in the PCP group on day 1. (*d*) Social interaction test. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way ANOVA were SB treatment × PCP treatment interaction: $F_{1,50}=6.698$, $p<0.05$. * $p<0.05$ compared to the findings in the Sal-treated mice in the Sal group. # $p<0.05$ compared to the results in the Sal-treated mice in the PCP group. (*e*) Novel object recognition test. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way repeated-measures ANOVA were group: $F_{3,50}=3.802$, $p<0.01$; session: $F_{1,50}=69.24$, $p<0.01$; group × session interaction: $F_{3,50}=4.652$, $p<0.01$. ** $p<0.01$ compared to the results in the Sal-treated mice in the Sal group in the retention session. ## $p<0.01$ compared to the results in the Sal-treated mice in the PCP group in the retention session. $$ $p<0.01$ compared to the results in the training session in the same group.

behaviour, we administered a non-selective HDAC inhibitor, SB (1 g/kg.d i.p.), to mice for 4 wk after weaning (Fig. 5*a*). SB treatment during adolescence did not affect the gain in body weight (Fig. 5*b*). Similar to the effect of EE exposure, SB treatment potentiated the hyperlocomotion induced by acute PCP treatment on day 1 (group: $F_{3,54}=88.34$, $p<0.01$; day: $F_{2,54}=35.08$, $p<0.01$; group × day interaction: $F_{6,54}=12.75$, $p<0.01$; two-way repeated-measures ANOVA and $p<0.05$ by *post-hoc* comparison) (Fig. 5*c*). SB treatment

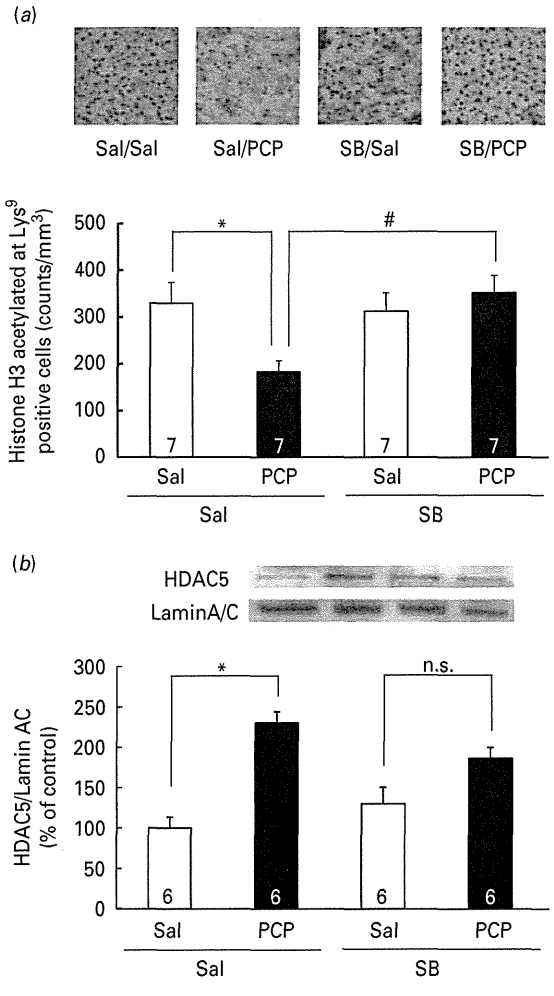

Fig. 6. Sodium butyrate (SB) treatment during adolescence prevented a decrease in the number of cells positive for histone H3 acetylated at Lys[9] and increase in the histone deacetylase (HDAC)5 protein expression induced by phencyclidine (PCP) in the prefrontal cortex (PFC). Mice were killed 24 h after the last PCP treatment. (*a*) Representative photomicrographs of immunostaining and quantification of the changes in the number of cells with histone H3 acetylated at Lys[9] in the PFC. Three sequential sections for the PFC were examined for counting the positive cells. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way analysis of variance (ANOVA) were SB treatment: $F_{1,24}=4.363$, $p<0.05$; SB treatment × PCP treatment interaction: $F_{1,24}=6.551$, $p<0.05$. * $p<0.05$ compared to the results in the saline (Sal)-treated mice in the Sal group. # $p<0.05$ compared to the results in the Sal-treated mice in the PCP group. Scale bar: 50 μm. (*b*) HDAC5 expression levels in the nuclear fraction of the PFC. The value was corrected by the level of Lamin AC. Values indicate the means ± S.E. The number of mice is indicated within the columns. Results of two-way ANOVA were PCP treatment: $F_{1,20}=11.97$, $p<0.01$. * $p<0.05$ compared to the results in the Sal-treated mice in the Sal group.

during adolescence prevented PCP-induced impairments in the social interaction test (SB treatment × PCP treatment interaction: $F_{1,50}=6.698$, $p<0.05$; two-way ANOVA and $p<0.05$ by *post-hoc* comparison) (Fig. 5*d*). In addition, SB treatment also prevented PCP-induced impairments in the novel object recognition test (group: $F_{3,50}=3.802$, $p<0.01$; session: $F_{1,50}=69.24$, $p<0.01$; group × session interaction: $F_{3,50}=4.652$, $p<0.01$; two-way repeated-measures ANOVA and $p<0.05$, $p<0.01$ by *post-hoc* comparison) (Fig. 5*e*).

