were of different shapes and colors, but of similar sizes. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. An animal was considered to be exploring an object when its head was facing the object or when an animal was sniffing an object at a distance of <2 cm and/or was touching it with its nose. At the conclusion of the training session, the mouse was immediately returned to its home cage. On Day 5, 1 d after the training session, the animals were placed back into the same box with one of the familiar objects used in the training session and one novel object. They were allowed to explore freely for 5 min and the time spent exploring each object was recorded (retention session). Exploratory preference, the ratio of time spent exploring each of the two objects (training session) or the novel object (retention session) to the total amount of time spent exploring both objects, was used to assess cognitive function.

PPI test. PPI of the acoustic startle response was measured with 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 16 decibel (dB) above the 70 dB background noise. The amount of prepulse inhibition 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.

Statistical analysis

The Mann–Whitney *U* test or paired *t* test was used for comparisons between two sets of data. Differences among three or more groups were analyzed for statistical significance by one-way ANOVA followed by Bonferroni's multiple-comparison test. A p value < 0.05 was considered statistically significant. All tests were two-tailed. All data were expressed as mean ± SEM.

Results

Most cortical GABAergic interneurons can be classified into the following four distinct classes based on the molecular markers: PV-positive cells, SST-positive cells, Reelin-positive/SST-negative cells, and VIP-positive cells (Gelman and Marín, 2010). A majority of cortical GABAergic interneurons are either PV-positive cells or SST-positive cells and originate from the MGE (Xu et al., 2004; Fogarty et al., 2007). We first investigated which classes of neurons would differentiate from MGE cells transplanted into the neonatal PFC. MGE cells were dissected from E13.5 mouse embryos expressing GFP and grafted into the mPFC of neonatal wild-type recipient mice (Fig. 1*A*). One week after transplantation, many GFP-expressing cells were found dispersed from the injection site (Fig. 1*B*). Six weeks after transplantation, many GFP-expressing cells were found in the mPFC (Fig. 1*C*,*D*), and our quantitative estimation revealed that the cells survived accounted for ~25% of the cells injected ($2.5 \pm 0.2 \times 10^4$ cells in each hemisphere, $n = 4$ hemispheres). Many of the GFP-expressing cells were positive for the neuronal marker NeuN (90.0 ± 3.0%, $n = 230$ GFP-expressing cells, 6 hemispheres) and GABA (65.7 ± 4.0%, $n = 147$ GFP-expressing cells, 6 hemispheres). In contrast, no or only a few cells expressed the oligodendrocyte marker CNPase (0.0 ± 0.0%, $n = 119$ GFP-expressing cells, 6 hemispheres) or the astrocyte marker GFAP (0.6 ± 0.6%, $n = 221$ GFP-expressing cells, 6 hemispheres), suggesting that a majority of the transplanted cells had differentiated into GABAergic interneurons. Examination of the interneuron subtype markers revealed that many GFP-expressing cells were positive for SST and Reelin (Fig. 1*E*,*F*) but negative for PV and VIP (Fig. 1*F*) and that most GFP/Reelin-double-positive cells were also positive for SST (Fig. 1*E*,*F*). The density of the GFP-expressing cells (Fig. 1*D*) and their interneuron subtypes (Fig. 1*F*) were largely constant across the subregions within the mPFC. We also found that GFP-expressing cells accounted for 7% (7.0 ± 1.5%, $n = 1651$ GABA-positive cells, 6 hemispheres) of the total number of recipient GABA-positive cells within the mPFC and 11% (10.9 ± 2.4%, $n = 611$ SST-positive cells, 6 hemispheres) of the total number of recipient SST-positive cells within the mPFC. Thus, a majority of the transplanted MGE cells had differentiated into an SST/Reelin-expressing class of GABAergic interneurons and constituted a substantial number of the interneurons within the mPFC of the recipient mice.

The neurochemical differentiation into cortical GABAergic interneurons by the MGE cells that had been transplanted prompted us to investigate whether these cells would be functionally integrated into the PFC neuronal circuitry of the host. Since c-Fos, an immediate early gene product, is known to be expressed in animals in response to various physiological stimuli, including exposure to a novel enriched environment (Staiger et al., 2000; Carlén et al., 2002; Tashiro et al., 2006; Kee et al., 2007; Ohira et al., 2010), we next investigated c-Fos expression in transplanted cells within the mPFC in response to exposure to a novel enriched environment 6 weeks after transplantation (Fig. 2*A*). When mice were housed in their original cage, only a few GFP-expressing cells in the mPFC showed c-Fos immunoreactivity (Fig. 2*B*,*E*). However, 1 h after exposure to a novel enriched environment for 10 min (Fig. 2*A*), the percentage of GFP-positive cells that expressed c-Fos was significantly higher than in the controls (Fig. 2*C–E*). In view of the fact that most of the transplanted cells differentiated into GABAergic interneurons (Fig. 1), these results suggest that at least some of the transplanted GABAergic interneurons had become functionally integrated into the PFC neuronal circuitry of the host.

We then investigated whether transplantation prevented the PCP-induced cognitive deficits by measuring the cognitive function of mice with the NORT (Ennaceur and Delacour, 1988; Tang et al., 1999; Niwa et al., 2010). The mice were exposed to two objects during the training session, and 1 d later they were tested for their ability to discriminate between a familiar object and a novel object during a retention session in which one of the two objects used in the training session had been replaced by a novel object (see Materials and Methods). PCP or vehicle was administered 30 min before the training session (Fig. 3*A*). The results showed that PCP-treated mice that had not undergone cell transplantation exhibited impaired performance in exploratory preference during the retention sessions (Fig. 3*C*), but that the PCP-induced impairment was significantly prevented in the mice that had undergone prior MGE cell transplantation (Fig. 3*C*). This preventive effect of MGE cell transplantation was not reproduced by transplantation of cells from E13.5 rostromedial cortex, which is the presumptive mPFC region and should mainly contain the excitatory projection neuron precursors/progenitors (Fig. 3*C*). Indeed, at 6 weeks after transplantation the rostromedial cortex cells that had been transplanted into the neonatal mPFC expressed the callosal projection neuron markers Satb2 (72.6 ± 6.7%, $n = 491$ GFP-expressing cells, 5 hemispheres) and the subcortical projection neuron marker Ctip2 (33.0 ± 4.2%, $n = 521$ GFP-expressing cells, 5 hemispheres), suggesting that most of them had differentiated into cortical projection neurons. Thus, the preventive effect of MGE cell transplantation against the impaired performance in exploratory preference (Fig. 3*C*) was not due to a nonspecific effect of transplantation of any neuronal precursors. There were no significant differences in exploratory time between the groups that showed significant differences in performance in the exploratory preference (Fig. 3*C*,*D*), suggesting that the differences in the performance in exploratory preference (Fig. 3*C*) were not due to the differences between the groups in curiosity and/or motivation to explore objects. MGE

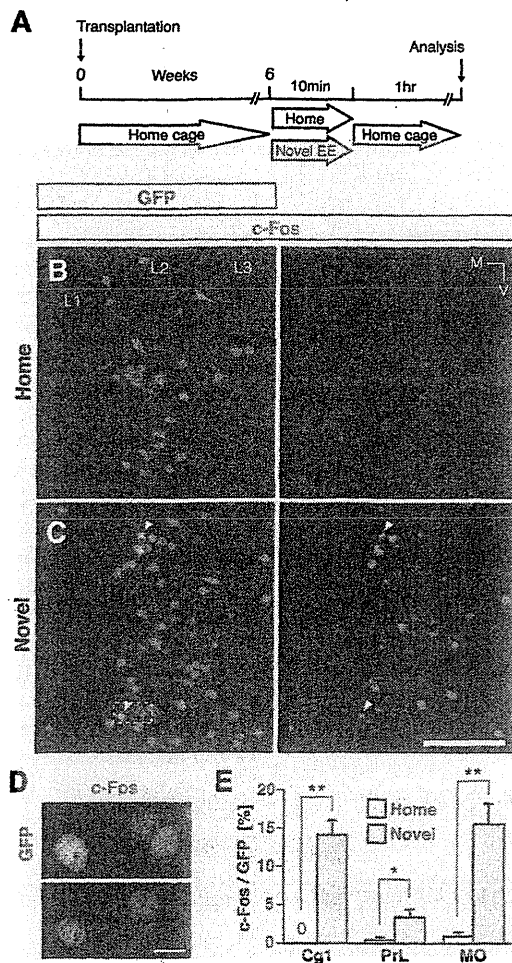

Figure 2. Some transplanted MGE cells were functionally integrated into the host neuronal circuitry within the mPFC. ***A***, Schema of the experimental design for investigating the functional integration of the transplanted cells into the neuronal circuitry of the host. Both the control mice and experimental mice underwent MGE cell transplantation. The experimental mice were exposed to a novel enriched environment for 10 min at 6 weeks after transplantation (yellow arrow), then returned to their home cages, and their brains were analyzed 1 h later. Control mice continued to be housed in their home cages. ***B***, ***C***, Expression of c-Fos (magenta) in GFP-expressing cells (green) within the PrL in the control mice (***B***) and mice exposed to a novel enriched environment (***C***). Some GFP-expressing cells in the mice exposed to a novel enriched environment expressed c-Fos (arrowheads). ***D***, Enlarged single-optical-sectional view of the boxed region in ***C***. ***E***, The percentages of GFP-expressing cells that expressed c-Fos within the Cg1 ($n = 345$ GFP-expressing cells), the PrL ($n = 522$ GFP-expressing cells), and the MO ($n = 500$ GFP-expressing cells) of the control mice (white bars) ($n = 6$ hemispheres) and within the Cg1 ($n = 733$ GFP-expressing cells), the PrL ($n = 962$ GFP-expressing cells), and the MO ($n = 1088$ GFP-expressing cells) of mice exposed to a novel enriched environment (yellow bars) ($n = 8$ hemispheres). $^{**}p = 0.0007$; $^{*}p = 0.042$ (Mann–Whitney U test). EE, Enriched environment; L1–3, layers 1–3; M, medial; V, ventral. Scale bars: ***B***, ***C***, 200 μm; ***D***, 10 μm.

cell transplantation alone did not enhance performance in exploratory preference or exploratory time (Fig. 3*C*,*D*). Thus, MGE cell transplantation into the mPFC had preventive effects against induction of the cognitive deficits by PCP.

