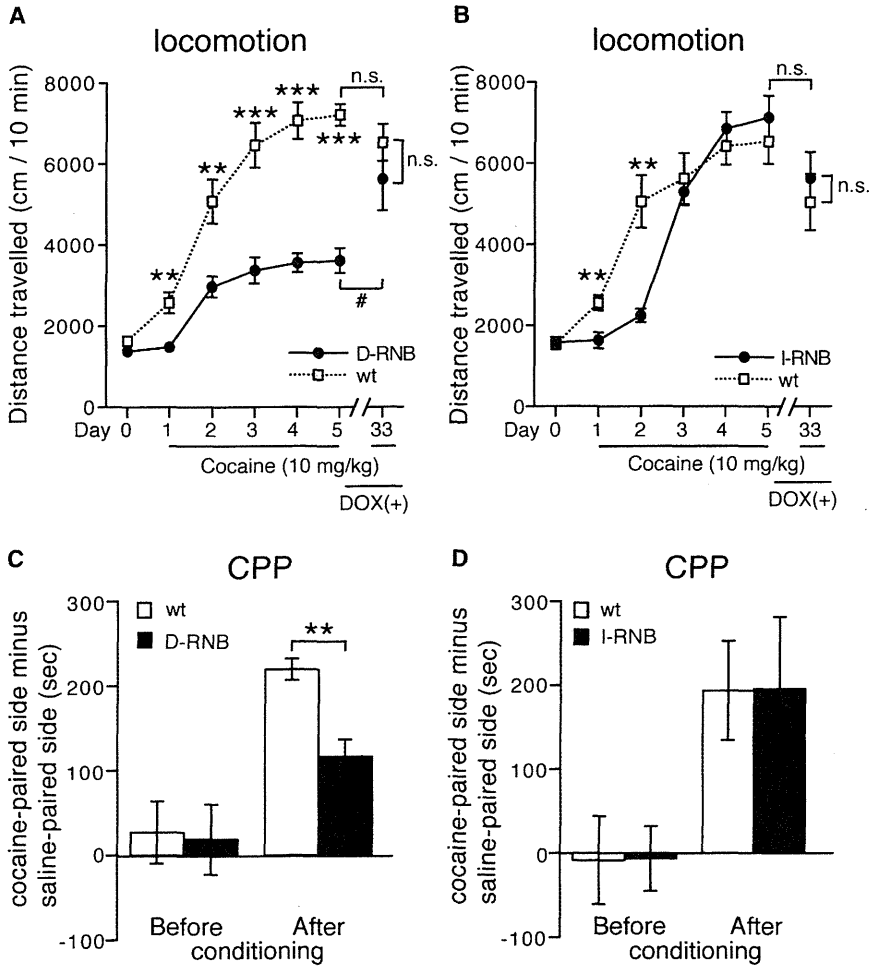

Figure 6. Impairment of Cocaine Sensitization in the D-RNB Mice

The V-S-tTA virus (A and C) or the V-E-tTA virus (B and D) was bilaterally injected into the NAc of wild-type and TN mice.

(A and B) Two weeks after the viral injection, the animals received i.p. saline once a day and were habituated to a novel chamber for 3 days. Cocaine (10 mg/kg) was then i.p. injected once a day from day 1 to day 5, and immediately after the cocaine injection, the locomotor activity was counted for a 10 min period. These animals were treated with DOX from day 6 to day 33, and on day 33, locomotor activity was counted for a 10 min period immediately after 10 mg/kg cocaine injection. Marks and error bars represent the mean ± SEM (n = 8 each). **p < 0.01, ***p < 0.001 (WT versus RNB); #p < 0.05, (D-RNB on day 5 versus day 33); n.s., not significant. Repeated-measured ANOVA showed a significant difference between the virus-injected wild-type and RNB mice (in A, for genotype, $p < 0.001$; for day, $p < 0.001$; interaction genotype × day, $p < 0.001$; in B, for genotype, $p = 0.723$, for day, $p < 0.001$, interaction genotype × day, $p < 0.001$).

(C and D) CPP was developed by repeated cocaine (10 mg/kg) administration for 3 days and measured on day 4. Columns and error bars represent the mean ± SEM (C, n = 8 each; D, n = 6 or 7). **p < 0.01.

It has been reported that repeated cocaine administration induces LTD in local field potentials of the NAc via D2 receptor stimulation (Goto and Grace, 2005a). We tested whether the cocaine sensitization could disrupt the synaptic plasticity involved in aversive behavior. In this test, cocaine was administered to wild-type mice for 5 days, and after a 3 day cocaine-free interval, aversive behavior was tested by the one-trial inhibitory avoidance task (Figure 7F). This test clearly indicated that cocaine sensitization severely impaired aversive behavior as compared with saline-injected control animals. Thus, similar to blockade of the indirect pathway, cocaine sensitization that alters the adaptive mechanisms of striatopallidal transmission causes impairment of aversive behavior. The striatonigral and striatopallidal transmission thus plays differential roles in reward-based and aversive behavior, respectively.

DISCUSSION

This study has established genetic manipulation that allowed determination of distinct roles of striatonigral and striatopallidal transmission in the basal ganglia circuitry. The expression of TN under the control of the SP and Enk promoters was mutually exclusive in the two types of MSNs; and the DOX-dependent TN expression was reflected in reversible cleavage of VAMP2, which is indispensable for transmitter release from the synaptic vesicles (Wada et al., 2007). The electrophysiological and c-*fos* mRNA analyses indicated that the selective expression of TN separately blunted transmission in the direct and indirect pathways. Furthermore, abnormal but reverse turning was induced by unilateral injection of the recombinant viruses, in agreement with the classical regulatory model of the two pathways in motor balance (Gerfen et al., 1990; Graybiel, 2000; Pycock, 1980). Thus, our gene-manipulating technique allowed selective and reversible blockade of striatonigral and striatopallidal transmission in a DOX-dependent manner.

This investigation has disclosed not only the coordinated function of these two pathways in acute psychostimulant responses but also unexpected, distinct roles of each pathway in reward-based and aversive behavior. The predominant role of the direct pathway in cocaine-induced adaptive responses is generally consistent with previous reports based on gene targeting and pharmacological analyses (Baker et al., 1996, 1998; Caine et al., 2007; Smith et al., 2002; Welter et al., 2007; Xu et al., 2000; but see also Miner et al., 1995). However, if the coordinated regulation by these two pathways, as is generally accepted, is a key mechanism of rewarding and aversive learning behavior, blunting one of the pathways would impair the learning ability of both rewarding and aversive behavior.

