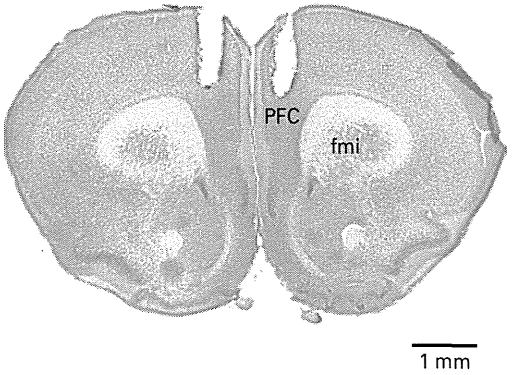


Fig. 1. Representative figure of mPFC local injection site. PFC; prefrontal cortex, fmi; forceps minor of the corpus callosum.

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 $B/(B+A) \times 100$ (%),

retention session: B or $C/(B+C) \times 100$ (%).

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

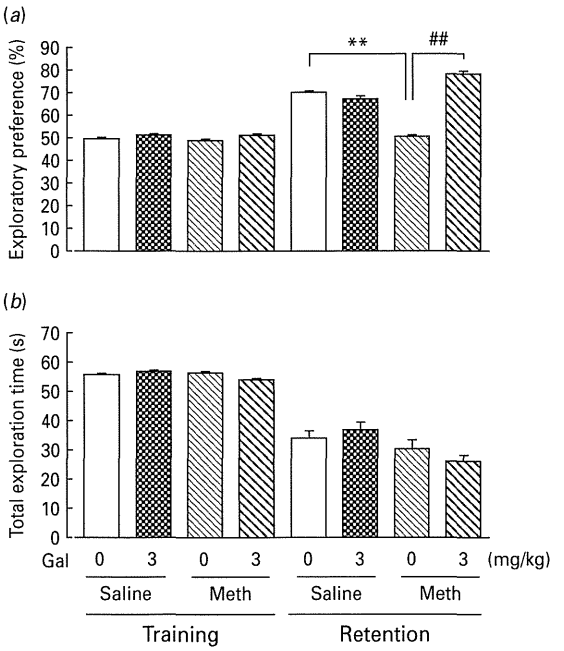


Fig. 2. Effect of galantamine on methamphetamine (Meth)-induced impairment of recognition memory in mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object recognition test. Galantamine (3 mg/kg p.o.) or saline was administered 1 h before the training session. Values indicate the mean ± 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

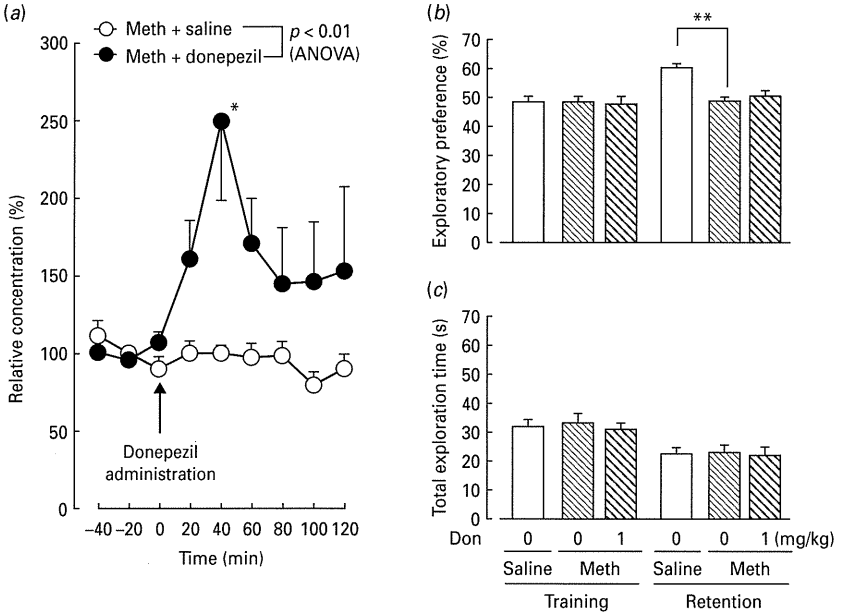


Fig. 3 Effect of donepezil on the extracellular acetylcholine (ACh) levels of the prefrontal cortex (PFC) and the impairment of recognition memory in methamphetamine (Meth)-treated mice. (*a*) Extracellular ACh levels of PFC in microdialysis. *In-vivo* microdialysis was performed 3 d after the final 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