To investigate whether this preventive effect could be obtained by MGE cell transplantation into other cortical regions, we then transplanted MGE cells into the neonatal occipital cortex, the presumptive region of the primary visual cortex (Fig. 3*B*). Similar to the results of transplantation into the mPFC (Fig. 1), a majority of the transplanted cells expressed GABA (64.1 ± 3.1%, $n = 328$ GFP-expressing cells, 6 hemispheres) at 6 weeks after transplantation, suggesting that most of them had differentiated into cortical GABAergic interneurons. Interestingly, however, the interneuron subtypes of the cells transplanted into the occipital cortex were significantly different from the subtypes after transplantation into the mPFC (Fig. 1): more transplanted cells expressed PV (50.6 ± 3.6%, $n = 291$ GFP-expressing cells, 6 hemispheres; $p = 0.0043$) compared with the cells in the MO after transplantation into the mPFC [11.2 ± 4.9% (Fig. 1*F*), Mann–Whitney U test] and fewer transplanted cells expressed SST (37.1 ± 3.2%, $n = 328$ GFP-expressing cells, 6 hemispheres; $p = 0.0022$) compared with the cells in the MO after transplantation into the mPFC [64.1 ± 4.3% (Fig. 1*F*), Mann–Whitney U test]. Even though the grafted cells had differentiated into GABAergic interneurons, MGE cell transplantation into the occipital cortex had no significant effect on the induction of the cognitive deficits by PCP (Fig. 3*E*,*F*). Thus, the preventive effect of MGE cell transplantation into the mPFC on PCP-induced cognitive deficits (Fig. 3*C*,*D*) was not a nonspecific effect of MGE cell transplantation into any cortical regions.

The finding that MGE cell transplantation into the mPFC significantly prevented impairment of a cortex-dependent task (Fig. 3*C*,*D*) raised the possibility that it might also prevent the impairment of other cortex-dependent functions that are known to be impaired by PCP administration. We therefore measured PPI, which reflects the sensory-motor gating function, a major indicator of information processing involving the cortex that is frequently impaired in schizophrenia (Arguello and Gogos, 2006). The results showed that PCP-treated mice that had not undergone cell transplantation displayed decreased PPI (Fig. 3*G*,*I*), but that the PCP-induced decrease was significantly prevented in mice that had undergone MGE cell transplantation into the mPFC (Fig. 3*G*). This preventive effect of MGE cell transplantation was not reproduced by either transplantation of rostromedial cortex cells into the mPFC (Fig. 3*G*) or of MGE cells into the occipital cortex (Fig. 3*I*). No statistically significant differences were observed in acoustic startle amplitude among any of the conditions (Fig. 3*H*,*J*), and MGE cell transplantation into the mPFC alone did not enhance PPI or the acoustic startle response (Fig. 3*G*,*H*). Thus, MGE cell transplantation into the mPFC had a preventive effect on the PCP-induced sensory-motor gating deficit.

The significant preventive effects of MGE cell transplantation into the mPFC on PCP-induced behavioral deficits raised the possibility that transplantation of the cells had altered mPFC activity in response to PCP. We focused on the preventive effect on PCP-induced cognitive deficits detected by NORT and on mPFC activity during NORT. Since NMDA receptor antagonists impair the encoding of recognition memory in mice, but not their memory consolidation and retrieval processes, and the encoding process should occur during the training session in the NORT (Nilsson et al., 2007), we then focused on mPFC activity during the training session in the NORT. To evaluate mPFC activity during the training session, brains were fixed 1 h after the training session (Fig. 4*A*), and the mPFC was analyzed for c-Fos expression. The results showed that the numbers of c-Fos-positive cells within both the PrL and the MO were significantly higher in the PCP-treated mice that had undergone MGE cell transplantation than in the PCP-treated mice that had not undergone MGE cell transplantation (Fig. 4*B*–*F*). Most of the c-Fos-positive cells in both of these regions were positive for Satb2, a marker of callosal projection neurons (Alcamo et al., 2008; Britanova et al., 2008) (Fig. 4*G*,*H*). These results suggested that prior MGE cell transplantation into the mPFC modulates the activity

of callosal projection neurons in the mPFC in response to PCP during the training session in the NORT.

Discussion

Different interneuron subtypes in different cortical regions

Approximately 10% and 60% of the MGE cells transplanted into the mPFC differentiated into PV-positive interneurons and SST-positive interneurons, respectively (Fig. 1), as opposed to ~50% and 35%, respectively, when transplanted into the occipital cortex. Previous studies have shown that the fate of distinct classes of GABAergic interneurons is already largely determined in their progenitor cells in the subpallium (Gelman and Marín, 2010). Thus, the differences in interneuron subtypes between the mPFC and the occipital cortex may have been caused by the differences in the survival and/or differentiation of interneuron subtypes after transplantation, which reflects the differences between the environments in different cortical regions of the host. Identification of environmental factors that affect the final interneuron subtypes may enable us to control the number of a specific interneuron subtype and modulate a specific neuronal circuitry of the host after MGE cell transplantation in the future.

MGE cell transplantation did not cause behavioral effects in naive mice

Transplantation of MGE cells into the mPFC modified the response to PCP treatment, but did not cause significant behavioral effects in naive mice (Fig. 3). Thus, MGE cell transplantation into the mPFC has a certain degree of specificity in its effect on the response to PCP treatment. Although as stated above, there were differences in the tendency of the

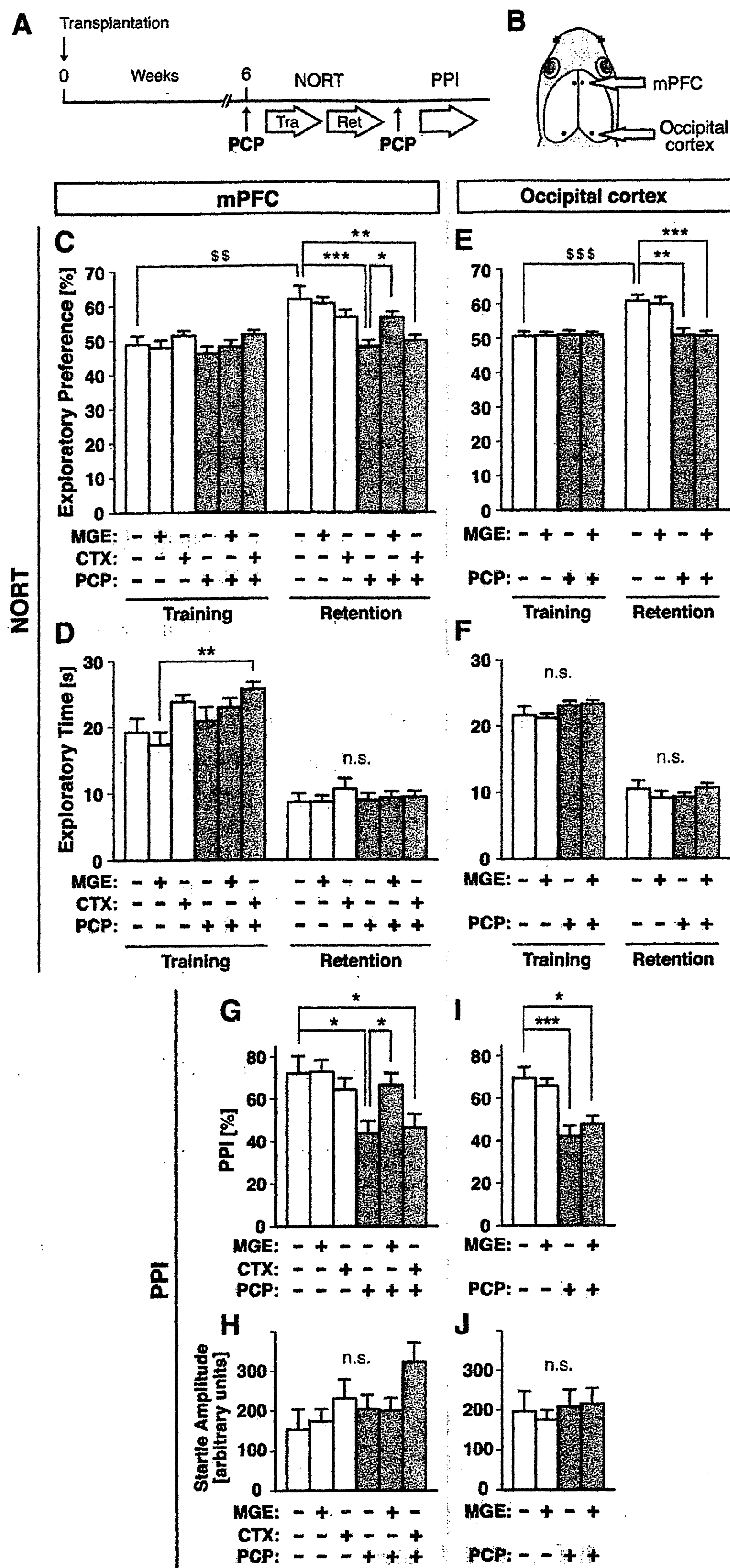

Figure 3. MGE cell transplantation into the mPFC prevented the induction of cognitive and sensory-motor gating deficits by PCP. ***A***, Schema of the experimental design for behavioral analysis of the recipient mice. MGE cells, rostromedial cortex cells, or vehicle were transplanted into the mPFC (***B***, ***C***, ***F***, ***G***) or occipital cortex (***D***, ***E***, ***H***, ***I***) of P0 neonates. PCP or vehicle was injected subcutaneously 6 weeks after transplantation, 30 min before each behavioral analysis. ***B***, Schema of bilateral transplantation into the mPFC or the occipital cortex of P0 wild-type recipient mice (dots). Dorsal view of a head. Exploratory preference (***C***, ***E***) and exploratory time (***D***, ***F***) in the NORT during the training and retention sessions. PPI with an 86 dB prepulse (***G***, ***I***) and acoustic startle amplitude (***H***, ***J***) in the PPI test. Gray bars indicate the results obtained under PCP-treated conditions. MGE-/CTX-/PCP- (−/−/−) group, $n = 14$; +/−/− group, $n = 14$; −/+/− group, $n = 11$; −/−/+ group, $n = 13$; +/−/+ group, $n = 17$; and −/+/+ group, $n = 12$ in ***C***, ***D***, ***G***, ***H***. MGE-/PCP- (−/−) group, $n = 10$; +/− group, $n = 15$; −/+ group, $n = 14$; and +/+ group, $n = 19$ in ***E***, ***F***, ***I***, ***J***. $^{\$\$\$}p < 0.001$; $^{\$\$}p < 0.01$ (paired *t* test); $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ (Bonferroni's test). Tra, Training; Ret, retention; CTX, cortex; n.s., not significant.