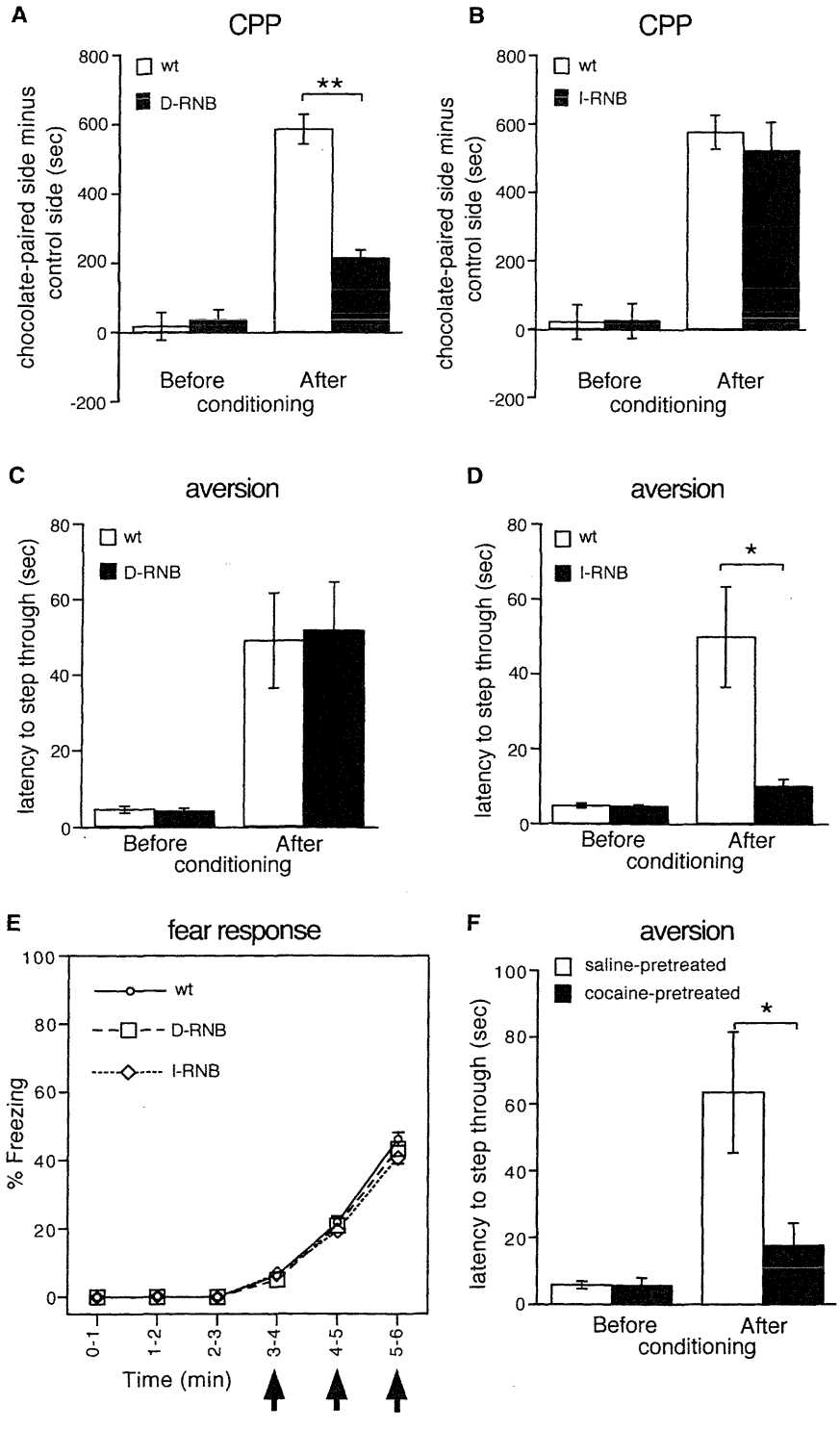

Figure 7. Analysis of Striatonigral and Striatopallidal Transmission in Reward-Based and Aversive Learning Behavior

(A and B) Two weeks after the viral injection into the NAc, animals were trained by pairing a standard food with one chamber and a chocolate food with the other chamber for 3 days. CPP for appetitive reward was then measured on day 4 (n = 6 each).

(C and D) Two weeks after the viral injection into the NAc, retention of aversive memory was tested by the one-trial inhibitory avoidance task. When mice moved from a light chamber to a preferred dark chamber, electric shocks were delivered. Memory retention was tested 24 hr later by measuring latencies for the animals to enter the dark chamber (n = 6 or 7).

(E) Percentage freezing was determined for the 1 min period by giving electric shocks at 3, 4, and 5 min (arrows) (n = 4–9). ANOVA with repeated-measures revealed a significant increase in post-stimulus freezing but no statistical difference among the three groups of mice (for genotype, $p = 0.297$; for time, $p < 0.001$; interaction genotype × time, $p = 0.53$).

(F) Wild-type mice received cocaine (10 mg/kg) for 5 days. After a cocaine-free interval for 3 days, memory retention for aversive stimuli was analyzed as described in (C) and (D) (n = 6 or 7). Columns and error bars represent the mean ± SEM. *$p < 0.05$, **$p < 0.01$.

In contrast, the present investigation has disclosed that each pathway has a differential and selective role in rewarding and aversive behavior. Because the aversive and rewarding behaviors were tested 1 day after footshocks and 3 days after training, respectively, impairments of these behaviors could have resulted from different temporal effects of transmission blockade on these behaviors. However, this possibility is unlikely, because blockade of each pathway abolished the initial stage of cocaine-induced hyperlocomotion of naive mice. In reward experiments, animals were allowed to choose freely between two chambers. In contrast, in aversive experiments, animals need to inhibit a prepotent response to enter into a preferred dark room. Impairments of aversive behavior could thus reflect an impulsivity when animals are faced with decision conflict. The indirect pathway may thus be more widely involved in behaviors of self-control in the so-called "NoGo" pathway (Frank, 2005; Frank et al., 2007).

The cocaine-induced adaptive response is exerted by multiple processes, at least triggering, execution, and storage of addictive behaviors (Hyman et al., 2006). The present study has indicated that the adaptive mechanism is endowed and saved during blockade of the direct pathway and induces normal levels of cocaine-mediated hyperlocomotion upon recovery of this transmission. Interestingly, it has been reported that the pharmacological inactivation of the striatum impairs the conditioned

orienting response involving an amygdalo-nigrostriatal pathway but that this response appeared immediately after the inactivation process is terminated (Han et al., 1997). Repeated cocaine administration evokes neural plasticity in not only the striato-SNr axis but also many other brain regions (Hyman et al., 2006). The study of reversible blockade of striatonigral transmission thus suggests that neural plasticity in the striato-SNr axis is involved in the expression of cocaine sensitization, but other brain regions may also be necessary for acquisition and storage of the cocaine-induced adaptive response.

Recent studies using BAC transgenic mice have indicated that D1 and D2 receptors are almost exclusively expressed in striatonigral and striatopallidal neurons, respectively (Heiman et al., 2008; Lobo et al., 2006; Surmeier et al., 2007). D1 and D2 receptors exhibit a marked difference in their dopamine binding affinity, i.e., a respective μM and nM order of affinity for dopamine (Maeno, 1982; Richfield et al., 1989). Furthermore, accumulated evidence has indicated that dopaminergic transmission within the striatum is segregated into functionally dissociable compartments, in which phasic and tonic firings of dopamine neurons differentially modulate D1 and D2 receptors (Grace et al., 2007; Hikosaka, 2007). On the basis of these characteristic features of dopamine transmission, we propose a mechanistic model for the roles of the two pathways in relation to the regulation and dysfunction of the basal ganglia (Figure S6). When naive animals encounter unexpected rewards, dopamine neurons emit a phasic burst of firings that considerably raises dopamine concentrations within synapses of the NAc (Mirenowicz and Schultz, 1994). Similarly, psychostimulants increase dopamine levels in the NAc (Di Chiara and Imperato, 1988). This increase not only activates the low-affinity D1 receptor in striatonigral neurons but also saturates the high-affinity D2 receptor in striatopallidal neurons. By contrast, tonic firings of dopamine neurons are not sufficient to activate the D1 receptor and modulate only the high-affinity D2 receptor (Grace et al., 2007; Hikosaka, 2007). The dual stimulation of D1 and D2 receptors is thus essential for facilitating the basal ganglia-cortical circuitry that triggers the early stage of reward-directed or psychostimulant-induced behaviors in naive animals (Figure S6A).

Then, how is the modulation by the striatonigral and striatopallidal transmission shifted to play predominant roles in reward-directed and aversive learning, respectively? Goto and Grace (2005b) demonstrated that the stimulation of D1 and D2 receptors produces behaviorally selective effects (in this case, learning versus set shifting of response strategy) that correspond to specific afferents derived from the hippocampus and the prefrontal cortex, respectively. Furthermore, they reported that D1 and D2 receptors differentially induce LTP and LTD, depending on different afferents in the NAc (Goto and Grace, 2005a). Importantly, the photogenetic study by Tsai et al. (2009) revealed that the phasic activation of dopamine neurons evokes CPP associated with the dopamine-activated environment but that tonic activation fails to derive such CPP. Conversely, aversive stimuli have been shown to reduce tonic firings of most or regionally confined dopamine neurons (Brischoux et al., 2009; Coizet et al., 2006; Mirenowicz and Schultz, 1996; Ungless et al., 2004), and blunting the tonic dopamine release in the ventromedial striatum leads to conditioned place aversion (Liu et al., 2008). Our model holds that the substantial increase in dopamine by reward-related, phasic dopamine release or repetitive cocaine administration induces LTP and LTD at striatonigral and striatopallidal neurons, respectively (Figure S6B). The striatonigral input thus becomes predominant over the striatopallidal input in the processes of reward-directed behavior and cocaine sensitization. Consequently, the blockade of striatonigral transmission should severely impair the adaptive responses of both appetitive reward behavior and cocaine sensitization. On the other hand, blockade of striatopallidal transmission produces the situation equivalent to the LTD-mediated reduction in input in striatopallidal synapses and would thus have no further effect on reward-directed behavior, once LTP is postsynaptically induced in striatonigral neurons. In contrast, this blockade or the cocaine-induced LTD would disrupt the modulatory mechanism by tonic dopamine release and would impair aversive behavior (Figure S6C). Because the induction of LTP and LTD in MSNs has been reported to depend on not only synaptic input integration but also the cellular processes sensitive to timing of pre- and postsynaptic activity (Goto and Grace, 2005a; Shen et al., 2008), the model discussed here needs to be more substantiated. It should, however, be pointed out that animals need to rapidly form memory against aversive stimuli to avoid uncomfortable or dangerous environments. In contrast, animals have to distinguish between associative and nonassociative rewarding stimuli to acquire such rewards efficiently and correctly. The modulatory switch of the two pathways thus plays an essential role in properly driving behavioral responses to rewarding and aversive stimuli.