Inhibition of HDACs during adolescence prevented a decrease in the number of cells with histone H3 acetylated at Lys[9] and increase in the HDAC5 protein expression induced by PCP in the PFC

Finally, to investigate whether the inhibition of HDACs during adolescence might prevent a PCP-induced decrease in the number of cells with a histone H3 acetylated at Lys[9] and increase in the HDAC protein expressions in the PFC, we administered SB (1 g/kg.d i.p.) for 4 wk to the mice after weaning, as shown in the schedule of Fig. 5*a*. As shown in Fig. 6*a*, the numbers of cells with histone H3 acetylated at Lys[9] were markedly decreased by PCP treatment in the Sal-treated mice (SB treatment: $F_{1,24}=4.363$, $p<0.05$; SB treatment × PCP treatment interaction: $F_{1,24}=6.551$, $p<0.05$; two-way ANOVA and $p<0.05$ by *post-hoc* comparison). In contrast, this decrease induced by PCP treatment was not observed in the SB-treated mice ($p<0.05$ by *post-hoc* comparison). Furthermore, the HDAC5 protein expression level was increased by PCP treatment in the Sal-treated mice (PCP treatment: $F_{1,20}=11.97$, $p<0.01$; two-way ANOVA and $p<0.05$ by *post-hoc* comparison), whereas no such increase of the HDAC5 protein expression induced by PCP treatment was observed in the SB-treated mice (Fig. 6*b*).

Discussion

In this study, we showed that exposure to an EE during adolescence prevented the impairments of sociality and recognition memory and also occurrence of the epigenetic abnormalities induced by chronic PCP treatment. SB treatment during adolescence also prevented the aforementioned behavioural impairments. Our results suggest that the effect of EE exposure of preventing PCP-induced abnormal behaviour is mediated by changes in the histone acetylation levels in the brain.

Pharmacological blockade of NMDA receptors with non-selective NMDA receptor antagonists such as PCP and MK-801 has been demonstrated to induce

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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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

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.

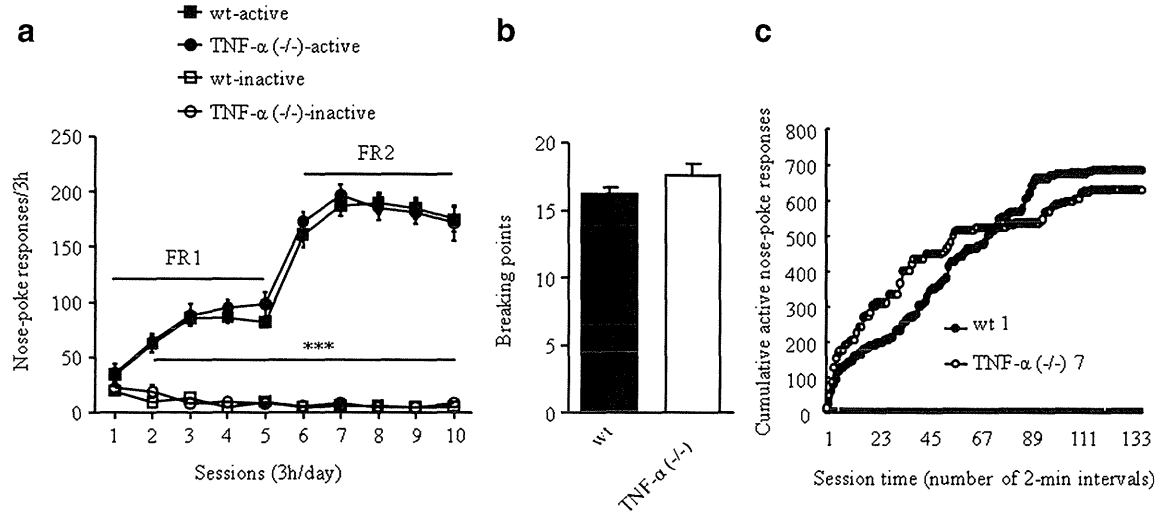

Fig. 1 Food-reinforced operant behavior and natural motivation in TNF-α (−/−) and wild-type mice. **a** Active and inactive nose-poke responses for food self-delivery under the FR2 schedule. ***P<0.001 versus inactive nose-poke responses in the same genotype. **b** Breaking points (total food pellets 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

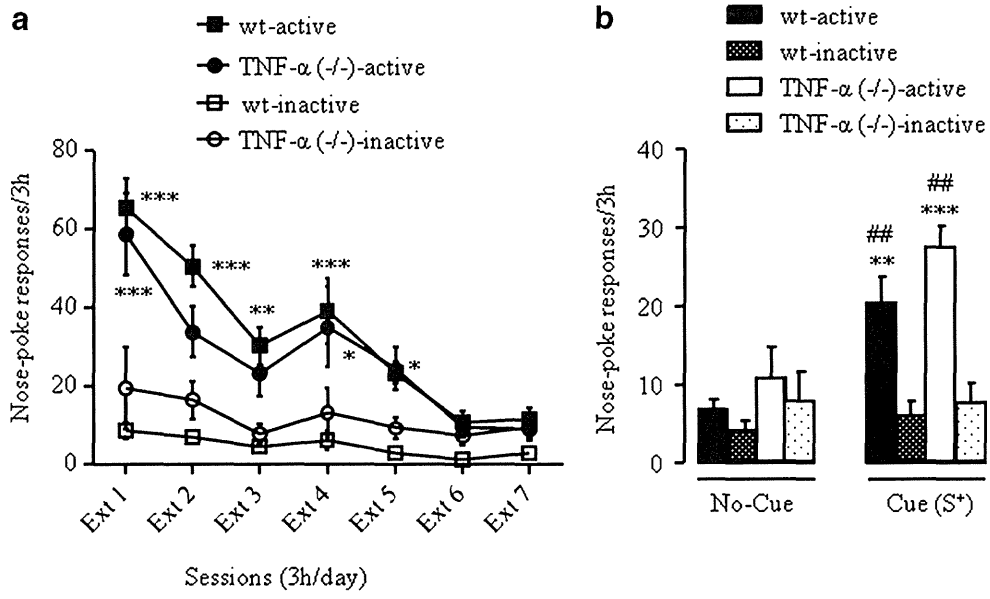

Fig. 2 Extinction and cue-induced food-seeking behavior in TNF-α (−/−) and wild-type mice. **a** Active or inactive nose-poke responses during extinction training. *P<0.05, **P<0.01, ***P<0.001 versus inactive nose-poke responses in the same genotype. **b** Active or inactive nose-poke responses during 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.