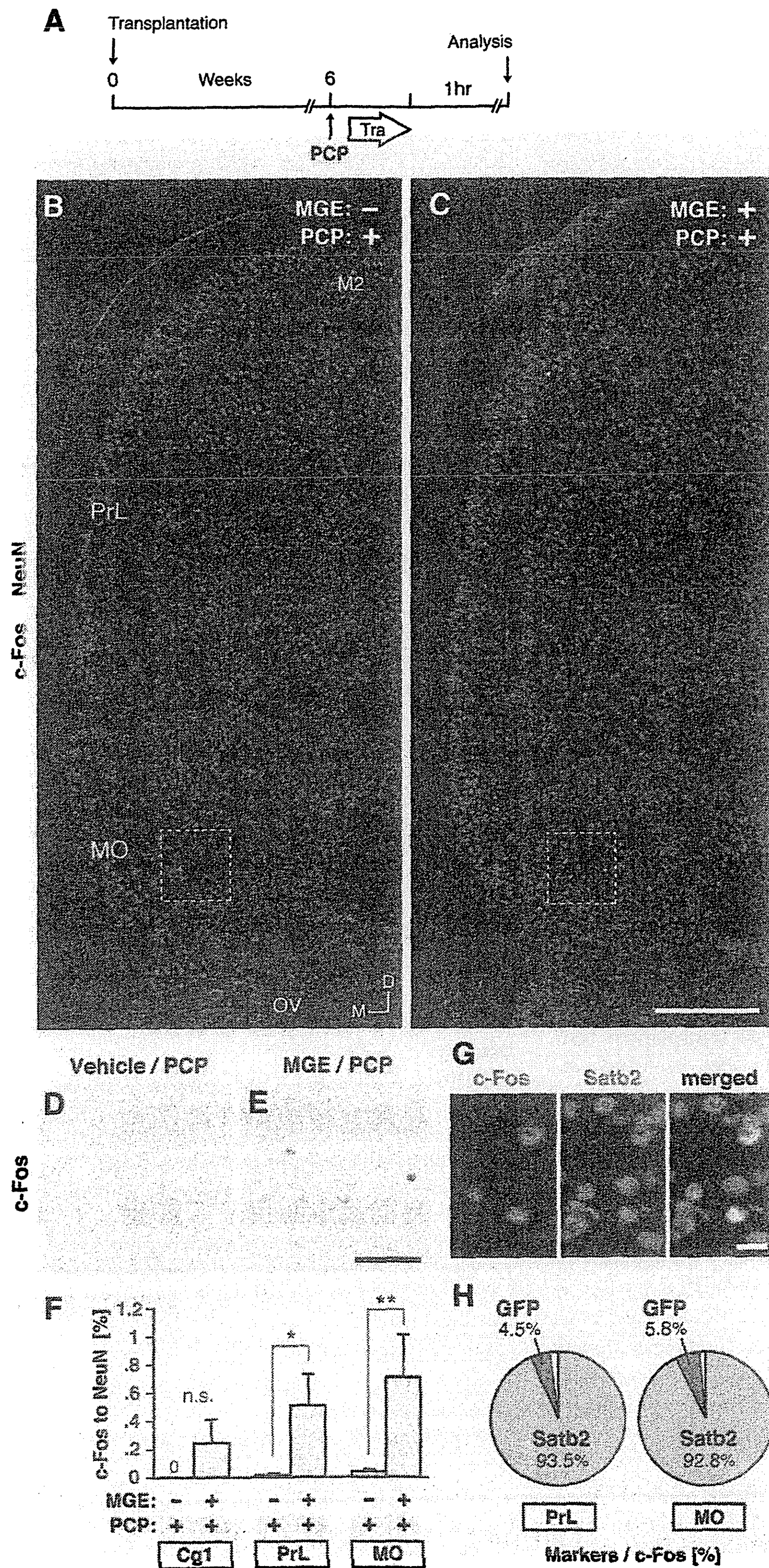

Figure 4. Prior MGE cell transplantation into the mPFC increased the activity of callosal projection neurons in the mPFC in response to the PCP. ***A***, Schema of the experimental design to investigate neuronal activity during the training session in the NORT. The design was the same as that used for the behavioral analysis (Fig. 3*A*), but brains were analyzed 1 h after the training session in the NORT. ***B***, ***C***, c-Fos expression (magenta) within the mPFC in mice injected with vehicle at P0 and with PCP at 6 weeks (MGE−/PCP+ group) (***B***) and in mice that underwent MGE cell transplantation at P0 and were injected with PCP at 6 weeks (MGE+/PCP+ group) (***C***). ***D***, ***E***, c-Fos expression (black) in enlarged views of the boxed regions in ***B*** and ***C***, respectively. ***F***, Ratio of the number of c-Fos-positive cells to the number of NeuN-positive cells within the Cg1 ($n = 0$ c-Fos-positive cell, 5951 NeuN-positive cells), the PrL ($n = 2$ c-Fos-positive cells, 15,904 NeuN-positive cells), and the MO ($n = 13$ c-Fos-positive cells, 34,047 NeuN-positive cells) in the MGE−/PCP+ group ($n = 12$ hemispheres) and within the Cg1 ($n = 13$ c-Fos-positive cells, 6373 NeuN-positive cells), the PrL ($n = 88$ c-Fos-positive cells, 17,520 NeuN-positive cells), and the MO ($n = 226$ c-Fos-positive cells, 35,591 NeuN-positive cells) in the MGE+/PCP+ group ($n = 12$ hemispheres). $**p = 0.0023$; $*p = 0.0387$ (Mann–Whitney *U* test). ***G***, Expression of Satb2 (light blue) in c-Fos-positive cells (magenta) in the MO in the MGE+/PCP+ mouse. ***H***, Calculation of the percentage of c-Fos-positive cells expressing Satb2 and GFP within the PrL ($n = 153$ c-Fos-positive cells) and the MO ($n = 277$ c-Fos-positive cells) in the MGE+/PCP+ group ($n = 6$ hemispheres). Tra, training; M2, secondary motor cortex; OV, olfactory ventricle; M, medial; D, dorsal. Scale bars: ***B***, ***C***, 500 μm; ***D***, ***E***, 100 μm; ***G***, 20 μm.

transplanted cells to survive and/or differentiate into certain interneuron subtypes according to the cortical areas into which they had been transplanted, the transplanted MGE cells were highly motile, dispersed widely, and intermingled with the endogenous neurons in both neonatal and adult brain (Wichterle et al., 1999; Alvarez-Dolado et al., 2006; Martínez-Cerdeño et al., 2010; Southwell et al., 2010; present study). We hypothesize that the cortical neuronal network is largely permissive for functional integration of transplanted MGE cells, and that the basal excitation/inhibition ratio in the PFC can be readjusted to prevent behavioral abnormalities in the host. This readjustment may be accompanied by structural alterations of the neuronal circuit (Southwell et al., 2010) in the PFC, and that may modify the behavioral response to PCP treatment. We cannot rule out the possibility, however, that MGE cell transplantation into the mPFC may also have behavioral effects on naive mice (Martínez-Cerdeño et al., 2010) that were not detected in the present study.

Mechanisms by which MGE cell transplantation increased the c-Fos level in the PrL and the MO

MGE cell transplantation into the neonatal mPFC significantly increased the activity of callosal projection neurons within the PrL and the MO in response to PCP (Fig. 4). The mechanism responsible for the absence of a significant increase in activity in the Cg1 alone (Fig. 4*F*) remains unclear. The properties of the transplanted MGE cells in the different subregions of the mPFC were indistinguishable

in terms of cell density (Fig. 1*D*), interneuron subtypes (Fig. 1*F*), and functional integration (Fig. 2*E*), suggesting that differences between the transplanted cells according to the subregions of the mPFC may not explain the mechanism.

Another interesting question in regard to mechanisms is how the increase in callosal projection neuron activity in the mPFC was induced by MGE cell transplantation. Since previous studies have shown that systemic administration of NMDA receptor antagonists disinhibits projection neurons in the PFC by decreasing the activity of GABAergic interneurons (Homayoun and Moghaddam, 2007) and that MGE cell transplantation increases GABA-mediated inhibition on host projection neurons and modulates host neuronal circuitry and its activity (Alvarez-Dolado et al., 2006; Baraban et al., 2009; Southwell et al., 2010; Zipancic et al., 2010), one might expect MGE cell transplantation to increase the level of inhibition on host projection neurons and decrease the amount of disinhibition produced by PCP, thereby diminishing c-Fos level relative to level observed in the absence of MGE cell transplantation. In reality, however, MGE cell transplantation increased the c-Fos level in host mPFC callosal projection neurons relative to its level in the absence of MGE cell transplantation (Fig. 4). One possible explanation for this unexpected result is that, rather than contributing to general suppression of the activities of callosal projection neurons, the transplanted MGE cells had contributed to generation of their rhythmic activity. This may then facilitate signal transduction of callosal projection neurons and efficiently activate their target cells, including homotopic callosal projection neurons in the contralateral hemisphere (Audinat et al., 1988; Cassell et al., 1989; Kuroda et al., 1995; Reep et al., 1996; Carr and Sesack, 1998), which, in turn, may ultimately lead to activation of the reciprocal network between homotopic callosal projection neurons across hemispheres and expression of c-Fos in Satb2-positive cells within the mPFC (Fig. 4). In support of this hypothesis, most MGE cells transplanted into the neonatal mPFC differentiate into a SST-expressing class of interneurons (Fig. 1) that may be involved in cortical rhythmogenesis (Gibson et al., 1999, 2005; Beierlein et al., 2000). Interestingly, most transplanted SST-expressing interneurons expressed Reelin (Fig. 1), which enhances signal transduction through NMDA receptors (Herz and Chen, 2006; Knuesel, 2010). The Reelin secreted by the transplanted cells may enhance the NMDA receptor function of surrounding cells, including the function of the endogenous GABAergic interneurons of the host. Thus, transplanted SST-positive cells and/or other GABAergic interneurons whose NMDA receptor-mediated signaling has been upregulated by Reelin might increase the rhythmic activity of callosal projection neurons. Since c-Fos expression appears to reflect the activity level but not the activity pattern of neurons, direct measurement of the activity pattern of callosal projection neurons, by electrophysiological recordings, for example, may be required to assess their rhythmic activity. A previous study has shown that the activity of SST-positive cells appears to be blocked by NMDA receptor antagonists more easily than the activity of PV-positive interneurons (Lu et al., 2007). The above-described possible effect of Reelin on surrounding cells may explain how transplanted SST-positive interneurons could change mPFC activity even in the presence of PCP. It will be important to determine whether the Reelin secreted by transplanted MGE cells is involved in the effect of the MGE cell transplantation by transplanting MGE cells derived from the Reelin-deficient reeler mutants (D'Arcangelo et al., 1995).

Mechanisms by which MGE cell transplantation prevents PCP-induced behavioral deficits

It remains unclear how MGE cell transplantation prevented PCP-induced behavioral deficits. Although we found that the preventive effect of MGE cell transplantation on the PCP-induced behavioral deficits was accompanied by an increase in the c-Fos level in the mPFC (Fig. 4), it remains unclear whether the increases of c-Fos levels in the mPFC are causally related to the effects of MGE cell transplantation on the behavioral deficits induced by PCP. The next intriguing question, therefore, is whether the activation of the callosal projection neurons within the PrL and the MO is required and/or sufficient to produce significant behavioral effects of MGE cell transplantation. It might be possible to answer this question by specifically inactivating and/or activating Satb2-postive cells within the PrL and the MO, for example, by means of an optogenetic technique (Zhang et al., 2007).