Recently, the biochemical characterization of D1-receptor-expressing and D2-receptor-expressing MSNs has revealed that these two subpopulations exhibit vast differences in their expression of functional and signaling molecules (Heiman et al., 2008; Surmeier et al., 2007; Valjent et al., 2009). The distinct functions of striatonigral and striatopallidal transmission could thus result from different functional molecules in these two subpopulations of MSNs. The present study will facilitate more informed and effective approaches to the treatment of the basal ganglia dysfunction that occurs in drug addiction and Parkinson's disease.

EXPERIMENTAL PROCEDURES

Animals

The TN transgenic mice were generated as described previously (Yamamoto et al., 2003) and used together with their wild-type littermates for all experiments. DOX was administered in food pellets containing 6 mg/g DOX and in drinking water containing 2 mg/ml DOX and 10% sucrose. All animal handling procedures were performed according to the guidelines of Osaka Bioscience Institute.

Construction of the Recombinant AAVs

The 2.1 kb promoter region of the mouse *PPTA* gene (residues from −1525 to +543, NCBI accession number NT_039340) or the 2.0 kb promoter region of the mouse *PPE* gene (residues −1834 to +148, NCBI accession number NT_039258) was isolated from BAC DNA. The promoter regions were attached to the *flag-tTA* cDNA and inserted into the Multiple Cloning Sequence of the pAAV-MCS vector of the AAV Helper−Free System (Stratagene, La Jolla, CA). Both constructs were packaged and serotyped with the AAV capsid protein with the use of the AAV Helper-Free System. The AAV was purified

by using a ViraTrap AAV Purification kit (Omega Bio-Tek Inc., Norcross, GA), yielding 10^{12} particles per milliliter when titrated by ELISA for the AAV2 capsids (PROGEN, Heidelberg, Germany). The viral injection and treatment with DOX were conducted according to the following protocols, unless otherwise stated: the recombinant AAV or the control AAV free of the inserted tTA construct was unilaterally or bilaterally injected into 11 sites of the striatum or into four sites of the NAc by stereotaxic techniques (Hikida et al., 2001). Two weeks after the viral injection, animals were continuously treated with DOX or left untreated.

Retrograde Tracing

The V-S-tTA or V-E-tTA virus was injected into the striatum of wild-type mice. The CTB-Alexa 594 conjugate (Molecular Probes, Eugene, OR) was then stereotaxically injected into the GP or SNr of these mice (for GP, 0.5 mm posterior to the bregma, 2 mm lateral from the midline, 3.5 mm depth from the dura; for SNr, 3.5 mm posterior to the bregma, 1.5 mm lateral from the midline, 4 mm depth from the dura). Two weeks after the injection, the mice were deeply anesthetized, and coronal sections (40 μm) of the striatum were prepared and immunostained with anti-flag M2 monoclonal antibody (Sigma, St. Louis, MO), followed by Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). Immunofluorescence and CTB-Alexa 594 fluorescence were detected with a BZ-9000 digital fluorescence microscope (Keyence, Osaka, Japan).

Immunohistochemistry and Immunoblotting

Coronal sections (40 μm) of the striatum from animals 2 weeks after viral injection were prepared and immunostained (Hikida et al., 2001). The primary antibodies used were obtained as described previously (Hikida et al., 2001). Signals were visualized with secondary antibodies (Yamamoto et al., 2003). Immunoblotting was performed as described previously (Yamamoto et al., 2003).

In Situ Hybridization Histochemistry

In situ hybridization analysis of coronal sections (10 μm) prepared from a fresh-frozen mouse brain was performed as described previously (Kaneko et al., 2000). Specific antisense riboprobes were [^{35}S]-labeled from the corresponding cDNAs (*SP* cDNA, residues from +1 to +390, NCBI accession number NT_039340; *Enk* cDNA, residues from +1 to +807, NCBI accession number NT_039258; c-*fos* cDNA, residues from +1 to +1143, NCBI accession number NM_010234) (Kaneko et al., 2000). Four to six coronal sections taken every 100 μm were used to calculate the mean radioactivity of *SP* and *Enk* mRNAs in the striatum and to count c-*fos* mRNA-positive cells in the VP or the SNr. Radioactivity of each section was quantified by using a BAS5000 image processing system (Fujifilm, Tokyo, Japan).

Electrophysiology

After anesthesia using 0.9% ketamine and 0.1% xylazine, a mouse was positioned in a stereotaxic apparatus. A pair of stimulating glass-coated metal electrodes (Elgiloy) was implanted into the right side of the striatum (one at 1.5 mm anterior, 1 mm lateral and the other at 0.5 mm anterior, 2 mm lateral to the bregma, 3 mm depth for both). A short pulse at 2 Hz (duration, 200 μs; amplitude, 50–75 μA) was applied for striatal stimulation. Local field potentials in response to the stimulation were recorded at the ipsilateral SNr (3.5 mm posterior, 1.5 mm lateral to the bregma, 3.5–4.2 mm depth) by using a glass electrode (tip diameter, ~8 μm) filled with ACSF (in mM, NaCl 160, KCl 5, $CaCl_2$ 2.5, $MgCl_2$ 1, HEPES 5, glucose 10). A reference electrode (Ag-AgCl) was placed in the cervical subcutaneous tissue. The short-latency response was gained from 50 pulses of either polarity between the two stimulating electrodes, separately averaged, and added thereafter. Bicuculline methochloride (Tocris, Ellisville, MO) was perfused with the use of a triple-barreled glass electrode. One barrel was filled with bicuculline methochloride, which was then ionophoretically injected. The other two barrels were filled with ACSF and each used for recording and a return path (retain current, 20 nA; injection current, 40 nA). Recorded sites were confirmed to be the SNr by histological reconstruction after fixation with 4% paraformaldehyde.

Behavior Tests

For the rotation test, mice were placed in a round-bottomed bowl (25 cm in diameter), and rotations were counted for a 5 min period by visual observation (Kaneko et al., 2000). One rotation was defined by the animal completing a 360° circle without turning back in the opposite direction. Locomotor activity was measured with an infrared activity monitor (MED Associates, St. Albans, VT) for a 60 min period immediately after i.p. injection of saline or 2 mg/kg methamphetamine. For cocaine experiments, locomotor activity was measured for a 10 min period immediately after i.p. injection of saline or 10 mg/kg cocaine (Hikida et al., 2001, 2003). The CPP test was performed as described previously (Hikida et al., 2001, 2003). Methamphetamine and cocaine were obtained from Dainippon Sumitomo Pharma (Osaka, Japan) and Shionogi (Osaka, Japan), respectively. Chocolate and food consumptions were measured after 24 hr free access to both chocolate and a standard food.

In the aversion test, the step-through inhibitory avoidance apparatus consisted of a straight alley divided into a small, light chamber (8 cm long) and a large, dark chamber (16 cm long). The light chamber was made of gray Plexiglas and illuminated by a lamp. The dark chamber was made of black Plexiglas covered by a black cloth and a grid floor connected to an electric source. The two chambers were separated by a black sliding door. On the training day, each mouse was placed in the light chamber and the door leading to the dark chamber was raised. Once the mouse had stepped with all four paws into the dark chamber, the door was closed; and then an electric footshock (0.5 mA, 60 Hz, 1 s) was delivered. Memory retention was tested 24 hr later following a similar procedure, expect that no shock was delivered. Latency to step into the dark chamber was measured. Fear responses were analyzed as described previously (Masugi et al., 1999). The percentage of freezing response was determined by scoring the number of positive freezing responses divided by the total number of samples at 2 s intervals in a 1 min time period.

Statistical Analysis

Statistical analysis was conducted by using STATVIEW. Data were analyzed by two-way ANOVA or repeated-measured ANOVA and were presented as the mean ± SEM.

SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and can be found with this article online at doi:10.1016/j.neuron.2010.05.011.

ACKNOWLEDGMENTS

We thank R. Sprengel and M. Yokoi for plasmids and T. Kaneko and M. Takahashi for antibodies. This work was supported by Grants KAKENHI 17002016 (S.N.) and 20790264 (T.H.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan; JST PRESTO program (T.H.); and by grants from the Takeda Science Foundation (S.N.) and Uehara Memorial Foundation (T.H.).

Accepted: May 7, 2010
Published: June 23, 2010

REFERENCES

Albin, R.L., Young, A.B., and Penney, J.B. (1989). The functional anatomy of basal ganglia disorders. Trends Neurosci. *12*, 366–375.

Alexander, G.E., and Crutcher, M.D. (1990). Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci. *13*, 266–271.

Baker, D.A., Khroyan, T.V., O'Dell, L.E., Fuchs, R.A., and Neisewander, J.L. (1996). Differential effects of intra-accumbens sulpiride on cocaine-induced locomotion and conditioned place preference. J. Pharmacol. Exp. Ther. *279*, 392–401.

Baker, D.A., Fuchs, R.A., Specio, S.E., Khroyan, T.V., and Neisewander, J.L. (1998). Effects of intraaccumbens administration of SCH-23390 on cocaine-induced locomotion and conditioned place preference. Synapse *30*, 181–193.