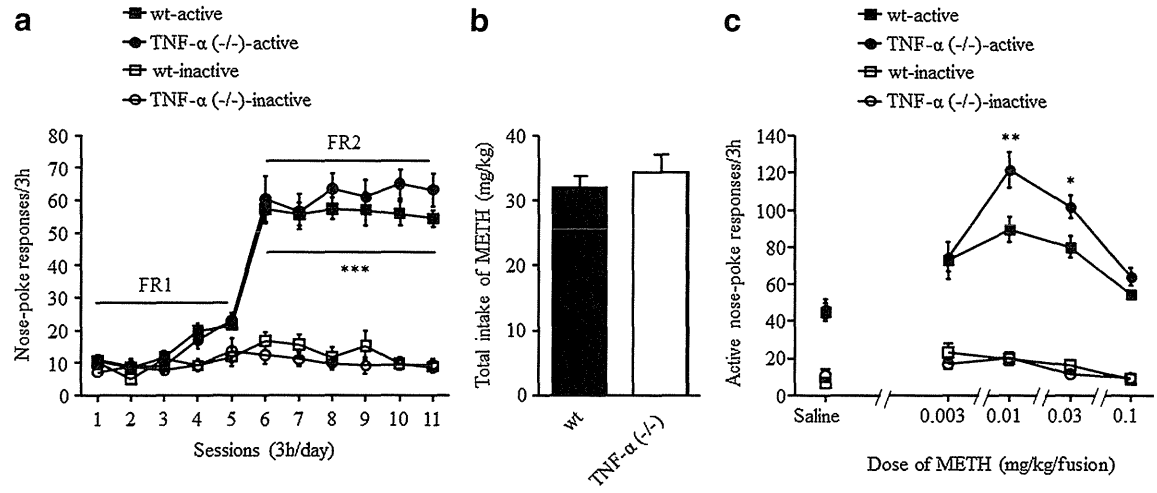

Fig. 3 Acquisition of METH self-administration and dose–response curve for METH-taking behavior in TNF-α (−/−) and wild-type mice. **a** Acquisition of METH self-administration in TNF-α (−/−) and wild-type mice. $P<0.001$ versus inactive nose-poke response in the same genotype. **b** Total intake of METH during METH self-administration training in TNF-α (−/−) and wild-type mice. **c** indicates active nose-poke responses for METH infusions during the tests for dose–response curve in TNF-α (−/−) and wild-type mice. *$P<0.05$, **$P<0.01$ versus active nose-poke responses in wild-type mice. Data are presented as the mean±SEM. $N=16$ for each genotype

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

We also determined extinction performance and cue-induced reinstatement of extinguished METH-seeking behavior in the two genotypes of mice. 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 [$F_{(1, 56)}=7.15$, $P<0.01$], day [$F_{(5, 28)}=14.54$, $P<0.001$], but no effect of genotype [$F_{(1, 56)}=0.37$, $P=0.55$]. There was a significant nose-poke×day interaction [$F_{(5, 280)}=8.31$, $P<0.001$], but there were no significant interactions with genotype (all had $P>0.59$). At the early phase of extinction training, both TNF-α (−/−) and wild-type mice exhibited more active than inactive nose-poke responses (Fig. 5a). An LSD post hoc analysis revealed significant differences between active and inactive nose-pokes in wild-type mice ($P<0.01$ on Ext 1) and in TNF-α (−/−) mice ($P<0.001$ on Ext 1 and $P<0.05$ on Ext 2). As shown in Fig. 5a, both TNF-α (−/−) and wild-type mice reduced their active nose-pokes, and there was no significant difference during the last three sessions of extinction training (L1–L3). Throughout the extinction training, there was no significant difference between groups in the number of training sessions needed for extinction criterion (data not shown). Once the animals were subjected to the test for cue-induced reinstatement, both TNF-α (−/−) and wild-type mice showed METH-seeking behavior induced by METH-associated cues (Fig. 5b; active–inactive nose-poke $F_{(1,64)}=14.21$, $P<0.001$; no-cue–cue

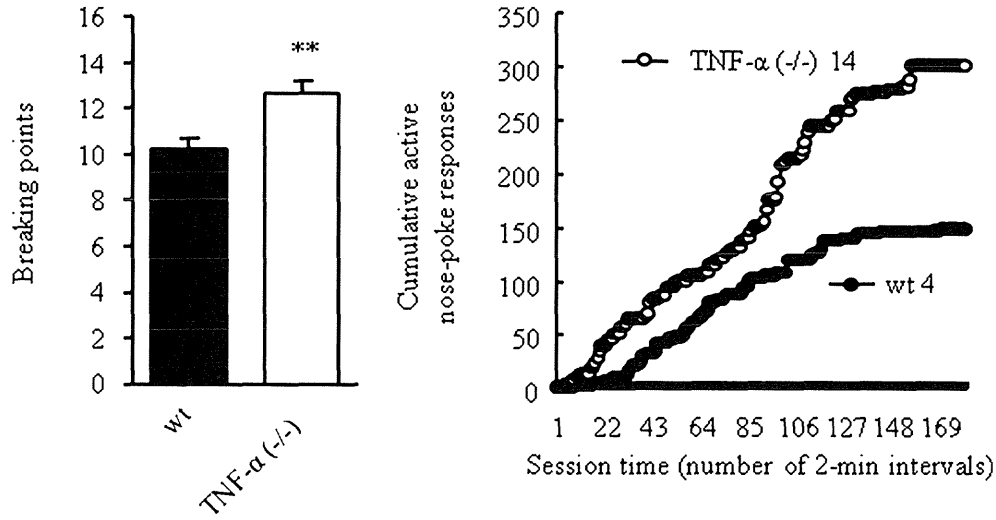

Fig. 4 Motivation to METH self-administration in TNF-α (−/−) and wild-type mice. **a** Breaking points (the final ratio) under the PR schedule of reinforcement. **b** Representative curves for cumulative active nose-poke responses for METH-taking under the PR schedule in the two genotypes of animals. *wt 4* wild-type mouse no. 4; *TNF-α (−/−) 14* TNF-α (−/−) mouse no. 14. **$P<0.01$ versus wild-type mice (Mann–Whitney test, $P<0.01$). Data are presented as the mean±SEM. $N=16$ for each genotype