One might argue that the mechanisms by which MGE cell transplantation prevents PCP-induced behavioral effects are unlikely to be related to modulating the alterations in PFC excitatory/inhibitory balance that are usually produced by PCP. In support of this argument, in contrast to a previous study (Kargieman et al., 2007), c-Fos expression was rarely detected after PCP administration alone in the present study (Fig. 4). Furthermore, as mentioned above, MGE cell transplantation increased, not decreased, the c-Fos level in the mPFC (Fig. 4). How, then, might MGE cell transplantation prevent PCP-induced behavioral effects? One possible explanation is that rather than simply augmenting the strength of the endogenous, mature inhibitory connections, the transplanted MGE cells might reorganize the PFC circuitry by introducing a new set of weak, but numerous, inhibitory synapses on host projection neurons (Southwell et al., 2010). Such a structural and functional reorganization of the PFC as a result of the transplantation may increase tolerance to PCP and thereby lead to the prevention of PCP-induced behavioral deficits.

Alternative means of increasing the number of interneuron precursors in the mPFC

Although we transplanted embryonic MGE cells to increase the number of interneuron precursors in the neonatal mPFC, an increase could be achieved by other means as well. For example, prospective MGE cells generated by pluripotent stem cells *in vitro* (Maroof et al., 2010; Danjo et al., 2011) could be used as graft cells. Manipulation of endogenous cells might also be possible, including by proliferative activation of interneuron progenitors present even in the adult cortex (Ohira et al., 2010) and directing postmitotic migrating interneurons to the PFC by, for example, injecting their *in vivo* attractant CXCL12 (Li et al., 2008; Tanaka et al., 2009) into the mPFC.

Clinical implications

The cognitive symptoms of schizophrenia are largely resistant to current pharmacological treatments and preventive approaches to their development have not been adequately explored (Keefe et al., 2007). Our findings in this study may contribute to the development of a cell-based approach as a novel means of preventing and possibly of treating the schizophreniform cognitive deficits.

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ARTICLE

CINP

Galantamine ameliorates the impairment of recognition memory in mice repeatedly treated with methamphetamine: involvement of allosteric potentiation of nicotinic acetylcholine receptors and dopaminergic-ERK1/2 systems

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Abstract

Galantamine, a drug used to treat Alzheimer's disease, inhibits acetylcholinesterase (AChE) and allosterically modulates nicotinic acetylcholine receptors (nAChRs) resulting in stimulation of catecholamine neurotransmission. In this study, we investigated whether galantamine exerts cognitive-improving effects through the allosteric modulation of nAChRs in an animal model of methamphetamine (Meth) psychosis. The mice treated with Meth (1 mg/kg.d) for 7 d showed memory impairment in a novel object recognition test. Galantamine (3 mg/kg) ameliorated the memory impairment, and it increased the extracellular dopamine release in the prefrontal cortex (PFC) of Meth-treated mice. Donepezil, an AChE inhibitor (1 mg/kg) increased the extracellular ACh release in the PFC, whereas it had no effect on the memory impairment in Meth-treated mice. The nAChR antagonist, mecamylamine, and dopamine D_1 receptor antagonist, SCH 23390, blocked the ameliorating effect of galantamine on Meth-induced memory impairment, whereas the muscarinic AChR antagonist, scopolamine, had no effect. The effects of galantamine on extracellular dopamine release were also antagonized by mecamylamine. Galantamine attenuated the defect of the novelty-induced activation of extracellular signal-regulated kinase 1/2 (ERK1/2). The ameliorating effect of galantamine on recognition memory in Meth-treated mice was negated by microinjection of an ERK inhibitor, PD98059, into the PFC. These results suggest that the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1 receptor-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could be a useful therapeutic agent for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Key words: Allosteric potentiation of nicotinic acetylcholine receptors, cognitive impairment, dopamine, extracellular signal-regulated kinase 1/2, galantamine, methamphetamine.

Introduction

Galantamine, a potent allosteric potentiating ligand (APL) and a drug approved for treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nicotinic acetylcholine receptors (nAChRs) (Eisele *et al.* 1993; Santos *et al.* 2002). We have found that galantamine has ameliorating effects on the impairment of performance in the novel object recognition (NOR) and/or conditioned fear learning tasks caused by a single intracerebroventricular infusion of amyloid-β peptide

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(Aβ) fragment (as an animal model of Alzheimer's disease) (Wang *et al.* 2007*a*) and by repeated treatment with a non-competitive *N*-methyl-D-asparatate receptor antagonist, phencyclidine (PCP) (as an animal model of schizophrenia) (Wang *et al.* 2007*b*). It increases the extracellular dopamine release in the hippocampus and prefrontal cortex (PFC) of $A\beta_{25-35}$-infused and PCP-treated mice, respectively. The ameliorating effects of galantamine on $A\beta_{25-35}$- and PCP-induced cognitive impairment are mediated through the augmentation of dopaminergic neurotransmission following activation of nAChRs (Wang *et al.* 2007*a*, *b*). These studies provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the hippocampus/PFC through the allosteric activation of nAChRs. Thus, galantamine shows potential as a novel therapeutic agent for cognitive impairments associated with schizophrenia, as well as Alzheimer's disease, although the molecular mechanism of action remains to be determined in detail.

Methamphetamine (Meth) is a highly addictive drug of abuse, and addiction to Meth has increased to epidemic proportions worldwide (Cretzmeyer *et al.* 2003; Rawson *et al.* 2002). Chronic Meth users show psychotic signs such as hallucinations and delusions, which are indistinguishable from paranoid schizophrenia (Sato *et al.* 1983; Srisurapanont *et al.* 2003; Yui *et al.* 2002). Recent studies have suggested that chronic use of Meth causes long-term cognitive deficits (Kalechstein *et al.* 2003; Nordahl *et al.* 2003; Simon *et al.* 2000). We have found that repeated Meth treatment in mice impairs long-term recognition memory after withdrawal, which is associated with the dysfunction of the extracellular signal-regulated kinase 1/2 (ERK1/2) pathway in the PFC, and that Meth-induced cognitive impairment is reversed by an atypical antipsychotic, clozapine, but not haloperidol (Kamei *et al.* 2006). Meth-induced cognitive impairment in mice may be a useful animal model for cognitive deficits in Meth abusers and/or schizophrenia patients.

The present study was designed to test the hypothesis that galantamine improves cognitive deficit in the Meth-treated animal model of Meth psychosis and/or schizophrenia (Kamei *et al.* 2006), and that such cognitive-improving effects are mediated via activation of nAChR-dopaminergic-ERK1/2 pathways. We attempted to investigate: (1) whether cognitive-improving effects of galantamine are mediated via nAChRs in Meth-treated mice and (2) whether galantamine augments dopamine neurotransmission in the PFC by activation of nAChRs.

Methods

Animals

Male mice of the ICR strain (Japan SLC Inc., Japan), aged 6 wk at the beginning of experiments, were used. They were housed in plastic cages, received food (CE2; Clea Japan Inc., Japan) and water *ad libitum*, and were maintained on a 12-h light/dark cycle (lights on 08:00 hours). Behavioural experiments were performed in a sound-attenuated and air-regulated experimental room, to which mice were habituated for at least 1 h. All experiments were conducted blind to treatment and in accordance with the Guidelines for Animal Experiments of Nagoya University Graduate School of Medicine. The procedures involving animals and their care conformed to the international guidelines set out in the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Drugs

Galantamine hydrobromide (4a,5,9,10,11,12-hexahydro-3-methoxy-11-methyl-6H-benzofuro [3a,3,2-ef] benzazepin-6-ol hydrobromide) was supplied by Janssen Pharmaceutica (Tokyo, Japan). Galantamine, donepezil hydrochloride (Toronto Research Chemicals Inc., Canada), methamphetamine hydrochloride (Dainippon Sumitomo Pharma Co. Ltd, Japan), mecamylamine hydrochloride (Sigma-Aldrich, USA), (−)scopolamine hydrobromide (Sigma-Aldrich) and R(+)-SCH 23390 hydrochloride [R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine hydrochloride] (Sigma-Aldrich) were dissolved in saline. PD98059 (Sigma-Aldrich) was dissolved in 60% dimethylsulfoxide (DMSO) saline.

Drug treatment

The mice were administered Meth (1 mg/kg.d s.c.) or saline once a day for 7 consecutive days (Kamei *et al.* 2006). The NOR test and microdialysis experiment were started 1 d and 3 d, respectively after the withdrawal of Meth treatment. The saline- or Meth-treated mice were administered galantamine (3 mg/kg p.o.) or donepezil (1 mg/kg p.o.) 1 h before the training session of the NOR test, or immediately after baseline collections in the microdialysis experiment. Mecamylamine (3 mg/kg s.c.), scopolamine (0.1 mg/kg s.c.) and SCH 23390 (0.02 mg/kg s.c.) were injected 20, 20 and 30 min, respectively, after treatment with galantamine. The doses of galantamine and donepezil used in the present study were as determined in previous experiments (Wang *et al.* 2007*a*, *b*) and in the report by Geerts *et al.* (2005), in which donepezil is

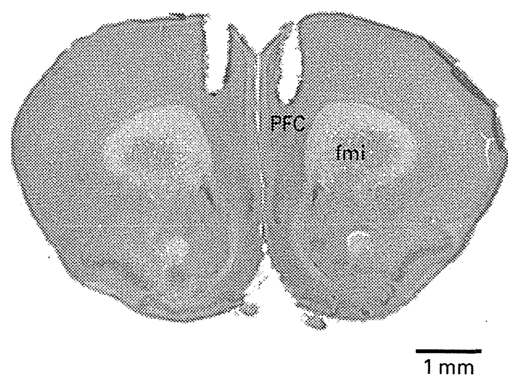

Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

3–15 times more potent than galantamine in inhibiting brain AChE *in vivo*. The doses of antagonists were selected based on our previous publications (Kamei *et al.* 2006; Wang *et al.* 2007*a*, *b*). All compounds except for PD98059 were systemically administered at a volume of 0.1 ml/10 g body weight. Control mice received the same volume of saline.

For local microinjection into the PFC, mice were anaesthetized with diethyl ether and fixed on the stereotactic apparatus (Narishige, Japan) 30 min before the training session. An L-shaped injection cannula (27 gauge) with a bevel tip at its short end was grasped with forceps and implanted into the PFC (+0.3 mm mediolateral from the midpoint on the line linking the two rear canthi, −2.5 mm in depth). PD98059 at a dose of 2 μg/1 μl/bilateral or vehicle (60% DMSO/2 μl/bilateral) was infused into the PFC for 45 s using a Hamilton microsyringe connected to the cannula via a Teflon tube, and the connection was maintained for another 45 s after the injection. After the behavioural experiments, the mice were decapitated, and the brains were removed. The brains were transversely cut along the direction of the vertical insertion of the cannula to confirm the injection site, which was obvious due to its dark red colour, and easily recognized as shown in Fig. 1. Misinjected mice were excluded from subsequent data analysis.