Brischoux, F., Chakraborty, S., Brierley, D.I., and Ungless, M.A. (2009). Phasic excitation of dopamine neurons in ventral VTA by noxious stimuli. Proc. Natl. Acad. Sci. USA *106*, 4894–4899.

Caine, S.B., Thomsen, M., Gabriel, K.I., Berkowitz, J.S., Gold, L.H., Koob, G.F., Tonegawa, S., Zhang, J., and Xu, M. (2007). Lack of self-administration of cocaine in dopamine D_1 receptor knock-out mice. J. Neurosci. *27*, 13140–13150.

Chesselet, M.F., and Delfs, J.M. (1996). Basal ganglia and movement disorders: an update. Trends Neurosci. *19*, 417–422.

Coizet, V., Dommett, E.J., Redgrave, P., and Overton, P.G. (2006). Nociceptive responses of midbrain dopaminergic neurones are modulated by the superior colliculus in the rat. Neuroscience *139*, 1479–1493.

Cuello, A.C., and Kanazawa, I. (1978). The distribution of substance P immunoreactive fibers in the rat central nervous system. J. Comp. Neurol. *178*, 129–156.

Deniau, J.M., Mailly, P., Maurice, N., and Charpier, S. (2007). The pars reticulata of the substantia nigra: a window to basal ganglia output. Prog. Brain Res. *160*, 151–172.

Di Chiara, G., and Imperato, A. (1988). Drugs abused by humans preferentially increase synaptic dopamine concentrations in the mesolimbic system of freely moving rats. Proc. Natl. Acad. Sci. USA *85*, 5274–5278.

Frank, M.J. (2005). Dynamic dopamine modulation in the basal ganglia: a neurocomputational account of cognitive deficits in medicated and nonmedicated Parkinsonism. J. Cogn. Neurosci. *17*, 51–72.

Frank, M.J., Samanta, J., Moustafa, A.A., and Sherman, S.J. (2007). Hold your horses: impulsivity, deep brain stimulation, and medication in parkinsonism. Science *318*, 1309–1312.

Gerfen, C.R., Engber, T.M., Mahan, L.C., Susel, Z., Chase, T.N., Monsma, F.J., Jr., and Sibley, D.R. (1990). D_1 and D_2 dopamine receptor-regulated gene expression of striatonigral and striatopallidal neurons. Science *250*, 1429–1432.

Goto, Y., and Grace, A.A. (2005a). Dopamine-dependent interactions between limbic and prefrontal cortical plasticity in the nucleus accumbens: disruption by cocaine sensitization. Neuron *47*, 255–266.

Goto, Y., and Grace, A.A. (2005b). Dopaminergic modulation of limbic and cortical drive of nucleus accumbens in goal-directed behavior. Nat. Neurosci. *8*, 805–812.

Grace, A.A., Floresco, S.B., Goto, Y., and Lodge, D.J. (2007). Regulation of firing of dopaminergic neurons and control of goal-directed behaviors. Trends Neurosci. *30*, 220–227.

Graybiel, A.M. (2000). The basal ganglia. Curr. Biol. *10*, R509–R511.

Grimm, J.W., Hope, B.T., Wise, R.A., and Shaham, Y. (2001). Neuroadaptation. Incubation of cocaine craving after withdrawal. Nature *412*, 141–142.

Han, J.S., McMahan, R.W., Holland, P., and Gallagher, M. (1997). The role of an amygdalo-nigrostriatal pathway in associative learning. J. Neurosci. *17*, 3913–3919.

Heiman, M., Schaefer, A., Gong, S., Peterson, J.D., Day, M., Ramsey, K.E., Suárez-Fariñas, M., Schwarz, C., Stephan, D.A., Surmeier, D.J., et al. (2008). A translational profiling approach for the molecular characterization of CNS cell types. Cell *135*, 738–748.

Hikida, T., Kaneko, S., Isobe, T., Kitabatake, Y., Watanabe, D., Pastan, I., and Nakanishi, S. (2001). Increased sensitivity to cocaine by cholinergic cell ablation in nucleus accumbens. Proc. Natl. Acad. Sci. USA *98*, 13351–13354.

Hikida, T., Kitabatake, Y., Pastan, I., and Nakanishi, S. (2003). Acetylcholine enhancement in the nucleus accumbens prevents addictive behaviors of cocaine and morphine. Proc. Natl. Acad. Sci. USA *100*, 6169–6173.

Hikosaka, O. (2007). Basal ganglia mechanisms of reward-oriented eye movement. Ann. N Y Acad. Sci. *1104*, 229–249.

Hyman, S.E., Malenka, R.C., and Nestler, E.J. (2006). Neural mechanisms of addiction: the role of reward-related learning and memory. Annu. Rev. Neurosci. *29*, 565–598.

Kalivas, P.W., and Stewart, J. (1991). Dopamine transmission in the initiation and expression of drug- and stress-induced sensitization of motor activity. Brain Res. Brain Res. Rev. *16*, 223–244.

Kaneko, S., Hikida, T., Watanabe, D., Ichinose, H., Nagatsu, T., Kreitman, R.J., Pastan, I., and Nakanishi, S. (2000). Synaptic integration mediated by striatal cholinergic interneurons in basal ganglia function. Science *289*, 633–637.

Lerchner, W., Xiao, C., Nashmi, R., Slimko, E.M., van Trigt, L., Lester, H.A., and Anderson, D.J. (2007). Reversible silencing of neuronal excitability in behaving mice by a genetically targeted, ivermectin-gated Cl^- channel. Neuron *54*, 35–49.

Liu, Z.H., Shin, R., and Ikemoto, S. (2008). Dual role of medial A10 dopamine neurons in affective encoding. Neuropsychopharmacology *33*, 3010–3020.

Lobo, M.K., Karsten, S.L., Gray, M., Geschwind, D.H., and Yang, X.W. (2006). FACS-array profiling of striatal projection neuron subtypes in juvenile and adult mouse brains. Nat. Neurosci. *9*, 443–452.

Maeno, H. (1982). Dopamine receptors in canine caudate nucleus. Mol. Cell. Biochem. *43*, 65–80.

Marshall, J.F., Ruskin, D.N., and LaHoste, G.J. (1998). Dopaminergic regulation of immediate early gene expression in the basal ganglia. Adv. Pharmacol. *42*, 678–681.

Masugi, M., Yokoi, M., Shigemoto, R., Muguruma, K., Watanabe, Y., Sansig, G., van der Putten, H., and Nakanishi, S. (1999). Metabotropic glutamate receptor subtype 7 ablation causes deficit in fear response and conditioned taste aversion. J. Neurosci. *19*, 955–963.

Miller, R.J., and Pickel, V.M. (1980). The distribution and functions of the enkephalins. J. Histochem. Cytochem. *28*, 903–917.

Miner, L.L., Drago, J., Chamberlain, P.M., Donovan, D., and Uhl, G.R. (1995). Retained cocaine conditioned place preference in D1 receptor deficient mice. Neuroreport *6*, 2314–2316.

Mirenowicz, J., and Schultz, W. (1994). Importance of unpredictability for reward responses in primate dopamine neurons. J. Neurophysiol. *72*, 1024–1027.

Mirenowicz, J., and Schultz, W. (1996). Preferential activation of midbrain dopamine neurons by appetitive rather than aversive stimuli. Nature *379*, 449–451.

Pulipparacharuvil, S., Renthal, W., Hale, C.F., Taniguchi, M., Xiao, G., Kumar, A., Russo, S.J., Sikder, D., Dewey, C.M., Davis, M.M., et al. (2008). Cocaine regulates MEF2 to control synaptic and behavioral plasticity. Neuron *59*, 621–633.

Pycock, C.J. (1980). Turning behaviour in animals. Neuroscience *5*, 461–514.

Richfield, E.K., Penney, J.B., and Young, A.B. (1989). Anatomical and affinity state comparisons between dopamine D_1 and D_2 receptors in the rat central nervous system. Neuroscience *30*, 767–777.

Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B.R., and Montecucco, C. (1992). Tetanus and botulinum-B neurotoxins block neurotransmitter release by proteolytic cleavage of synaptobrevin. Nature *359*, 832–835.

Schultz, W. (2007). Behavioral dopamine signals. Trends Neurosci. *30*, 203–210.

Shen, W., Flajolet, M., Greengard, P., and Surmeier, D.J. (2008). Dichotomous dopaminergic control of striatal synaptic plasticity. Science *321*, 848–851.

Smith, J.W., Fetsko, L.A., Xu, R., and Wang, Y. (2002). Dopamine D2L receptor knockout mice display deficits in positive and negative reinforcing properties of morphine and in avoidance learning. Neuroscience *113*, 755–765.

Surmeier, D.J., Ding, J., Day, M., Wang, Z., and Shen, W. (2007). D1 and D2 dopamine-receptor modulation of striatal glutamatergic signaling in striatal medium spiny neurons. Trends Neurosci. *30*, 228–235.