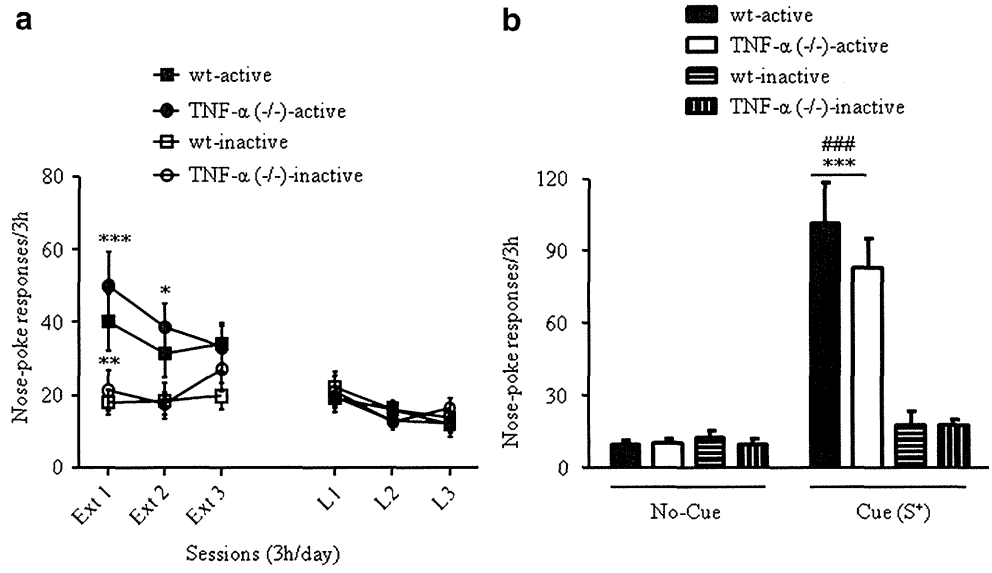

Fig. 5 Active and inactive nose-poke responses during extinction and cue-induced reinstatement of METH-seeking behavior in TNF-α (−/−) and wild-type mice. **a** Active or inactive nose-poke responses during the extinction training between TNF-α (−/−) and wild-type mice. *$P<0.05$, **$P<0.01$, ***$P<0.001$ versus inactive nose-poke responses in the same genotype. **b** Active or inactive nose-poke responses during the test for cue-induced reinstatement in TNF-α (−/−) and wild-type mice. ***$P<0.01$ versus inactive nose-poke responses in the same genotype (active–inactive nose-poke $F_{(1,64)}=14.21$, $P<0.001$; no-cue–cue $F_{(1,64)}=58.06$, $P<0.001$; interaction $F_{(3,646)}=15.03$, $P<0.001$). $^{\#\#\#}P<0.001$ versus the no-cue in the same genotype (ANOVAs for wild-type $F_{(1,44)}=35.63$, $P<0.001$; for TNF-α (−/−) $F_{(1,52)}=53.78$, $P<0.001$). Data are presented as the mean±SEM. $N=12$ for wild-type and 13 for TNF-α (−/−) mice

$F_{(1,64)}=58.06$, $P<0.001$; interaction $F_{(3,646)}=15.03$, $P<0.001$). There was no significant difference in cue-induced reinstatement of extinguished METH-seeking behavior between TNF-α (−/−) and wild-type mice (Fig. 5b; ANOVAs for genotype $F_{(1,48)}=2.83$, $P>0.05$). We conclude that the effect of TNF-α gene deletion in extinction training and cue-induced reinstatement of METH-seeking behavior is minimal.

Discussion

We found that targeted deletion of the TNF-α gene did not affect the motivation for natural reward and food-seeking behavior in mice. The deletion did alter the dose response to METH and motivation to self-administer METH. Interestingly, there was no significant difference in cue-induced reinstatement of METH-seeking behavior in the two genotypes of mice. TNF-α may affect the reinforcing property of METH, but not the relapsing behavior induced by METH-associated cues.

We have previously demonstrated that TNF-α activates vesicular dopamine uptake in vitro to attenuate dopamine transporter-mediated METH- or morphine-induced release of dopamine in the nucleus accumbens in vivo (Nakajima et al. 2004; Yamada and Nabeshima 2004; Niwa et al. 2007a; Yamada 2008). Exogenous TNF-α treatment decreases the reward property of METH or morphine, locomotor sensitization, and discriminative stimulus effects of METH. TNF-α (−/−) mice were more responsive to METH- or morphine-induced locomotor sensitization and conditioned place preference than wild-type mice (Nakajima et al. 2004; Niwa et al. 2007a, b). In the present study, TNF-α (−/−) mice responded with more active nose-pokes to self-administer METH at doses of 0.03 and 0.01 mg/kg/infusion than wild-type mice did. There are two conflicting interpretations for the upward shift of the dose–response curve for METH self-administration in TNF-α (−/−) mice. One is that the pharmacological effects of METH are reduced in TNF-α (−/−) mice, and the animals have to self-administer more METH to get the same effects. The other is that TNF-α gene deletion increases sensitivity to METH self-administration. Under a PR schedule of METH reinforcement, TNF-α (−/−) mice had higher breaking points (motivation) than wild-type mice. The upward shift of the dose–response curve for METH self-administration in TNF-α (−/−) mice may be caused by an increase in sensitivity to METH self-administration during the maintenance phase. These new observations, together with our previous findings, support the theory that the mesolimbic dopaminergic pathway in the brain is crucial in both the rewarding and reinforcing properties of addictive drugs (Koob 1992; Carlezon and Thomas 2009; Koob and Volkow 2009). There was no significant difference between

the TNF-α (−/−) and wild-type mice in the acquisition of METH self-administration. One explanation is that the dose of METH (0.1 mg/kg/infusion) for developing drug self-administration was high enough to mask the potential behavioral alternations induced by the targeted deletions of TNF-α gene. This explanation is supported by evidence of an upward shift in dose response for METH self-administration and higher motivation for METH in TNF-α (−/−) mice. Our results are also consistent with recent reports suggesting an association between TNF-α and cocaine or heroin self-administration (Kubera et al. 2008; Weber et al. 2009).