NOR test

The task was carried out on days 1–3 after the final injection of Meth in accordance with the method of Kamei *et al.* (2006) with a minor modification. The experimental apparatus consisted of a Plexiglas open-field box (40 × 40 × 29 high cm), the floor of which was covered with paper bedding. The apparatus was placed in a sound-isolated room. A light bulb, located in the upper part of the room and which could not be seen directly by the mice, provided constant illumination of about 40 lx at the level of the task apparatus.

The NOR task procedure consisted of three sessions: habituation, training, retention. Each mouse was individually habituated to the box, with 10 min exploration in the absence of objects on day 1 (habituation session). During the training session on day 2, two objects (A and B) were placed in the back corner of the box, 10 cm away from the side wall. A mouse was then placed in the middle front of the box and the total time spent in exploring the two objects was recorded for 10 min by the experimenter using two stopwatches. Exploration of an object was defined as directing the nose to the object at a distance of <2 cm and/or touching it with the nose. During the retention session on day 3, the animals were returned to the same box 24 h after the training session, in which one of the familiar objects (e.g. object A) used during the training session was replaced by a novel object C. The animals were then allowed to explore freely for 10 min and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a balanced manner in terms of their physical complexity and emotional neutrality. A preference index, the ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function, e.g.

training session: A or B/(B + A) × 100 (%),

retention session: B or C/(B + C) × 100 (%).

Determination of extracellular acetylcholine (ACh) and dopamine levels in the PFC

In-vivo microdialysis was performed 3 d after the final injection of Meth. One day before microdialysis, mice were anaesthetized with sodium pentobarbital (50 mg/kg i.p.) and a guide cannula (MI-AG-6; Eicom Corp., Japan) was implanted into the mPFC (+1.9 mm anteroposterior, +1.0 mm mediolateral from bregma, −1.5 mm dorsoventral from the skull, +15° angle from vertical) according to the atlas of Franklin & Paxinos (1997). One day after the operation, the dialysis probe of ACh (A-I-4-02; 2 mm membrane length; Eicom Corp.) and dopamine (A-I-6-01; 1 mm membrane length; Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, 2.3 mM $CaCl_2$) at a flow rate of 1 μl/min (Mouri *et al.*

2006) and 1.2 μl/min (Shintani *et al.* 1993), respectively. The outflow fractions of ACh and dopamine were collected every 20 min and 10 min, respectively. When the difference of each fraction was <20%, we considered this a stable baseline. Following the collection of three stable baseline fractions of ACh and dopamine, mice were treated with donepezil, galantamine and/or mecamylamine, and then dialysates of ACh and dopamine were collected every 20 min for 120 min and every 10 min for 90 min, respectively. ACh and dopamine levels in the dialysates were analysed using an HPLC system equipped with an electrochemical detector (Mouri *et al.* 2007, 2006).

Western blotting

We examined activation of ERK1/2 in the brain of mice that were exposed to the novel objects during the training session. Phosphorylation of ERK1/2 was examined by Western blotting as described previously (Kamei *et al.* 2006; Mizoguchi *et al.* 2004). Immediately after a training session, the mice were sacrificed by decapitation, and the brain was immediately removed. The PFC was rapidly dissected out on an ice-cold plate, frozen, and stored at −80 °C until required. Tissue samples from the PFC were homogenized by sonication at 4 °C in a lysis buffer composed of 20 mM Tris–HCl, 150 mM NaCl, 50 mM NaF, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 1 mM sodium orthovanadate, 0.1% SDS, 1% sodium deoxycholate, 0.5 mM dithiothreitol, 10 mM sodium pyrophosphate decahydrate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 10 μg/ml pepstatin (pH 7.4). The homogenate was centrifuged at 13 000 *g* for 20 min and the supernatant was used. The protein concentration of tissue extracts was determined using a DC Protein Assay kit (Bio-Rad, USA). Samples (20 μg protein) were boiled in a sample buffer [0.125 M Tris–HCl (pH 6.8), 2% SDS, 5% glycerol, 0.002% Bromphenol Blue, and 5% 2-mercaptoethanol], applied onto a 10% polyacrylamide gel, subsequently transferred to a polyvinylidene difluoride membrane (Millipore Corporation, USA) or a nitrocellulose membrane (GE Healthcare Biosciences, USA), and blocked with a Detector Block kit (Kirkegaard and Perry Laboratories, USA). Membranes were incubated with anti-phospho-ERK1/2 [phospho-p44/42 mitogen-activated protein kinase (MAPK) (Thr^{202}/Tyr^{204}) Antibody no. 9101] (1:1000 dilution; Cell Signaling Technology Inc., USA) and washed with Tris-buffered saline (TBS)-Tween 20 [10 mM Tris–HCl (pH 7.5), 100 mM NaCl, and 1% Tween 20] three times for 10 min each. After incubation with a 1:2000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (secondary antibody) for 1 h, membranes were washed with TBS-Tween 20 three times for 10 min each. The immune complex was detected using ECL Western blotting detection reagents (GE Healthcare Biosciences). The same membranes were stripped with a stripping buffer (137 mM NaCl, 2.7 mM KCl, 8.1 mM disodium hydrogen phosphate, 12-water, 1.5 mM potassium dihydrogen phosphate, and 0.2% 2-mercaptoethanol) at 55 °C for 30 min, incubated with anti-ERK1/2 (1:1000 dilution, p44/42 MAPK Antibody no. 9102, Cell Signaling Technology Inc.), and treated as described above.

Statistical analysis

Statistical significance was determined using a one-way analysis of variance (ANOVA) or a two-way ANOVA with repeated measures, followed by Bonferroni's test for multigroup comparisons. Statistical differences between two sets of groups were determined with the Student's *t* test. *p* values <0.05 were taken to indicate statistically significant differences.

Results

Effect of galantamine on Meth-induced impairment of recognition memory in mice

We examined whether Meth-induced cognitive impairment was reversed by galantamine. One day after the cessation of repeated Meth (1 mg/kg.d s.c.) treatment for 7 d, mice were subjected to the NOR test. Galantamine (3 mg/kg p.o.) was acutely administered 1 h before the training session.

As shown in Fig. 2, repeated Meth treatment significantly reduced the exploratory preference for a novel object in the retention session ($p<0.01$) (Fig. 2*a*). Treatment with galantamine significantly improved cognitive impairment in Meth-treated mice ($p<0.01$) (Fig. 2*a*). Galantamine affected neither the level of exploratory preference for the objects in the training session [$F(3,36)=1.188$, $p=0.328$] (Fig. 2*a*) nor the total exploration time in either the training [$F(3,36)=1.241$, $p=0.309$] or retention [$F(3,36)=2.396$, $p=0.084$] sessions in Meth-treated mice (Fig. 2*b*).

Effect of donepezil on the extracellular ACh levels of the PFC and the impairment of recognition memory in Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment

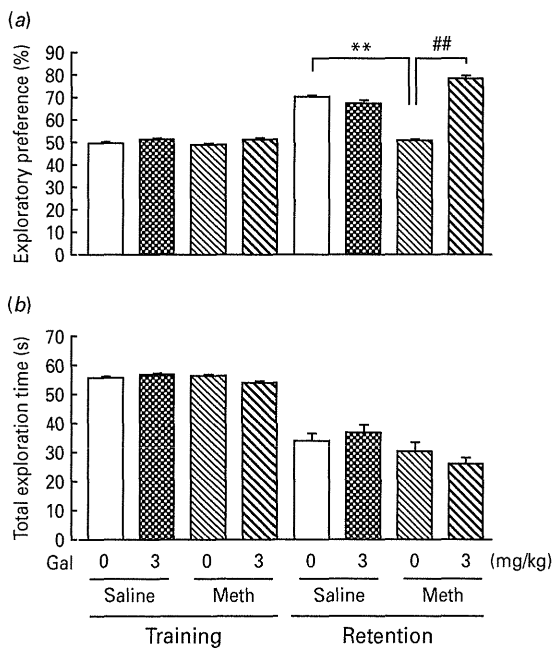

Fig. 2. Effect of galantamine on methamphetamine (Meth)-induced impairment of recognition memory in mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (3 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean$\pm$S.E. ($n=10$). One-way ANOVA, (*a*) training: $F(3,36)=1.188, p=0.328$; retention: $F(3,36)=63.849$, $p<0.01$; (*b*) training: $F(3,36)=1.241, p=0.309$; retention: $F(3,36)=2.396, p=0.084$. ** $p<0.01$ compared to saline+saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth+saline-treated group (Bonferroni's test).

are due to increase of ACh levels caused by inhibition of AChE, we examined the effect of donepezil, an AChE inhibitor, on the impairment of cognition in Meth-treated mice.

Donepezil at a dose of 1 mg/kg caused about a 2-fold increase in the levels of extracellular ACh in the PFC of Meth-treated mice [$F(1,35)=14.042$, $p<0.01$] (Fig. 3*a*). However, donepezil (1 mg/kg) had no effect on the level of exploratory preference for the objects in the retention sessions in Meth-treated mice (Fig. 3*b*). It also affected neither the level of exploratory preference for the objects in the training session [$F(2,40)=0.159$, $p=0.854$] (Fig. 3*a*) nor the total exploration time in either the training [$F(2,40)=0.296$, $p=0.746$] or retention [$F(2,40)=0.160$, $p=0.215$] sessions in Meth-treated mice (Fig. 3*c*).

Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on Meth-treated mice

To determine whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated via nAChRs, but not muscarinic AChRs (mAChRs), we examined the antagonism by using mecamylamine, a nAChR antagonist and scopolamine, a mAChR antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

In the training session of the NOR task, there were no differences in exploratory preference for the objects in any of the groups (Fig. 4*a, c*). The nAChR antagonist, mecamylamine (3 mg/kg) significantly and completely prevented the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice ($p<0.01$) (Fig. 4*a*). In saline-treated mice, mecamylamine alone at the dose used had no effect on the NOR performances (Fig. 4*a*). The antagonistic effect of mecamylamine on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(4,57)=0.516$, $p=0.725$; retention: $F(4,57)=2.403, p=0.060$] (Fig. 4*b*).

Scopolamine at a dose of 0.1 mg/kg impaired the performance of saline-treated mice in the NOR task (Fig. 4*c*). However, scopolamine failed to prevent the improving effects of galantamine on the impairment of recognition memory in Meth-treated mice (Fig. 4*c*). Treatment with any compound did not affect the total exploration time in either the training [$F(6,77)=2.193$, $p=0.053$] or retention [$F(6,77)=1.919$, $p=0.088$] sessions (Fig. 4*d*).