Tsai, H.C., Zhang, F., Adamantidis, A., Stuber, G.D., Bonci, A., de Lecea, L., and Deisseroth, K. (2009). Phasic firing in dopaminergic neurons is sufficient for behavioral conditioning. Science *324*, 1080–1084.

Tzschentke, T.M. (2007). Measuring reward with the conditioned place preference (CPP) paradigm: update of the last decade. Addict. Biol. *12*, 227–462.

Ungless, M.A., Magill, P.J., and Bolam, J.P. (2004). Uniform inhibition of dopamine neurons in the ventral tegmental area by aversive stimuli. Science *303*, 2040–2042.

Valjent, E., Bertran-Gonzalez, J., Hervé, D., Fisone, G., and Girault, J.A. (2009). Looking BAC at striatal signaling: cell-specific analysis in new transgenic mice. Trends Neurosci. *32*, 538–547.

Wada, N., Kishimoto, Y., Watanabe, D., Kano, M., Hirano, T., Funabiki, K., and Nakanishi, S. (2007). Conditioned eyeblink learning is formed and stored without cerebellar granule cell transmission. Proc. Natl. Acad. Sci. USA *104*, 16690–16695.

Welter, M., Vallone, D., Samad, T.A., Meziane, H., Usiello, A., and Borrelli, E. (2007). Absence of dopamine D2 receptors unmasks an inhibitory control over the brain circuitries activated by cocaine. Proc. Natl. Acad. Sci. USA *104*, 6840–6845.

Wickens, J.R., Reynolds, J.N.J., and Hyland, B.I. (2003). Neural mechanisms of reward-related motor learning. Curr. Opin. Neurobiol. *13*, 685–690.

Xu, M., Guo, Y., Vorhees, C.V., and Zhang, J. (2000). Behavioral responses to cocaine and amphetamine administration in mice lacking the dopamine D1 receptor. Brain Res. *852*, 198–207.

Yamamoto, M., Wada, N., Kitabatake, Y., Watanabe, D., Anzai, M., Yokoyama, M., Teranishi, Y., and Nakanishi, S. (2003). Reversible suppression of glutamatergic neurotransmission of cerebellar granule cells *in vivo* by genetically manipulated expression of tetanus neurotoxin light chain. J. Neurosci. *23*, 6759–6767.

Neuropsychopharmacology (2012) 37, 1387–1396

www.neuropsychopharmacology.org

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

Kazuya Toriumi[1,2], Akihiro Mouri[1,3], Shiho Narusawa[1], Yuki Aoyama[1], Natsumi Ikawa[1], Lingling Lu[1,2], Taku Nagai[3], Takayoshi Mamiya[1,2], Hyoung-Chun Kim[4] and Toshitaka Nabeshima*[,1,2]

[1]Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya, Japan; [2]The Academic Frontier Project for Private University, Comparative Cognitive Science Institutes, Meijo University, Nagoya, Japan; [3]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Japan; [4]Neurotoxicology Program, Department of Pharmacy, College of Pharmacy, Kangwon National University, Korea Institute of Drug Abuse, Chunchon, South Korea

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.
Neuropsychopharmacology (2012) **37,** 1387–1396; doi:10.1038/npp.2011.324; published online 18 January 2012

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

*Correspondence: Professor T Nabeshima, Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya, 468-8503, Japan, Tel: +81 52 839 2735, Fax: +81 52 839 2738,
E-mail: tnabeshi@meijo-u.ac.jp
Received 8 June 2011; revised 3 December 2011; accepted 5 December 2011

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.

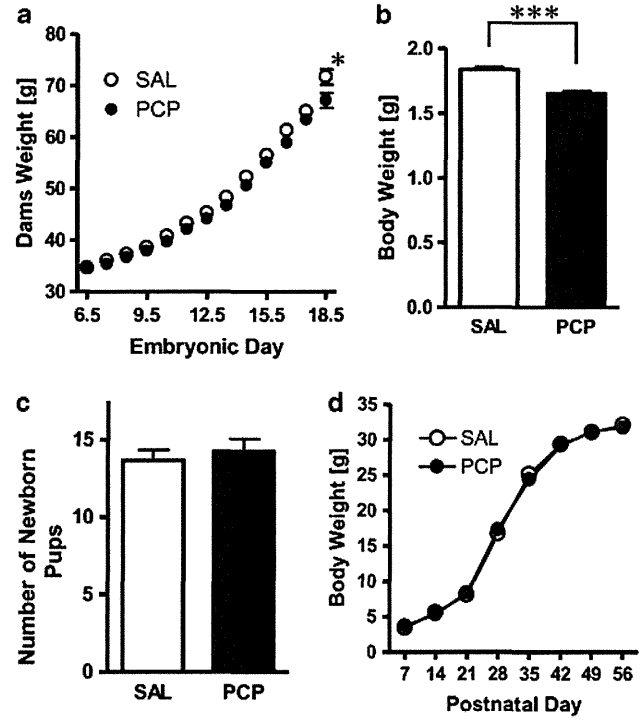

Figure 1 Prenatal PCP-treatment reduced body weight in newborn pups. Mice were administrated SAL or PCP from E6.5 to E18.5. (a) Changes in the body weight of the dams are shown (two-way ANOVA with repeated measures: $F_{Interaction(12, 132)}=3.01$; $p<0.05$, $F_{Time(12, 132)}=1190$; $p<0.0001$, $F_{Treatment(1, 132)}=1.71$; $p>0.05$). *$p<0.05$ 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

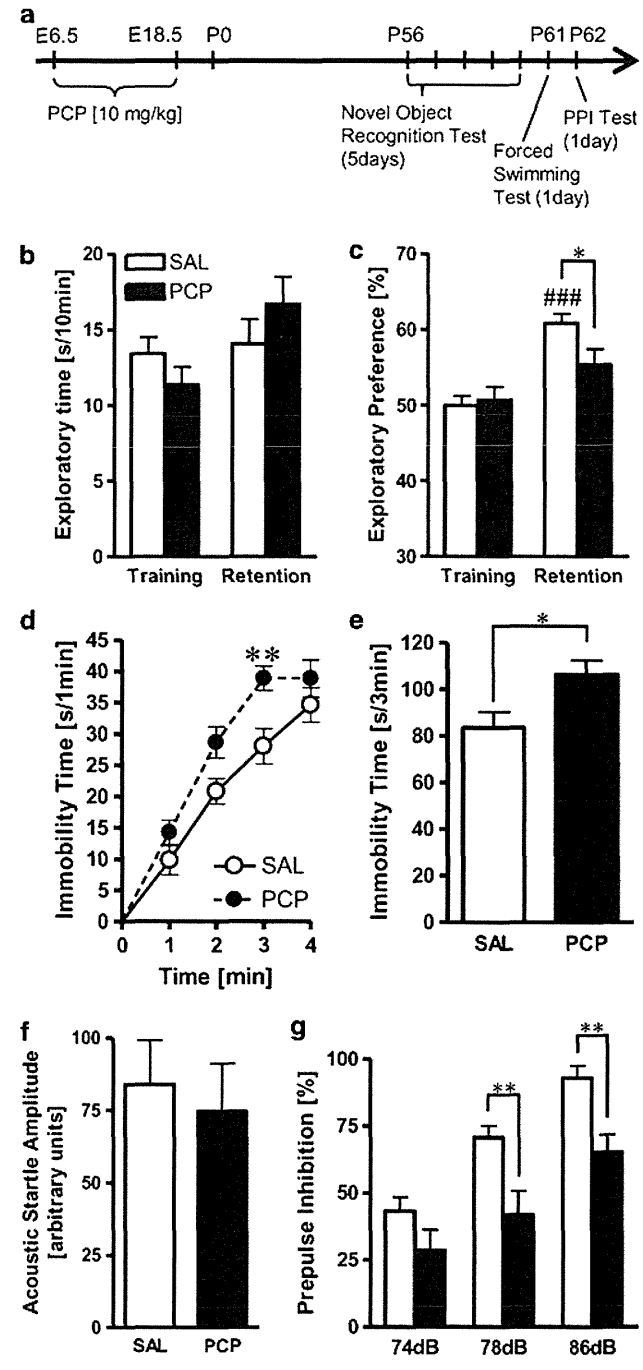

Figure 2 Prenatal PCP treatment induced behavioral deficits in adult. (a) Experimental schedule: mice were tested from P56. In the novel object recognition test, (b) exploratory time and (c) exploratory preference were determined. $^{\#\#\#}p<0.001$ compared with SAL-treated mice in the training session 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 ($n=20$).