We did not observe a significant difference in extinction training and cue-induced reinstatement of METH-seeking behavior between TNF-α (−/−) and wild-type mice. The dissociation of METH self-administration and cue-induced relapsing behavior in TNF-α (−/−) mice may result from dissociable neural substrates for drug self-administration and drug relapsing behavior (Grimm and See 2000). Neural substrates for drug reward/reinforcement are different from those of drug relapse although they may overlap (Self 1998; Feltenstein and See 2008; Koob and Volkow 2009). It has been widely accepted that the mesolimbic dopaminergic pathway is pivotal in the development of drug self-administration and motivation to self-administer addictive drugs (Pierce and Kumaresan 2006). In contrast, much more extensive and complex brain areas and transmitter systems are needed for drug extinction and relapsing behavior. Among these transmitter pathways, glutamate transmission is critical for relapse to many addictive drugs (Gass and Olive 2008; Knackstedt and Kalivas 2009). Previously, we found no correlation between cue-induced relapsing behavior or extinction and the total amount of METH intake during drug self-administration (Yan et al. 2007; Yan and Nabeshima 2009). Given that TNF-α has been identified as an endogenous modulator for the addictive drug-induced release of dopamine in the nucleus accumbens, it seems reasonable that TNF-α affects METH self-administration and motivation but not cue-induced relapsing behavior. The precise mechanisms underlying this behavioral dissociation remain unclear.

Previously, we have shown that exogenous TNF-α inhibits METH-associated discriminative effects in rats (Nakajima et al. 2004). TNF-α or TNF-α inducer attenuates METH- or morphine-induced conditioned place preference in mice (Niwa et al. 2007a, b). In human opiate addicts, the level of TNF-α expression in the brain (locus coeruleus) is higher than that in control subjects (Dyuizen and Lamash 2009). Levels of circulating TNF-α are altered in alcoholics, opiate addicts, and cocaine or marijuana abusers (Baldwin et al. 1997; Peng et al. 1999; Irwin et al. 2007, 2009; Gonzalez-Quintela et al. 2008; Sacerdote et al. 2008; Li et al. 2009; Franchi et al. 2010), and TNF-α may be a candidate biomarker of alcohol abuse and alcoholism (Achur et al. 2010). In our study in animal, TNF-α (−/−) mice were more sensitive to METH self-administration and had a higher motivation for getting METH than wild-type mice. The TNF-α (−/−) and wild-type mice had a similar sensitivity for natural reward and cue-induced seeking. METH self-administration and METH relapsing behavior are dissociable in TNF-α (−/−) mice. TNF-α may be a valuable target for the development of potential therapeutic agents to prevent drug abuse or help cessation. It is also a potential diagnostic biomarker for drugs of abuse, although more extensive clinical investigations and studies of neurobiological mechanisms are warranted.

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Neurobiology of Disease

GABAergic Precursor Transplantation into the Prefrontal Cortex Prevents Phencyclidine-Induced Cognitive Deficits

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Phencyclidine (PCP) is a noncompetitive NMDA receptor antagonist, and it induces schizophreniform cognitive deficits in healthy humans and similar cognitive deficits in rodents. Although the PCP-induced cognitive deficits appear to be accompanied and possibly caused by dysfunction of GABAergic inhibitory interneurons in the prefrontal cortex (PFC), the potential benefit(s) of GABAergic interneuron manipulations on PCP-induced cognitive deficits remains unexplored. In this study we show that when embryonic medial ganglionic eminence (MGE) cells, many of which differentiate into cortical GABAergic interneurons *in situ*, were grafted into the medial PFC (mPFC) of neonatal mice, they differentiated into a specific class of GABAergic interneurons and became functionally integrated into the host neuronal circuitry in adults. Prior MGE cell transplantation into the mPFC significantly prevented the induction of cognitive and sensory-motor gating deficits by PCP. The preventive effects were not reproduced by either transplantation of cortical projection neuron precursors into the mPFC or transplantation of MGE cells into the occipital cortex. The preventive effects of MGE cell transplantation into the mPFC were accompanied by activation of callosal projection neurons in the mPFC. These findings suggest that increasing GABAergic interneuron precursors in the PFC may contribute to the development of a cell-based approach as a novel means of modulating the PFC neuronal circuitry and preventing schizophreniform cognitive deficits.

Introduction

Noncompetitive NMDA receptor antagonists, including phencyclidine (PCP), evoke schizophreniform cognitive deficits in healthy humans (Javitt and Zukin, 1991) and similar cognitive deficits in rodents (Mouri et al., 2007a). The cognitive deficits induced by the NMDA receptor antagonists are associated with altered rhythmic activities (Sebban et al., 2002; Kiss et al., 2011) and alterations of projection neuron activity in the prefrontal cortex (PFC) (Jackson et al., 2004; Kargieman et al., 2007). The alterations of projection neuron activity appear to be preceded by (Homayoun and Moghaddam, 2007) and to be reproduced by (Sohal et al., 2009; Korotkova et al., 2010) decreased activity of fast-spiking parvalbumin (PV)-positive inhibitory GABAergic interneurons in the PFC. A majority of cortical GABAergic interneurons are classified into either PV-positive interneurons or somatostatin (SST)-positive interneurons (Kawaguchi and Kubota, 1997). PV-positive interneurons are involved in cortical rhythmogenesis (Klausberger et al., 2005; Fuchs et al., 2007; Cardin et al., 2009; Sohal et al., 2009; Korotkova et al., 2010), and SST-positive interneurons may also be involved in cortical rhythmogenesis, because they are extensively electrically coupled into networks that robustly synchronize their spiking activity (Gibson et al., 1999, 2005) in the theta range (4–7 Hz), which induces synchronized IPSPs in neighboring projection neurons (Beierlein et al., 2000). Thus, PCP may alter the rhythmic activity of projection neurons in the PFC, and that may be related to the cognitive deficits through the dysfunction of PV-positive interneurons and/or SST-positive interneurons in the PFC. However, the potential benefit(s) of manipulations of these interneurons in the PFC on PCP-induced cognitive deficits remains unexplored.