Effects of galantamine on the levels of extracellular dopamine in the PFC of Meth-treated mice

We examined whether galantamine at a dose of 3 mg/kg, which improved the cognitive deficit in Meth-treated mice, facilitated dopamine release in the PFC of Meth-treated mice.

There were no differences in the basal levels of extracellular dopamine in the PFC in any of the groups (Fig. 5 insert). As shown in Fig. 5, galantamine (3 mg/kg) caused a marked increase in the levels of extracellular dopamine in the PFC of Meth-treated mice (Fig. 5). The significant increase in the levels of extracellular dopamine was observed from 30 min after galantamine administration ($p<0.01$ by *post hoc* test, Fig. 5). When mecamylamine (3 mg/kg) was injected into Meth-treated mice 20 min after galantamine administration, galantamine-induced elevation of extracellular dopamine levels was significantly diminished

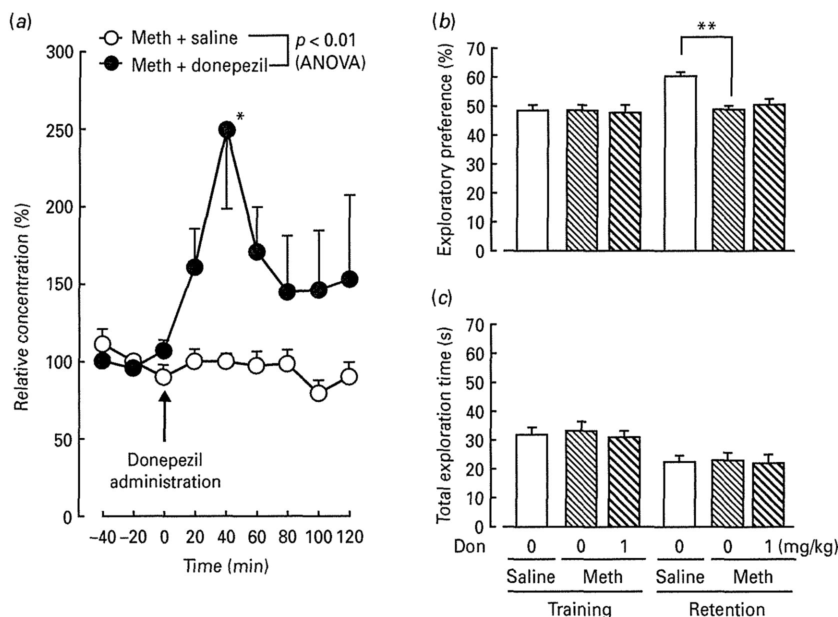

Fig. 3 Effect of donepezil on the extracellular acetylcholine (ACh) levels of the prefrontal cortex (PFC) and the impairment of recognition memory in methamphetamine (Meth)-treated mice. (*a*) Extracellular ACh levels of PFC in microdialysis. *In-vivo* microdialysis was performed 3 d after the final injection of Meth (1 mg/kg s.c.) treatment for 7 d. Donepezil (1 mg/kg p.o.) was administered to the Meth-treated mice (●, Meth + donepezil). In the control group, an equivalent amount of saline was given to the Meth-treated mice (○, Meth + saline). Values indicate the mean ± S.E. ($n = 4$–5). Results with the repeated ANOVA were: time [$F(5, 35) = 1.111$, $p = 0.37$]; treatment [$F(1, 35) = 14.042$, $p < 0.01$]; time × treatment interaction [$F(5, 35) = 0.677$, $p = 0.64$]. * $p < 0.05$ compared to Meth + saline-treated group (Bonferroni's test). The basal levels of ACh in the PFC of the Meth + saline- and Meth + donepezil-treated mice were 0.17 ± 0.05 and 0.12 ± 0.06 pmol/20 μl per 20 min, respectively. (*b*) Exploratory preference in novel object recognition (NOR) test. (*c*) Total exploration time in NOR test. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the NOR test. Donepezil (1 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean ± S.E. ($n = 13$–15). One-way ANOVA, (*b*) training: $F(2, 40) = 0.159$, $p = 0.854$; retention: $F(2, 40) = 9.400$, $p < 0.01$; (*c*) training: $F(2, 40) = 0.296$, $p = 0.746$; retention: $F(2, 40) = 0.160$, $p = 0.215$. ** $p < 0.01$ compared to saline + saline-treated group (Bonferroni's test).

(Fig. 5). However, mecamylamine alone did not affect the extracellular dopamine levels in saline-treated mice (data not shown).

Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on Meth-treated mice

Previous studies have shown that the ERK1/2 signalling pathway linked to dopamine D_1 receptors (D_1Rs) (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in Meth-associated contextual memory in rats (Mizoguchi *et al.* 2004) and that repeated Meth treatment induces cognitive impairment in the NOR test in mice, which is accompanied by dysfunction of the dopamine D_1R-ERK1/2 pathway in the PFC (Kamei *et al.* 2006). To clarify whether the improving effects of galantamine on Meth-induced cognitive impairment are mediated through the activation of dopamine D_1Rs, we investigated the antagonism by using SCH 23390, a dopamine D_1R antagonist, against the cognitive-improving effects of galantamine in Meth-treated mice.

SCH 23390 (0.02 mg/kg) significantly and completely prevented the improving effects of galantamine on Meth-induced cognitive impairment without affecting the exploratory preference for the objects in the training session (Fig. 6*a*). In saline-treated mice, SCH 23390 alone had no effect on NOR performance (Fig. 6*a*). SCH 23390 also had no effect on the total exploration time in either the training [$F(4, 50) = 1.520$, $p = 0.211$] or retention [$F(4, 55) = 1.943$, $p = 0.116$] sessions of Meth-treated mice (Fig. 6*b*).

Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of Meth-treated mice

Kamei *et al.* (2006) have demonstrated novelty-induced ERK1/2 activation in the PFC when mice are

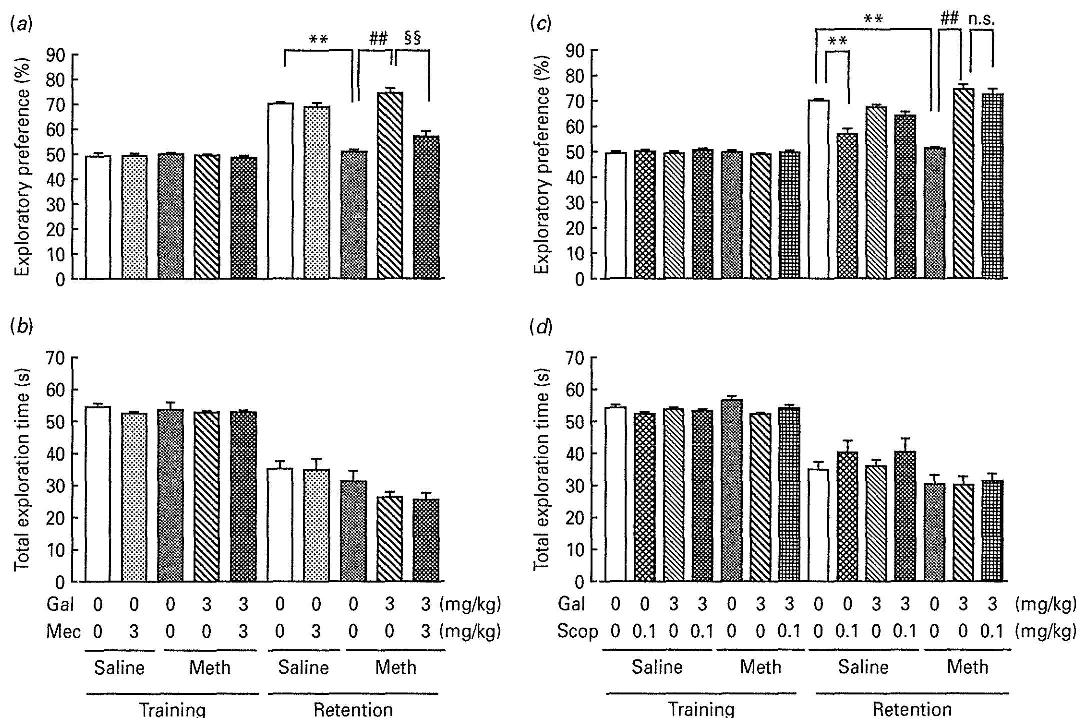

Fig. 4. Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a, c*) exploratory preference and (*b, d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.), mecamylamine (Mec; 3 mg/kg s.c.) and/or scopolamine (Scop; 0.1 mg/kg s.c.) were administered to saline- or Meth-treated mice 1 h, 40 min and/or 40 min, respectively, before the training session. Values indicate the mean $\pm$ S.E. ($n=10$–15). One-way ANOVA, (*a*) training: $F(4,57)=0.255$, $p=0.906$; retention: $F(4,57)=28.901$, $p<0.01$; (*b*) training: $F(4,57)=0.516$, $p=0.725$; retention: $F(4,57)=2.403$, $p=0.060$; (*c*) training: $F(6,77)=0.429$, $p=0.858$; retention: $F(6,77)=20.277$, $p<0.01$; (*d*) training: $F(6,77)=2.193$, $p=0.053$; retention: $F(6,77)=1.919$, $p=0.088$. ** $p<0.01$ compared to saline+saline/saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth+saline/saline-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth+galantamine/saline-treated group (Bonferroni's test). n.s., Not significant.

exposed to novel objects, leading to the formation of long-lasting object recognition memory. Further, memory impairment in Meth-treated mice was associated with dysfunction of ERK1/2 signalling in the PFC. In order to examine the mechanism by which galantamine ameliorates the impairment of recognition memory in Meth-treated mice, we examined the effect of galantamine on ERK1/2 phosphorylation in the PFC of Meth-treated mice when they were exposed to novel objects.

A significant increase in phosphorylation of ERK1/2 levels was observed in the PFC of saline-treated mice immediately after a 10-min exposure to novel objects (Fig. 7*a, b*) ($p<0.01$ *vs.* baseline in saline-treated mice, Student's *t* test), and repeated Meth treatment abolished novelty-induced ERK1/2 activation in the PFC in accord with the previous study (Kamei *et al.* 2006) ($p<0.01$) (Fig. 7*a*). Galantamine (3 mg/kg) significantly recovered the defect of novelty-induced activation of ERK1/2 in the PFC of Meth-treated mice ($p<0.01$) (Fig. 7*a*). SCH 23390 (0.02 mg/kg) significantly blocked the improving effects of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC ($p<0.01$) (Fig. 7*a*). SCH 23390 alone had no effect on the levels of phosphorylation and total ERK1/2 in either the baseline or exposure of saline-treated mice (Fig. 7*b*). The levels of total ERK1/2 did not differ in the exposed groups examined [$F(3,16)=1.629$, $p=0.222$].

Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on Meth-treated mice

We confirmed that PD98059 (2 μg/1 μl/bilateral) has no effect on the phosphorylation of ERK1/2 in the PFC and hippocampus of naive mice (data not shown). Then, we examined the effect of PD98059 (2 μg/1 μl/bilateral) administered before the training session on

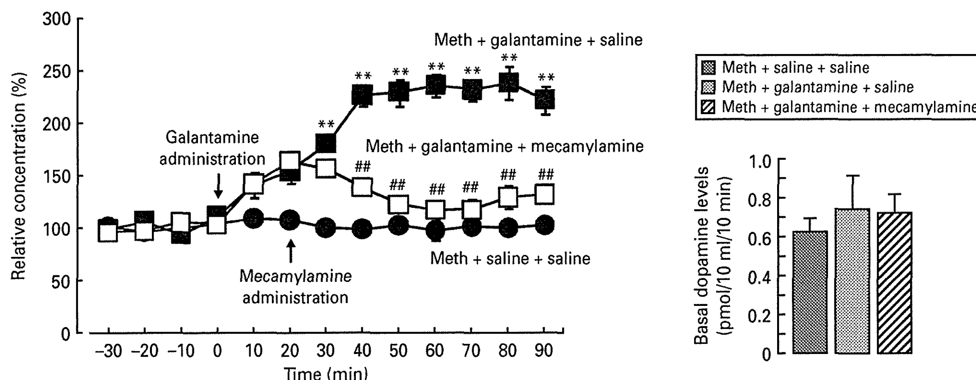

Fig. 5. Effects of galantamine on the levels of the extracellular dopamine in the PFC of methamphetamine (Meth)-treated mice. Meth (1 mg/kg, s.c.) was injected for 7 d, and 3 d after withdrawal, extracellular levels of dopamine were measured in the PFC by *in-vivo* microdialysis. Galantamine (3 mg/kg p.o.) was administered to the Meth-treated mice (■, Meth+galantamine+saline). In the control group, an equivalent amount of saline was given (●, Meth+saline+saline) to the Meth-treated mice. Mecamylamine (3 mg/kg s.c.) was injected 20 min after galantamine (□, Meth+galantamine+mecamylamine) to Meth-treated mice. The basal levels of dopamine in the PFC of the Meth+saline+saline (▩)-, Meth+galantamine+saline (▩)- and Meth+galantamine+mecamylamine (▨)-treated mice were 0.62 ± 0.08, 0.74 ± 0.18 and 0.72 ± 0.10 pmol/10 μl per 10 min, respectively (right-hand panel). Values indicate the mean±S.E. ($n=3$). Results with the repeated ANOVA were time [$F(9,54)=8.063$, $p<0.01$], treatment [$F(2,6)=73.188$, $p<0.01$], and time × treatment interaction [$F(18,54)=10.802$, $p<0.01$]. ** $p<0.01$ compared to Meth+saline+saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth+galantamine+saline-treated group (Bonferroni's test).

the cognitive-improving effect of galantamine in Meth-treated mice to determine the involvement of ERK1/2 activation in the mechanism of action of galantamine.

In the training session, bilateral microinjections of PD98059 into the PFC (1 μg/side) of saline-treated mice did not affect the exploratory preference for the objects (Fig. 8*a*). In the retention session, the level of exploratory preference in PD98059-treated mice was significantly increased as for vehicle-treated mice ($p<0.01$, Fig. 8*a*), but it was significantly decreased compared to that in vehicle-treated mice ($p<0.05$, Fig. 8*a*). PD98059 had no effect on the total exploration time in either the training or retention sessions of saline-treated mice (Fig. 8*b*).

In Meth-treated mice, PD98059 completely blocked the ameliorating effect of galantamine on the impairment of exploratory preference for a novel object in the retention session [$F(2,25)=27.986$, $p<0.01$] (Fig. 8*c*). The antagonistic effect of PD98059 on galantamine-induced improvement of exploratory preference in Meth-treated mice was not associated with changes in the total exploration time [training: $F(2,25)=0.399$, $p=0.676$; retention: $F(2,25)=0.015$, $p=0.985$] (Fig. 8*d*).

Discussion

We have reconfirmed that Meth-treated mice show impairments to their novelty discrimination ability in the NOR test that is consistent with previous reports (Ito *et al.* 2007; Kamei *et al.* 2006). It is unlikely that the impairment in performance of Meth-treated mice in learning and memory tasks is due to changes in motivation, although various motivations are involved in the behavioural task. The fact that Meth reduced the exploratory preference for the objects in the retention session could be interpreted as neophobia. However, the possible involvement of motivation and/or neophobia can be excluded because Meth treatment had no effect on total exploration time of novel objects during the training session. Therefore, it is likely that impairment of performance in Meth-treated mice is due to learning and memory deficits.

Galantamine, a drug approved for the treatment of Alzheimer's disease, has a dual mechanism of action; it inhibits AChE and allosterically modulates nAChR as a potent APL (Eisele *et al.* 1993; Santos *et al.* 2002). We have recently reported that galantamine reverses the impairment of object recognition in $A\beta_{25-35}$-infused mice as an animal model of Alzheimer's disease and in repeated PCP-treated mice as an animal model of schizophrenia (Wang *et al.* 2007*a*,*b*). In accord with these findings, in the present study, galantamine significantly ameliorated the cognitive impairments induced by Meth in the NOR test. Galantamine at a dose of 3 mg/kg had no effect on the total exploration time in the training session of the NOR test in Meth-treated

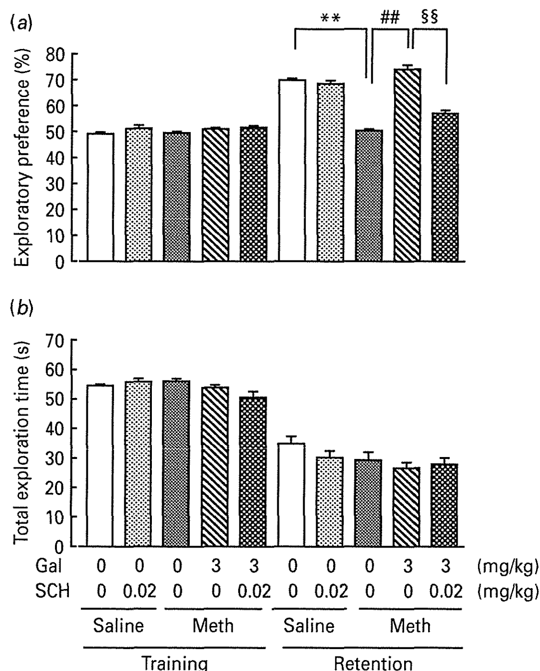

Fig. 6. Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and SCH 23390 (SCH; 0.02 mg/kg s.c.) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean±S.E. ($n=10$–15). One-way ANOVA, (*a*) training: $F(4,50)=1.422$, $p=0.240$; retention: $F(4,55)=40.622$, $p<0.01$; (*b*) training: $F(4,50)=1.520$, $p=0.211$; retention: $F(4,55)=1.943$, $p=0.116$. ** $p<0.01$ compared to saline+saline/saline-treated group (Bonferroni's test). ## $p<0.01$ compared to Meth+saline/saline-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth+galantamine/saline-treated group (Bonferroni's test).

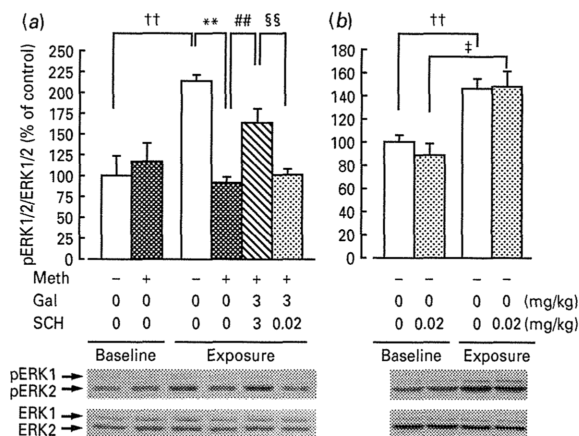

Fig. 7. Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of methamphetamine (Meth)-treated mice. One hour before exposure to novel objects, galantamine (Gal; 3 mg/kg p.o.) or saline was administered to mice that had been previously treated with either saline or Meth (1 mg/kg s.c.) for 7 d. SCH 23390 (SCH; 0.02 mg/kg s.c.) was administered 30 min before exposure to novel objects. Values indicate the mean±S.E. ($n=4$–5). †† $p<0.01$ compared to saline+saline/saline-treated group that was not exposed to novel objects (baseline) (Student's *t* test). ‡ $p<0.05$ compared to saline+saline/SCH23390-treated group that was not exposed to novel objects (baseline) (Student's *t* test). One-way ANOVA: $F(3,16)=28.286$, $p<0.01$. ** $p<0.01$ compared to saline+saline/saline-treated group (exposure) (Bonferroni's test). ## $p<0.01$ compared to Meth+saline/saline-treated group (exposure) (Bonferroni's test). §§ $p<0.01$ compared to Meth+galantamine/saline-treated group (exposure) (Bonferroni's test).

mice. Therefore, it is unlikely that the observed improvement in performance in the task brought about by galantamine is due to changes in motivation in Meth-treated mice, and it is apparently true that galantamine ameliorates learning and memory deficits caused by repeated Meth treatment in mice. The improving effects of galantamine on the performance of Meth-treated mice were prevented by treatment with mecamylamine, a nAChR antagonist, at a dose that did not significantly affect the performance of saline-treated mice. These findings support the notion that galantamine improves Meth-induced cognitive impairment via activation of nAChRs. Alternatively, the roles of mAChRs in the effects of galantamine were also investigated in the present study. The effects of galantamine on the performance of Meth-treated mice in the NOR task were not blocked by scopolamine at the dose that impaired the performance of saline-treated mice. Although mAChR agonists improve cognitive dysfunctions in patients with Alzheimer's disease and schizophrenia (Friedman, 2004), the present result indicated that mAChRs have little influence on the effects of galantamine for this particular cognitive task. On the other hand, the activation of nAChRs may be due to an increase in the levels of ACh caused by AChE inhibition of galantamine. We investigated the effect of donepezil, which is 3–15 times more potent in AChE inhibition than that of galantamine *in vivo* (Geerts *et al.* 2005), on Meth-induced cognitive impairment. Although donepezil at 1 mg/kg caused about a 2-fold increase from basal extracellular ACh levels in the PFC of Meth-treated mice, it had no effect on behavioural performance in Meth-treated mice. From the