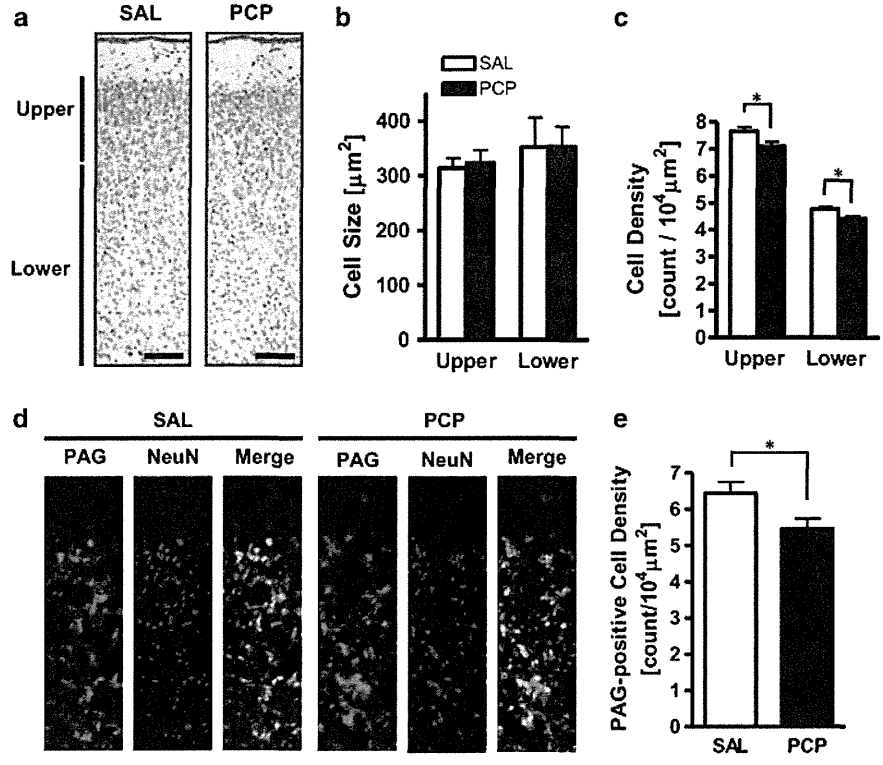

Figure 3 Prenatal PCP-treatment reduced the density of glutamatergic neurons in PFC. (a) Nissl-stained coronal sections of the PFC in SAL- and PCP-treated mice. Scale bar: 200 μm. Stereological analysis of (b) the size and (c) density of Nissl-stained cells in SAL- and PCP-treated mice ($n = 6$). (d) 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.

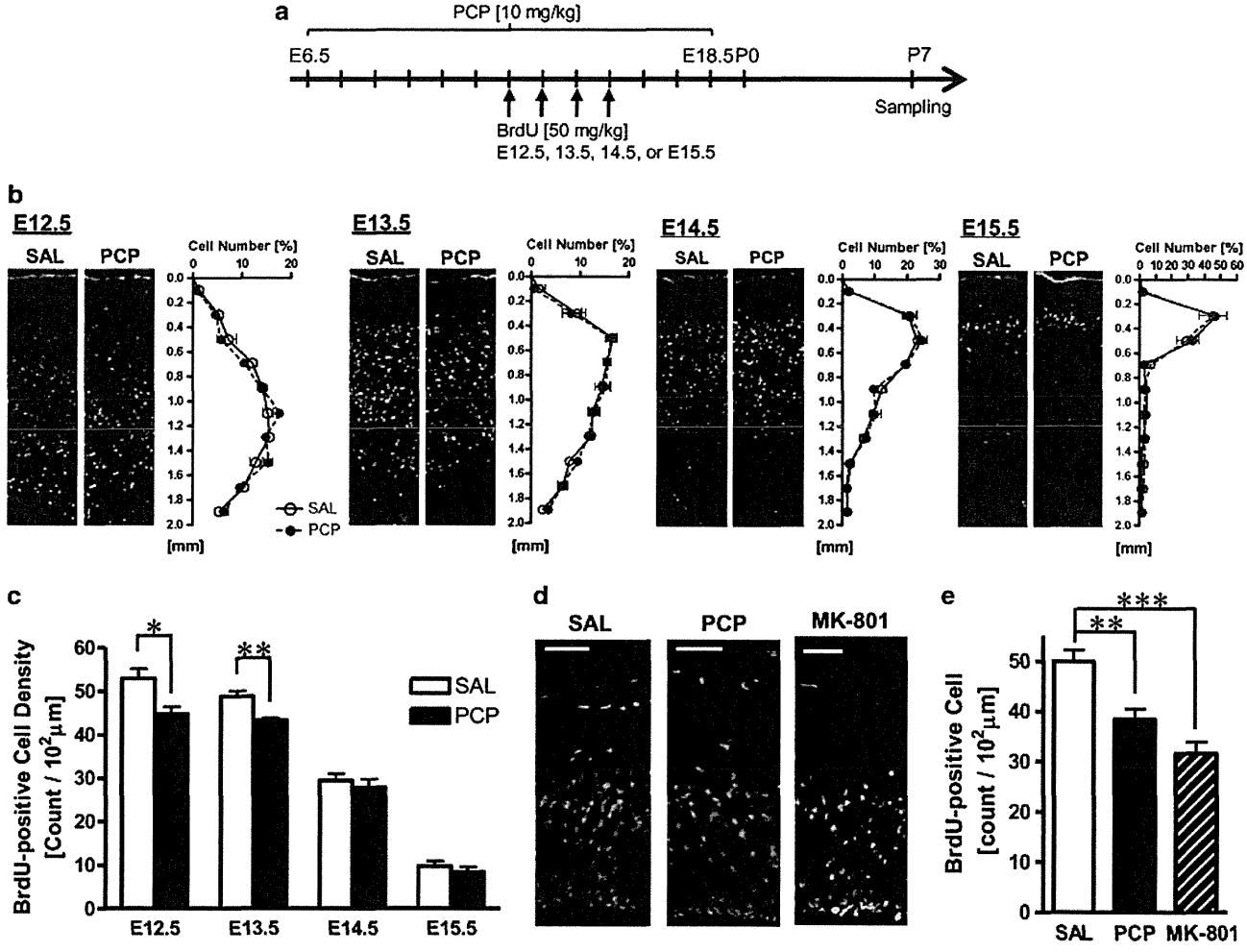

Figure 4 Prenatal PCP treatment affected the mitosis of neuronal progenitor but not neuronal migration. (a) Experimental schedule: the pregnant mice were given a single BrdU injection on E12.5, E13.5, E14.5, or E15.5, 3 h after the PCP/SAL administration to label mitotic progenitors in the embryonic 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,