During the development of the mouse cortex, most PV-positive interneurons and SST-positive interneurons are generated in the medial ganglionic eminence (MGE) of the embryonic ventral forebrain (Lavdas et al., 1999; Xu et al., 2004; Fogarty et al., 2007). A majority of SST-positive interneurons secrete Reelin (Miyoshi et al., 2010), which enhances NMDA receptor-mediated signaling (Herz and Chen, 2006; Knuesel, 2010). When transplanted into the cortex of older animals, the young MGE cells disperse widely and differentiate into either PV-positive interneurons or SST-positive interneurons (Wichterle et al., 1999; Alvarez-Dolado et al., 2006; Baraban et al., 2009; Southwell et al.,

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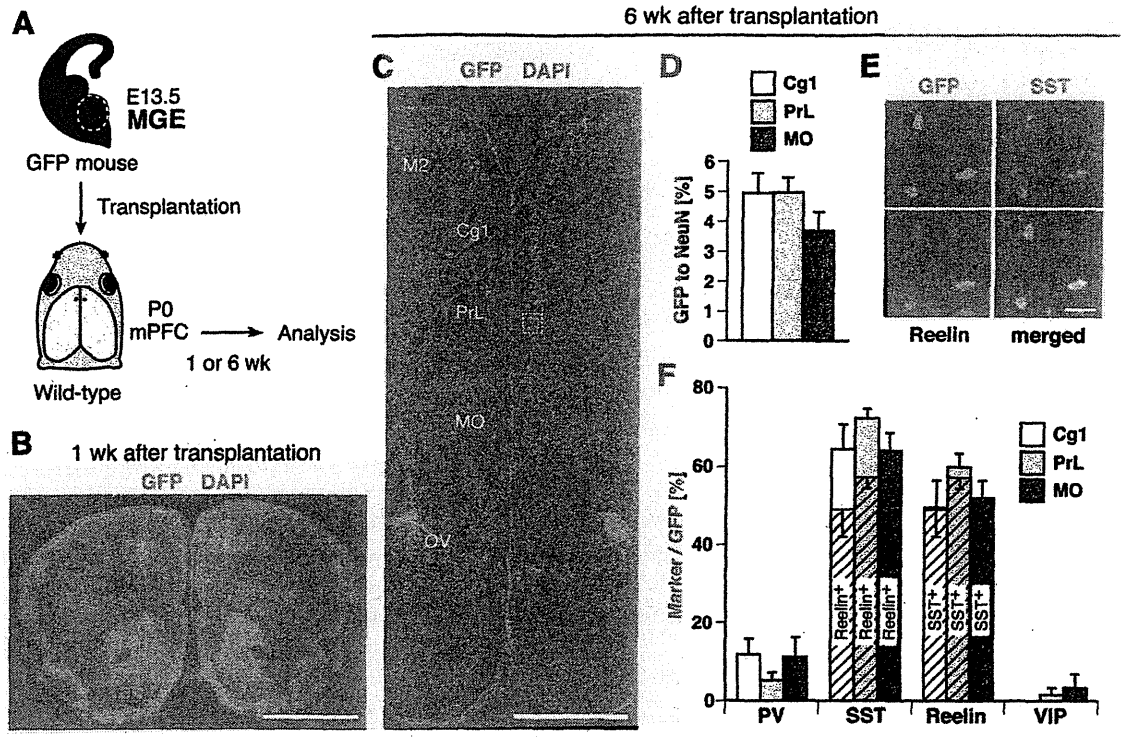

Figure 1. A majority of the MGE cells transplanted into the neonatal mPFC differentiated into an SST/Reelin-expressing class of GABAergic interneurons in adults. ***A***, Schema of MGE dissection (region within the dashed white line in E13.5 GFP-expressing telencephalic coronal slices) and bilateral transplantation into the mPFC of P0 wild-type recipient mice (filled red circles) (dorsal view of a head). Transplanted cells were analyzed 1 week or 6 weeks after transplantation. ***B***, ***C***, Representative distribution of GFP-expressing cells (green) on coronal sections (100 μm thick in ***B***, 8 μm thick in ***C***) at the level of the prospective injection site at 1 week (***B***) and 6 weeks (***C***) after transplantation. ***D***, Ratio of the number of GFP-expressing cells to the number of NeuN-positive cells within the Cg1 (white bar) (n = 311 GFP-expressing cells, 6373 NeuN-positive cells), the PrL (light gray bar) (n = 844 GFP-expressing cells, 17,520 NeuN-positive cells), and the MO (dark gray bar) (n = 1242 GFP-expressing cells, 35,591 NeuN-positive cells) at 6 weeks after transplantation (n = 12 hemispheres). There were no statistically significant differences between the regions. ***E***, Enlarged view of the boxed area in ***C***. Coexpression of SST (light blue) and Reelin (magenta) in GFP-expressing cells in the PrL. The "merged" panel is a merged figure of the other three. ***F***, Calculation of the percentage of GFP-expressing cells expressing an interneuron subtype marker(s) within the Cg1 (white bars) (n = 255 GFP-expressing cells), the PrL (light gray bars) (n = 497 GFP-expressing cells) and the MO (dark gray bars) (n = 343 GFP-expressing cells) at 6 weeks after transplantation (n = 5–6 hemispheres). Shaded bars indicate the percentage of GFP-expressing cells that are SST/Reelin-coexpressing cells. M2, Secondary motor cortex; OV, olfactory ventricle. Scale bars: ***B***, 500 μm; ***C***, 1 mm; ***E***, 20 μm.