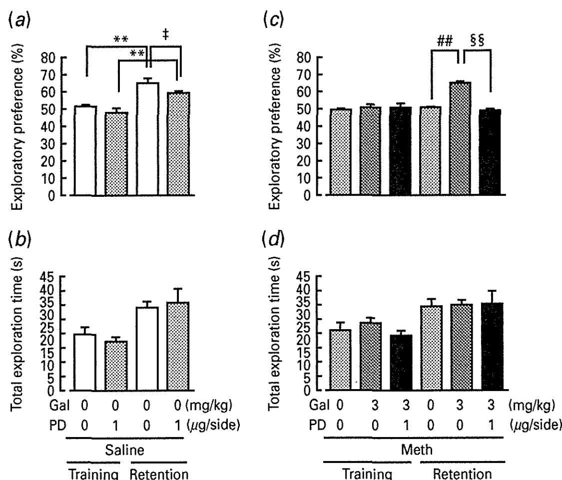

Fig. 8. Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (Gal; 3 mg/kg p.o.) and PD98059 (PD; 1 μg/0.5 μl per side) were administered 1 h and 30 min, respectively, before the training session. Values indicate the mean ± S.E. (*a*, *b*; $n=8$) (*c*, *d*; $n=9$–10). One-way ANOVA, (*c*) training: $F(2, 25)=0.309$, $p=0.737$; retention: $F(2, 25)=27.986$, $p<0.01$; (*d*) training: $F(2, 25)=0.399$, $p=0.676$; retention: $F(2, 25)=0.015$, $p=0.985$. ** $p<0.01$ compared to corresponding saline-treated training group (Student's *t* test). ‡ $p<0.05$ compared to saline + saline/vehicle-treated retention group (Student's *t* test). ## $p<0.01$ compared to Meth + saline/vehicle-treated group (Bonferroni's test). §§ $p<0.01$ compared to Meth + galantamine/vehicle-treated group (Bonferroni's test).

present results and a report that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts *et al.* 2005), our conclusion is that galantamine induces the ameliorating effect on impairment of memory mainly by allosterically modulating the function of nAChRs, but not by AChE inhibition. However, further experiments are needed to exclude the involvement of AChE inhibition by galantamine in the ameliorating effect of it on cognitive impairment in Meth-treated mice, since the allosteric potentiating effect of nAChRs can be detected at lower doses (Geerts *et al.* 2005).

Accumulating evidence suggests that the dopaminergic system in the PFC is involved in cognitive function. For instance, disruption of dopamine transmission in the PFC by infusions of dopamine D_1R antagonists or by excitotoxic lesions impairs the performance of object retrieval-detour tasks, as well as delayed response tasks in non-human primates (Dias *et al.* 1996*a*,*b*; Sawaguchi & Goldman-Rakic, 1991). A previous study with functional magnetic resonance imaging has shown that dysfunction in the PFC of Meth abusers is related to cognitive impairment (Paulus *et al.* 2002). Accordingly, cognitive impairment in Meth abusers may be associated with deficits in dopamine transmission in the PFC. Our previous findings in *in-vivo* microdialysis experiments demonstrated that galantamine increases the extracellular dopamine release in the hippocampus and PFC and that the increasing effects of galantamine on dopamine release in the hippocampus are potentiated by nicotine and antagonized by mecamylamine (Wang *et al.* 2007*a*). The present *in-vivo* microdialysis experiment show that galantamine significantly increased extracellular dopamine release in the PFC of Meth-treated mice. The effects of galantamine on increasing dopamine release were antagonized by mecamylamine. These results strongly suggest that galantamine ameliorates Meth-induced learning and memory deficits by activating nAChRs, and thereby stimulates release of dopamine in the PFC. Further, we found that the improving effects of galantamine were prevented by SCH 23390, a dopamine D_1R antagonist. Galantamine enhances dopaminergic neurotransmission *in vivo* via allosteric potentiation of nAChRs. These findings provide the *in-vivo* evidence that galantamine augments dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. The present results are supported by the results published by Schilström *et al.* (2007) that effects of galantamine on dopamine cell firing are mediated by allosteric potentiation of nAChRs. Taken together, our results suggest that the PFC-dependent behaviour task was impaired due to dysfunction of dopaminergic systems induced by Meth, since the PFC is involved in object recognition behaviour (Kamei *et al.* 2006). In fact, Kamei *et al.* (2006) have already demonstrated that repeated administration of Meth in mice induces object recognition impairment, which is associated with the dopamine D_1Rs, but not dopamine D_2Rs in the PFC. However, the object recognition memory is ascribed to the perirhinal cortex and its interactions with the hippocampus (Winters *et al.* 2008). We will investigate the functional role of the perirhinal cortex in Meth-induced cognitive deficits, in the ameliorating effects of galantamine and D_1R/ERK signalling in the NOR test.

Previous studies have demonstrated that the ERK1/2 signalling pathway linked to dopamine D_1Rs (Valjent *et al.* 2000; Zanassi *et al.* 2001) is involved in the rewarding effects induced by Meth (Mizoguchi *et al.* 2004) and the behavioural sensitization and

rewarding effects induced by cocaine (Valjent *et al.* 2000). Regarding the mechanism underlying the repeated Meth-induced memory impairment, Kamei *et al.* (2006) have already demonstrated dysfunction of the ERK1/2 pathway in the PFC. Hyperphosphorylation of ERK1/2 was found in the PFC when control mice were exposed to novel objects, whereas this activation was abolished in repeated Meth-treated mice. Inhibition of ERK1/2 by the microinjection of PD98059 (4 μg/mouse/bilateral), a selective MEK inhibitor, into the PFC resulted in cognitive impairment (Kamei *et al.* 2006). Ito *et al.* (2007) have also found that another MEK1/2 inhibitor, SL327 (30 and 50 mg/kg i.p.), significantly impairs long-term recognition memory 24 h after a training session in naive mice. In this study, galantamine ameliorated the Meth-induced defect of ERK1/2 hyperphosphorylation in the PFC of mice exposed to novel objects. In addition, the ameliorating effect of galantamine on Meth-induced object recognition impairment was completely blocked by pretreatment with the ERK inhibitor PD98059 at the dose used, slightly affecting the performance of saline-treated mice. Accordingly, these results suggest that the ameliorating effect of galantamine on Meth-induced cognitive impairment is related to the activation of ERK1/2 in the PFC.

As discussed above, our findings suggest that dopamine D_1R-ERK1/2 systems are required for the effects of galantamine. Since dopamine the D_1R antagonist and ERK inhibitor impaired recognition memory based on phosphorylation of ERK in the PFC of normal mice (Kamei *et al.* 2006), dopamine D_1R-ERK1/2 systems are critical in recognition memory. If the action site of galantamine is downstream of dopamine D_1R-ERK1/2 systems, dopamine D_1R antagonists or the ERK inhibitor would fail to reverse the effect of galantamine. Accordingly, our data suggest that galantamine acts upstream of dopamine D_1R-ERK1/2 systems.

In conclusion, the ameliorating effect of galantamine on Meth-induced memory impairment is associated with indirect activation of dopamine D_1R-ERK1/2 following augmentation with dopaminergic neurotransmission in the PFC through the allosteric activation of nAChRs. Galantamine could prove to be a useful therapeutic drug for treating cognitive deficits in schizophrenia/Meth psychosis, as well as Alzheimer's disease.

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Statement of Interest

None.

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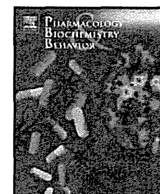

Increases of CRF in the amygdala are responsible for reinstatement of methamphetamine-seeking behavior induced by footshock

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ABSTRACT

Recent evidence suggests the involvement of corticotropin-releasing factor (CRF) in drug abuse. Here, we evaluated whether CRF modulates the reinstatement of methamphetamine (METH)-seeking behavior induced by stress using a drug-self administration paradigm in rats. Rats were trained to lever-press for intravenous METH (0.02 mg/infusion) accompanied by light and tone (drug-associated cues) and then underwent extinction training (saline substituted for METH without cues). Under the extinction condition, the inhibitory effects of a CRF receptor antagonist on the stress-induced reinstatement of METH-seeking behavior were assessed. Anxiety-like behaviors during METH withdrawal in METH self-administered rats were also evaluated. The non-selective CRF receptor antagonist α-helical CRF_{9-41} attenuated METH-seeking behavior induced by footshock stress. CRF levels both in the amygdala and in plasma were significantly increased on day 10 of withdrawal after METH self-administration. However, plasma corticosterone concentrations were unchanged during the withdrawal. In addition, METH-seeking behavior was not affected by an inhibitor of corticosterone synthesis, metyrapone. In the elevated plus maze test, METH self-administered rats showed a decrease in the duration of time spent in the open arms on day 10 of withdrawal. The increased CRF levels in the amygdala may, at least in part, contribute to the footshock-induced reinstatement of METH-seeking behavior and the increase in anxiety-like behavior. The present findings indicate that CRF receptor antagonists would be useful as a therapeutic agent for METH-dependence.

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1. Introduction

The biggest challenge to the successful treatment of drug dependence is preventing craving and relapse, which are hallmarks of the disease. Three different kinds of stimuli such as stress, drug associated-cues and drug-priming injections are capable of eliciting drug-seeking behavior in both human addicts (de Wit, 1996; Sinha et al., 2003) and animal reinstatement models (Shaham et al., 1996; Anggadiredja et al., 2004; Hiranita et al., 2008). Methamphetamine (METH) is a highly addictive psychostimulant, with reinforcing properties comparable to those of cocaine (Peltier et al., 1996; Shimosato and Ohkuma, 2000), but only one study has investigated the effect of stress on the reinstatement of METH-seeking behavior (Shepard et al., 2004). Furthermore, responses to stress such as footshock or the administration of corticosterone during drug self-administration differ between METH and cocaine (Moffett and Goeders, 2005). The aim of this study is to clarify mechanisms of stress-induced METH-seeking behavior.

The stress hormone corticotropin-releasing factor (CRF) plays a key role in the neuroendocrine and behavioral responses to stress, primarily via the hypothalamic–pituitary–adrenal (HPA) axis (Chalmers et al., 1996; Carrasco and Van de Kar, 2003). Centrally administered CRF produces several signs of increased anxiety and transgenic mice that over-express CRF exhibit increased anxiogenic behavior (Stenzel-Poore et al., 1994). Conversely, the central administration of either a CRF antisense oligodeoxynucleotide or a CRF receptor antagonist produces anxiolytic effects in the rat (Skutella et al., 1994; Griebel et al., 2002). In addition, clinical study also suggests that CRF_1 receptor antagonists are promising candidates for drug development in stress-related disorders such as depression and anxiety (Ising et al., 2007). Thus, additional functions of CRF as a contributing factor in psychiatric diseases are indicated.

We previously showed a critical role of the amygdala and nucleus accumbens (NAc) in the reinstatement of METH-seeking behavior attenuated by a reversible loss in function of the amygdala or NAc achieved using a local anesthetic, lidocaine (Hiranita et al., 2006). In the amygdala, CRF levels are indicated to rise during the early phase of withdrawal (the first 12 h) after cocaine self-administration (Richter and Weiss, 1999), but whether the levels change during withdrawal from METH in which cravings are elicited has not been elucidated. Therefore, the first aim of this study was to determine whether CRF could be involved in the reinstatement of stress-induced METH-seeking behavior, focusing on the amygdala and NAc.

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