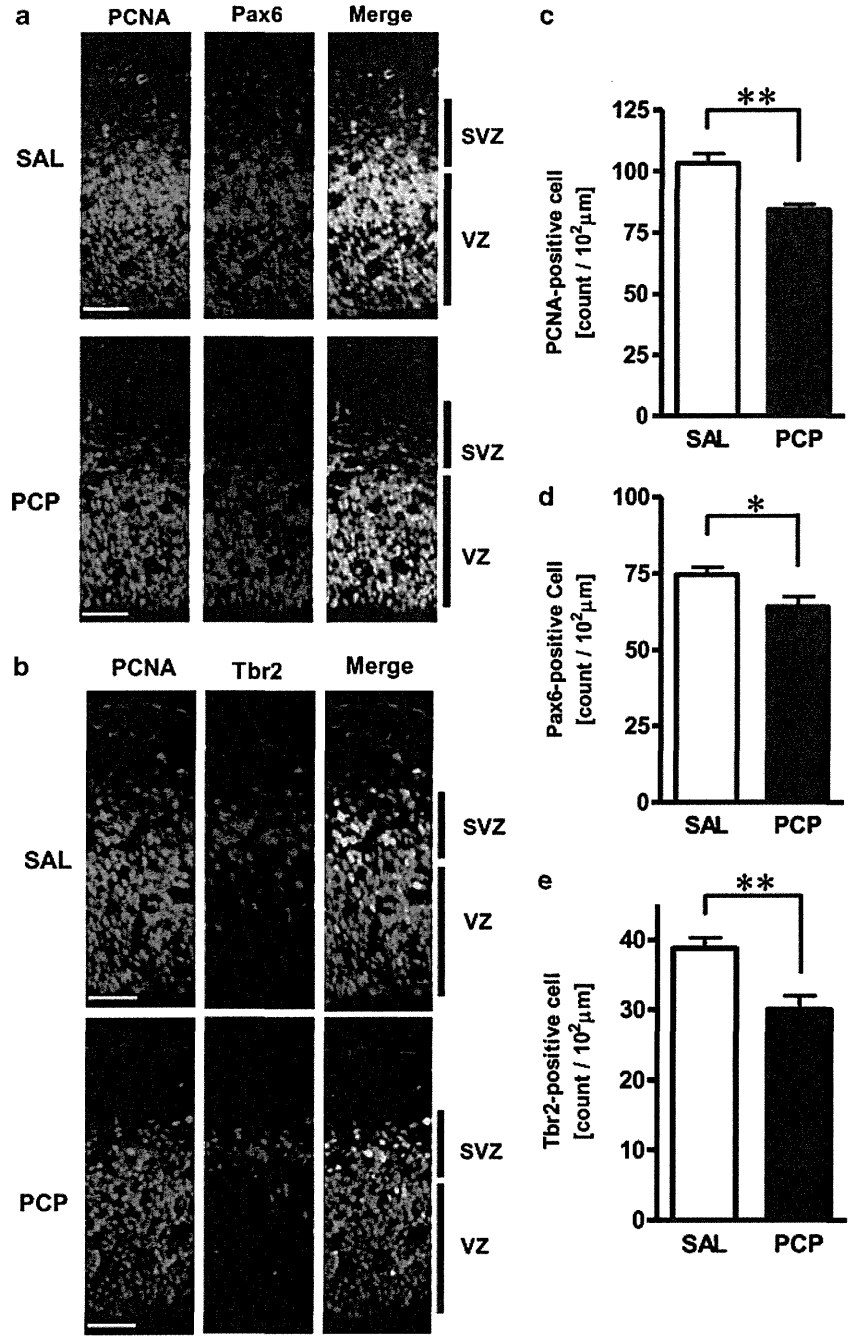

Figure 5 Prenatal PCP-treatment reduced the density of neuronal progenitors. On E13.5, mice were decapitated 6 h after the PCP/SAL injection. The pups were analyzed by immunohistochemical analysis using α-PCNA as a marker of proliferation, α-Pax6 antibody as a marker for neuronal progenitors in VZ, and 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.

ACKNOWLEDGEMENTS

We thank Dr Furukawa H for synthesizing PCP. This study was supported by Grants-in-aid for Scientific Research (A) (22248033), Scientific Research (B) (20390073) (21390045) and Exploratory Research from the JSPS (19659017) (22659213); by the 'Academic Frontier' Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan (MEXT); by Regional Joint Research Program supported by grants to Private Universities to Cover Current Expenses from MEXT; by Research on Regulatory Science of Pharmaceuticals and Medical Devices from the Ministry of Health, Labour and Welfare, Japan (MHLW); by Research on Risk of Chemical Substances, Health and Labour Science Research Grants supported by MHLW; and by the joint research project under the Japan-Korea basic scientific cooperation program (JSPS).

DISCLOSURE

The authors declare no conflict of interest.

REFERENCES

Abekawa T, Ito K, Nakagawa S, Koyama T (2007). Prenatal exposure to an NMDA receptor antagonist, MK-801 reduces density of parvalbumin-immunoreactive GABAergic neurons in the medial prefrontal cortex and enhances phencyclidine-induced hyperlocomotion but not behavioral sensitization to methamphetamine in postpubertal rats. *Psychopharmacology (Berlin)* **192**: 303–316.

Asahi M, Hoshimaru M, Hojo M, Matsuura N, Kikuchi H, Hashimoto N (1998). Induction of the N-methyl-D-aspartate receptor subunit 1 in the immortalized neuronal progenitor cell line HC2S2 during differentiation into neurons. *J Neurosci Res* **52**: 699–708.

Brazel CY, Nuñez JL, Yang Z, Levison SW (2005). Glutamate enhances survival and proliferation of neural progenitors derived from the subventricular zone. *Neuroscience* **131**: 55–65.

Cirulli V, Yebra M (2007). Netrins: beyond the brain. *Nat Rev Mol Cell Biol* **8**: 296–306.

Dehay C, Kennedy H (2007). Cell-cycle control and cortical development. *Nat Rev Neurosci* **8**: 438–450.

Fico TA, Vanderwende C (1988). Phencyclidine during pregnancy: fetal brain levels and neurobehavioral effects. *Neurotoxicol Teratol* **10**: 349–354.

Fitzgerald DP, Bradford D, Cooper HM (2007). Neogenin is expressed on neurogenic and gliogenic progenitors in the embryonic and adult central nervous system. *Gene Expr Patterns* **7**: 784–792.

Forrest D, Yuzaki M, Soares HD, Ng L, Luk DC, Sheng M *et al* (1994). Targeted disruption of NMDA receptor 1 gene abolishes NMDA response and results in neonatal death. *Neuron* **13**: 325–338.

Götz M, Huttner WB (2005). The cell biology of neurogenesis. *Nat Rev Mol Cell Biol* **6**: 777–788.

Hamada Y, Kadokawa Y, Okabe M, Ikawa M, Coleman JR, Tsujimoto Y (1999). Mutation in ankyrin repeats of the mouse

Notch2 gene induces early embryonic lethality. *Development* **126**: 3415–3424.
Heins N, Malatesta P, Cecconi F, Nakafuku M, Tucker KL, Hack MA *et al* (2002). Glial cells generate neurons: the role of the transcription factor Pax6. *Nat Neurosci* **5**: 308–315.
Hevner RF (2006). From radial glia to pyramidal-projection neuron: transcription factor cascades in cerebral cortex development. *Mol Neurobiol* **33**: 33–50.
Ikonomidou C, Bosch F, Miksa M, Bittigau P, Vöckler J, Dikranian K *et al* (1999). Blockade of NMDA receptors and apoptotic neurodegeneration in the developing brain. *Science* **283**: 70–74.
Joo JY, Kim BW, Lee JS, Park JY, Kim S, Yun YJ *et al* (2007). Activation of NMDA receptors increases proliferation and differentiation of hippocampal neural progenitor cells. *J Cell Sci* **120**: 1358–1370.
Kaneko T, Mizuno N (1994). Glutamate-synthesizing enzymes in GABAergic neurons of the neocortex: a double immunofluorescence study in the rat. *Neuroscience* **61**: 839–849.
Kaufman KR, Petrucha RA, Pitts Jr FN, Weekes ME (1983). PCP in amniotic fluid and breast milk: case report. *J Clin Psychiatry* **44**: 269–270.
Komuro H, Rakic P (1993). Modulation of neuronal migration by NMDA receptors. *Science* **260**: 95–97.
Koseki T, Mouri A, Mamiya T, Aoyama Y, Toriumi K, Suzuki S *et al* (2011). Exposure to enriched environments during adolescence prevents abnormal behaviors associated with histone deacetylation in phencyclidine-treated mice. *Int J Neuropsychopharmacol* (in press).
Lewis DA, Glantz LA, Pierri JN, Sweet RA (2003). Altered cortical glutamate neurotransmission in schizophrenia: evidence from morphological studies of pyramidal neurons. *Ann NY Acad Sci* **1003**: 102–112.
Llambi F, Causeret F, Bloch-Gallego E, Mehlen P (2001). Netrin-1 acts as a survival factor via its receptors UNC5H and DCC. *EMBO J* **20**: 2715–2722.
López T, López-Colomé AM, Ortega A (1997). NMDA receptors in cultured radial glia. *FEBS Lett* **405**: 245–248.
Louvi A, Artavanis-Tsakonas S (2006). Notch signalling in vertebrate neural development. *Nat Rev Neurosci* **7**: 93–102.
Lu L, Mamiya T, Lu P, Toriumi K, Mouri A, Hiramatsu M *et al* (2010). Prenatal exposure to phencyclidine produces abnormal behavior and NMDA receptor expression in postpubertal mice. *Int J Neuropsychopharmacol* **13**: 877–889.
Lu L, Mamiya T, Lu P, Toriumi K, Mouri A, Hiramatsu M *et al* (2011). Prenatal exposure to PCP produces behavioral deficits accompanied by the overexpression of GLAST in the prefrontal cortex of postpubertal mice. *Behav Brain Res* **220**: 132–139.
Luk KC, Kennedy TE, Sadikot AF (2003). Glutamate promotes proliferation of striatal neuronal progenitors by an NMDA receptor-mediated mechanism. *J Neurosci* **23**: 2239–2250.
Maddox VH, Godefroi EF, Parcell RF (1965). The synthesis of phencyclidine and other 1-arylcyclohexylamines. *J Med Chem* **8**: 230–235.
McConnell SK (1995). Constructing the cerebral cortex: neurogenesis and fate determination. *Neuron* **15**: 761–768.
Mochizuki N, Takagi N, Kurokawa K, Kawai T, Besshoh S, Tanonaka K *et al* (2007). Effect of NMDA receptor antagonist on proliferation of neurospheres from embryonic brain. *Neurosci Lett* **417**: 143–148.
Mouri A, Noda Y, Hara H, Mizoguchi H, Tabira T, Nabeshima T (2007). Oral vaccination with a viral vector containing Abeta cDNA attenuates age-related Abeta accumulation and memory deficits without causing inflammation in a mouse Alzheimer model. *FASEB J* **21**: 2135–2148.
Murai R, Noda Y, Matsui K, Kamei H, Mouri A, Matsuba K *et al* (2007). Hypofunctional glutamatergic neurotransmission in the prefrontal cortex is involved in the emotional deficit induced by repeated treatment with phencyclidine in mice: implications for abnormalities of glutamate release and NMDA-CaMKII signaling. *Behav Brain Res* **180**: 152–160.
Nicholas JM, Lipshitz J, Schreiber EC (1982). Phencyclidine: its transfer across the placenta as well as into breast milk. *Am J Obstet Gynecol* **143**: 143–146.
Pfaffl MW (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* **29**: e45.
Rakic P (1995). A small step for the cell, a giant leap for mankind: a hypothesis of neocortical expansion during evolution. *Trends Neurosci* **18**: 383–388.
Sah DW, Ray J, Gage FH (1997). Regulation of voltage- and ligand-gated currents in rat hippocampal progenitor cells *in vitro*. *J Neurobiol* **32**: 95–110.
Solecki DJ, Liu XL, Tomoda T, Fang Y, Hatten ME (2001). Activated Notch2 signaling inhibits differentiation of cerebellar granule neuron precursors by maintaining proliferation. *Neuron* **31**: 557–568.
Suzuki M, Nelson AD, Eickstaedt JB, Wallace K, Wright LS, Svendsen CN (2006). Glutamate enhances proliferation and neurogenesis in human neural progenitor cell cultures derived from the fetal cortex. *Eur J Neurosci* **24**: 645–653.
Wang C, McInnis J, Ross-Sanchez M, Shinnick-Gallagher P, Wiley JL, Johnson KM (2001). Long-term behavioral and neurodegenerative effects of perinatal phencyclidine administration: implications for schizophrenia. *Neuroscience* **107**: 535–550.

Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)

Behavioural Brain Research 220 (2011) 132–139

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

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

Lingling Lu[a,b], Takayoshi Mamiya[a], Ping Lu[a,b], Kazuya Toriumi[a], Akihiro Mouri[a,c], Masayuki Hiramatsu[a], Li-Bo Zou[b], Toshitaka Nabeshima[a,d,*]

[a] Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, Nagoya 468-8503, Japan
[b] Department of Pharmacology, School of Life Science and Biopharmaceutics, Shenyang Pharmaceutical University, Shenyang 110016, China
[c] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan
[d] Japanese Drug Organization for Appropriate Use and Research, Nagoya 468-0069, Japan

ARTICLE INFO

Article history:
Received 12 November 2010
Received in revised form 13 January 2011
Accepted 19 January 2011

Keywords:
Prenatal PCP
Behavior
GLAST
Glutamate transmission
Prefrontal cortex
Mice

ABSTRACT

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

1. Introduction

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

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

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

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

* Corresponding author at: Department of Chemical Pharmacology, Graduate School of Pharmaceutical Sciences, Meijo University, 150 Yagotoyama, Tenpaku-ku, Nagoya 468-8503, Japan. Tel.: +81 52 839 2735; fax: +81 52 839 2738.
E-mail addresses: tnabeshi@meijo-u.ac.jp, tnabeshi@ccmfs.meijo-u.ac (T. Nabeshima).

0166-4328/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbr.2011.01.035

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

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

2. Materials and methods

2.1. Animals

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

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

2.2. Drugs

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

2.3. Drug treatment

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

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

2.4. Novel object recognition test

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

2.5. Forced swimming test

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

2.6. Microdialysis analysis

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

2.7. Preparation of brain slice and staining

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

2.8. Western blotting analysis

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

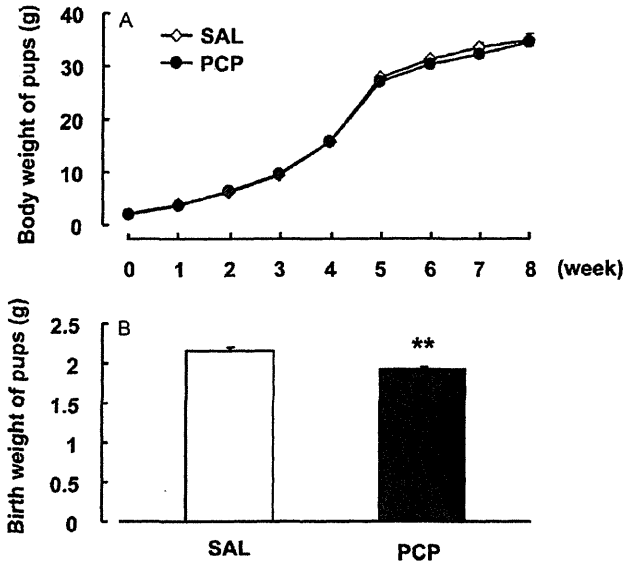

Fig. 1. Effects of prenatal PCP treatment on body weight of pups. The body weight of pups during the developing period from birth to 8 weeks old (A) (repeated one-way ANOVA with Bonferroni's test); the birth weight of pups at PD1 (B) (Student's *t*-test). $^{**}P<0.01$ compared with the prenatal SAL-treated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

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

2.9. Statistical analysis

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

3. Results

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

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

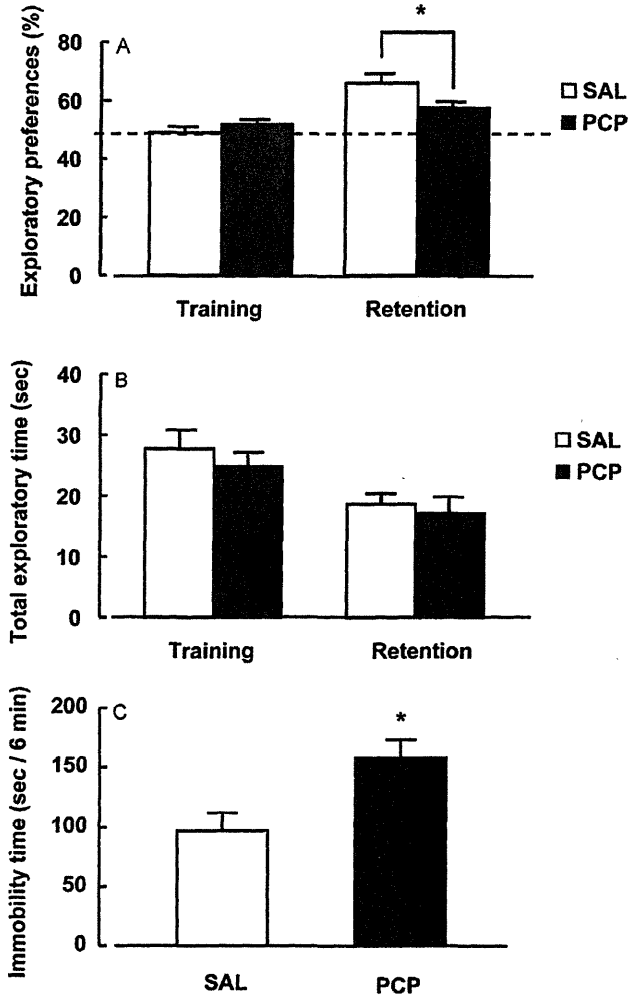

Fig. 2. Influences of prenatal PCP treatment on cognitive and emotional behavior in offspring at 8 weeks old. Exploratory preference (%) (A); and total time spent exploring the objects (s) (B) in training and retention sessions of the novel object recognition test. In the forced swimming test, immobility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

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

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

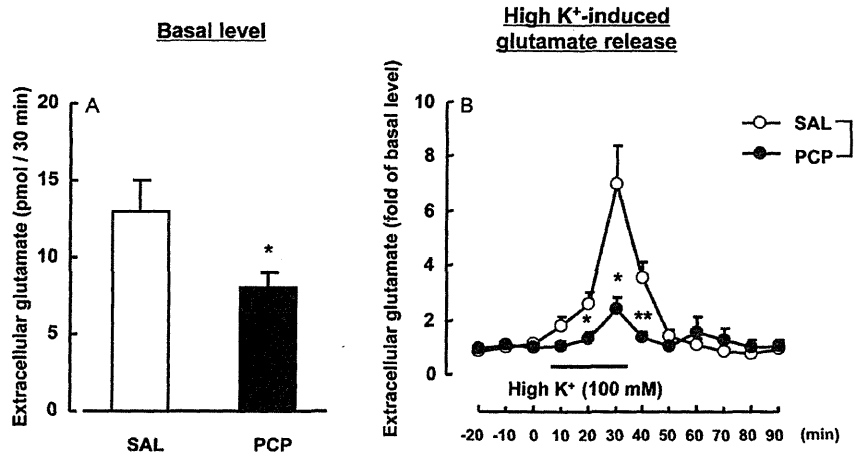

Fig. 3. Basal extracellular glutamate levels and high K^+-evoked glutamate release in the prefrontal cortex of prenatal PCP-treated mice. Basal extracellular levels of glutamate (A) and high K^+ (100 mM)-evoked glutamate release (B) in the prefrontal cortex of prenatal SAL- or PCP-treated mice were determined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

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

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

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

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

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

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

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

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

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

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