2010). MGE cell transplantation increases GABA-mediated inhibition on host projection neurons and modulates host neuronal circuitry and its activity (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Southwell et al., 2010; Zipancic et al., 2010). Their transplantation reduces seizures (Baraban et al., 2009; Zipancic et al., 2010), ameliorates motor deficits (Martínez-Cerdeño et al., 2010) and induces cortical plasticity (Southwell et al., 2010).

All of the above findings together raise the possibility that MGE cell transplantation into the PFC may modulate host medial PFC (mPFC) neuronal circuitry through GABA-mediated inhibition and/or Reelin-mediated signaling, which may then prevent the alteration of the rhythmic activity of projection neurons and the cognitive deficits induced by PCP. In the present study, we especially focused on an analysis of the expected final effect of MGE cell transplantation into the mPFC, i.e., prevention of the induction of cognitive deficits by PCP.

Materials and Methods

Animals

Embryonic donor tissue was obtained by crossing ICR wild-type female mice with homozygous green fluorescent protein (GFP)-expressing C57BL/6-Tg(CAG-EGFP)C14-Y01-FM131Osb male mice (Okabe et al., 1997), in which essentially all cell types express GFP. Breeder and host ICR wild-type mice were obtained from SLC Japan. Littermates were used as controls in all comparison studies, including the c-Fos expression and behavioral studies. All animals intended for use in the behavioral studies were maintained on a 12 h light/dark cycle (lights on at 8:00 A.M.) and given *ad libitum* access to food (CE2; Clea Japan Inc.). Noon on the day a vaginal plug was detected was recorded as embryonic day 0.5 (E0.5), and E19.5 was counted as postnatal day 0 (P0). All animal experiments were performed in accordance with the Guidelines for the Care and Use of Laboratory Animals of Keio University School of Medicine, 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.

Donor tissue dissection

Approximate ventricular and subventricular zones of the MGE or rostromedial cortical wall, the presumptive region of the mPFC, of E13.5 GFP-expressing embryos were dissected in chilled Hanks' balanced salt solution devoid of calcium and magnesium (Sigma). Explants were mechanically dissociated into a single cell suspension in Leibovitz's L-15 medium (Invitrogen) containing DNase I (100 μg/ml; Sigma) by repeated pipetting with a 200 μl tip (15–25 times). Dissociated cells were pelleted by centrifugation (5 min, 800 g). Cell suspensions (~10^6 cells/μl in L-15 medium containing 100 μg/ml DNase I) were kept on ice until used.

Cell transplantation

The donor cell suspensions were front-loaded into beveled glass micropipettes (~75 μm caliber at the tip) (GD-1; Narishige). P0 host mice were anesthetized by chilling on ice, and they were then fixed in position under a stereomicroscope. The x-y coordinates of the injection sites were estimated from the surface anatomy: for transplantation into the mPFC (Fig. 1*A*), the rostrocaudal level of the injection site was one fourth the dis-

tance from the rostrocaudal level of the center of the eye to the rostrocaudal level of the lambdoid suture. The mediolateral level of the injection site was one eighth the distance from the mediolateral level of the center of the eye to the midline. The injection site was defined as the point of intersection between a line passing through the rostrocaudal level of the injection site and a line passing through the mediolateral level of the injection site in each hemisphere; for transplantation into the occipital cortex (see Fig. 3*B*), the rostrocaudal level of the injection site was half the distance from the rostrocaudal level of the lambdoid suture to the rostrocaudal level of the caudal edge of the cerebral cortex. The mediolateral level of the injection site was half the distance from the mediolateral level of the center of the eye to the midline. The injection site was defined as the point of intersection between a line passing through the rostrocaudal level of the injection site and a line passing through the mediolateral level of the injection site in each hemisphere. The *z*-coordinate of the injection sites was −0.9 mm below the skin surface for transplantation into the mPFC and −0.7 mm below the skin surface for transplantation into the occipital cortex. Approximately 10^5 cells (~0.1 μl) were transcranially injected into the mPFC or occipital cortex of each hemisphere bilaterally (total of 2×10^5 cells per animal). Cell flow within the pipette during injection was confirmed visually during every injection. A similar volume of vehicle was injected into controls for the behavioral analysis. Vehicle injection is a standard control procedure for grafting studies and provides more reliable controls than injection of "dead" cells, which would cause toxic effects and exaggerate any positive graft effects under certain conditions (Modo et al., 2003). Immediately after the injections, the recipient mice were placed on a warm surface until they became active, and they were then returned to their mothers until they reached weaning age (4 weeks).

Immunohistochemistry

Animals were transcardially perfused with 4% paraformaldehyde (PFA) or 4% PFA containing 0.08% glutaraldehyde in phosphate buffer (0.1 M, pH 7.4). The brains were removed, postfixed at 4°C for 2 h to overnight in the same fixatives, and washed with PBS (0.1 M, pH 7.4). For cryosectioning, brains were cryoprotected in 30% sucrose in PBS at 4°C. Coronal sections were cut with a vibrating-blade microtome (50–100 μm) (VT-1000; Leica Microsystems) or a frozen sliding microtome (8–10 μm) (CM3050 M; Leica Microsystems). The sections were incubated in PBS with 0.3% Triton X-100 and 5% normal donkey serum (NDS) for 1–2 h at room temperature (RT) and then for 1–2 d at RT or for 2 d at 4°C in the primary antibody diluted in PBS with 0.3% Triton X-100 and 1% NDS. The primary antibodies used were as follows: chicken anti-GFP (1:500; Abcam), mouse anti-NeuN (1:50; Millipore), mouse anti-CNPase (1:500; Abcam), rabbit anti-GFAP (1:1000; DAKO), rabbit anti-GABA (1:1000; Sigma), mouse anti-PV (1:1000; Sigma); rabbit anti-PV (1:2000; Abcam), rat anti-SST (1:100; Millipore), mouse anti-Reelin (G10; 1:500; gift from Dr. André M. Goffinet, University of Louvain, Brussels, Belgium), rabbit anti-vasoactive intestinal polypeptide (VIP) (1:300; Immunostar), rabbit anti-c-Fos (1:500; Santa Cruz Biotechnology), mouse anti-Satb2 (1:50; Abcam), or rat anti-Ctip2 (1:500; Abcam). The sections were incubated for 1–3 h at RT in a secondary antibody diluted in 1% NDS in PBS. The secondary antibodies used were as follows: donkey DyLight488-conjugated anti-chicken IgY, donkey DyLight549- or TRITC-conjugated anti-rabbit IgG, donkey Cy5-, DyLight549-, or TRITC-conjugated anti-mouse IgG or donkey Cy5-conjugated anti-rat IgG (all at 1:100; all from Jackson ImmunoResearch). For nuclear staining, sections were incubated in 1% 4,6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS for 10–30 min at RT. Images were captured with a CCD camera (VB-7010; Keyence) attached to an epifluorescence microscope (BX60; Olympus) or with a confocal microscope (FV1000; Olympus).

Quantification of transplanted cells

MGE cell transplantation into the mPFC. To estimate the number of surviving cells after MGE cell transplantation into the mPFC, we counted the number of cortical GFP-positive cells in every eighth coronal slice from brains at 6 weeks after transplantation. The total number of surviving MGE cells was calculated by multiplying the number counted by eight. The GFP-expressing cells in the prelimbic cortex (PrL) (Franklin and Paxinos, 2008) were examined for expression of NeuN, GABA, SST, CNPase, and GFAP, and they were counted manually to calculate the ratio of GFP/NeuN-double-positive cells to GFP-positive cells, the ratio of GFP/GABA-double-positive cells to GFP-positive cells, the ratio of GFP/CNPase-double-positive cells to GFP-positive cells, the ratio of GFP/GFAP-double-positive cells to GFP-positive cells, the ratio of GFP/GABA-double-positive cells to GABA-positive cells, and the ratio of GFP/SST-double-positive cells to SST-positive cells. To examine the interneuron subtypes and the functional integration of the transplanted cells, the GFP-expressing cells in area 1 of the cingulate cortex (Cg1), the PrL, and the medial orbital cortex (MO) (Franklin and Paxinos, 2008) were examined for expression of several markers and were counted manually. To determine the ratio of the number of GFP-expressing cells or c-Fos-positive cells to the number of NeuN-positive cells in the Cg1, the PrL, and the MO (Franklin and Paxinos, 2008), the numbers of c-Fos-positive and NeuN-positive cells in these regions were counted with NIH ImageJ software (version 1.40 g), and the number of GFP-expressing cells was counted manually. To examine the cell-type of c-Fos-positive cells, the c-Fos-positive cells in the PrL and the MO (Franklin and Paxinos, 2008) were examined for expression of Satb2 and GFP manually and were counted with the NIH ImageJ software (version 1.40 g).

Rostromedial cortex cell transplantation into the mPFC. The bright GFP-expressing cells with DAPI staining around the center of the cell in the Cg1, the PrL, and the MO (Franklin and Paxinos, 2008) were examined for expression of Satb2 and Ctip2 at 6 weeks after transplantation and were counted manually. Most GFP-expressing cells were clustered in the Cg1 and the PrL, the presumptive injection site, and essentially no GFP-expressing cells were found in the MO.

MGE cell transplantation into the occipital cortex. The GFP-expressing cells in the primary visual cortex (Franklin and Paxinos, 2008) were examined for expression of GABA, PV, and SST at 6 weeks after transplantation and were counted manually. Most GFP-expressing cells were found in the caudolateral cortex and the hippocampus, and essentially no cells were found in the mPFC.

The SEM in the histological analyses is attributable to the variability across different hemispheres.

Behavioral analysis

We decided on ~6 weeks after transplantation (P40–P47) as the optimal time to perform the behavioral tests in this study, because synaptic integration of MGE cells transplanted into neonatal mouse visual cortex has been demonstrated at approximately that time (~36 d after transplantation) (Southwell et al., 2010) and because 6 weeks of age in mice appeared to largely correspond to mid-adolescence in humans (Spear, 2000), when the symptoms of schizophrenia usually are first manifested.

Novel enriched environment. The novel enriched environment consisted of a Plexiglas open field box (30 × 30 × 35 cm) whose floor was covered with sawdust, and two novel objects (half of a red plastic ball and a 100 ml culture flask with a light green cap) were placed on the floor. The experimental mice were placed into the box individually and allowed to explore for 10 min. Control mice continued to be housed in their home cage.

PCP administration. PCP hydrochloride was synthesized according to the method reported in the literature (Maddox et al., 1965) and checked for purity. PCP dissolved in saline was injected (1 mg/kg, s.c.) 6 weeks after transplantation, 30 min before the prepulse inhibition (PPI) test and the training session in the novel object recognition test (NORT). Controls were injected with an equal volume of vehicle (saline).

NORT. The NORT was conducted as described previously (Mouri et al., 2007b) with minor modifications. The experimental apparatus consisted of a Plexiglas open field box (30 × 30 × 35 cm) whose the floor was covered with sawdust. The test procedure consisted of three sessions: habituation, training, and retention. At 6 weeks after transplantation, each mouse was individually habituated to the box by allowing 10 min of exploration in the absence of any objects each day for 3 consecutive days (Day 1–3) (habituation session). On Day 4, two novel objects were symmetrically placed on the sawdust on the floor of the box, 8 cm from the walls. Each animal was allowed to explore the box for 10 min, and the time spent exploring each object was recorded (training session). The objects