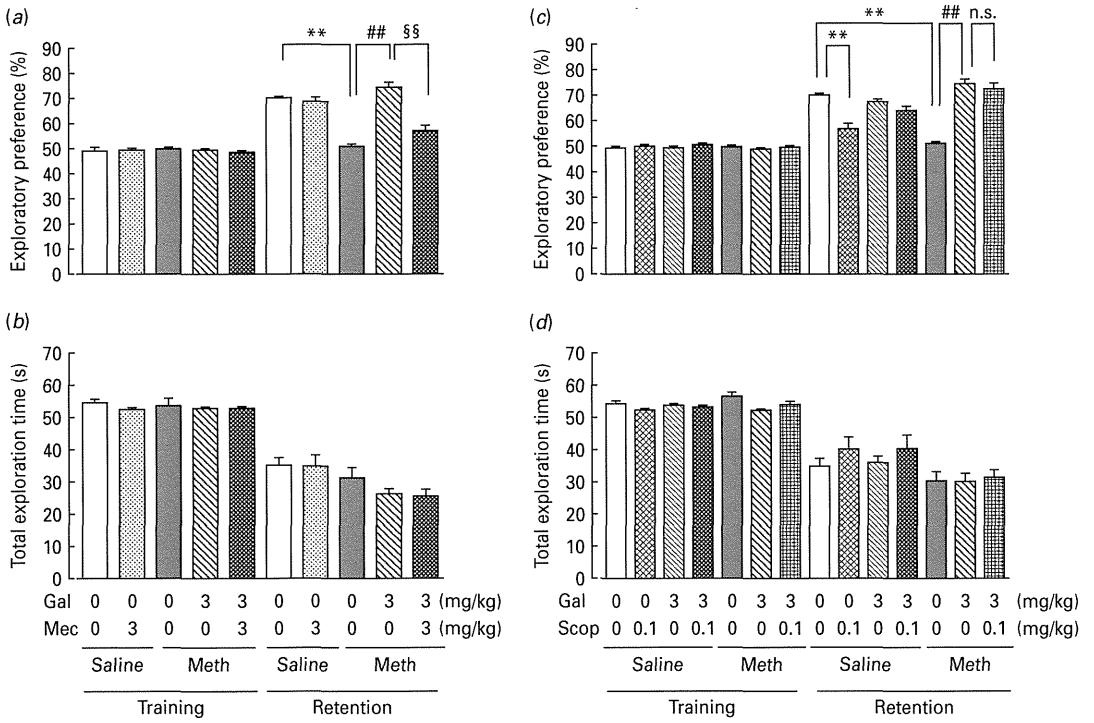


Fig. 4. Involvement of nicotinic receptors, but not muscarinic receptors in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment 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 ± 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

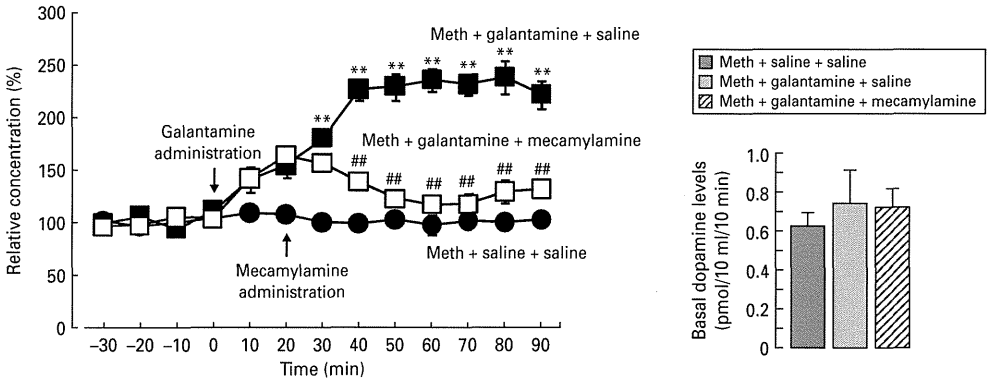


Fig. 5. Effects of galantamine on the levels of the extracellular dopamine in the PFC of methamphetamine (Meth)-treated mice. Meth (1 mg/kg, s.c.) was injected for 7 d, and 3 d after withdrawal, extracellular levels of dopamine were measured in the PFC by *in-vivo* microdialysis. Galantamine (3 mg/kg 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

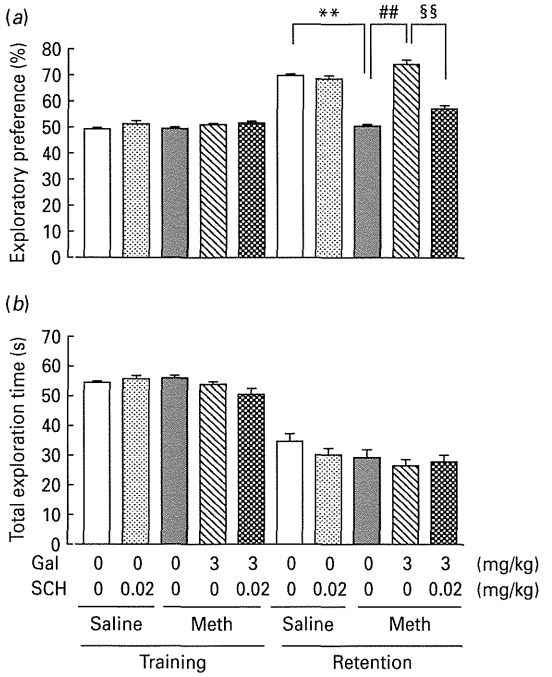


Fig. 6. Involvement of dopaminergic systems in the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*) exploratory preference and (*b*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel object 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).

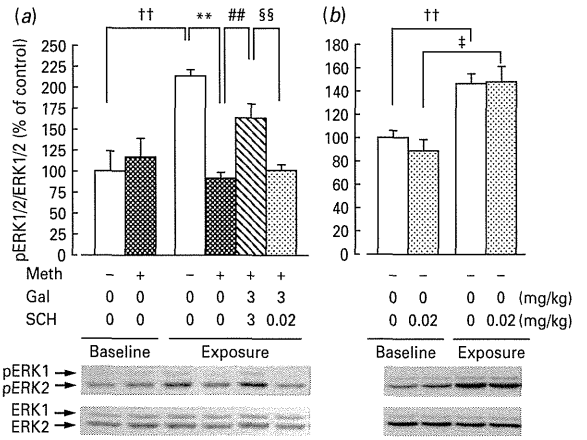


Fig. 7. Effect of galantamine on the defect of novelty-induced ERK1/2 phosphorylation in the PFC of methamphetamine (Meth)-treated mice. One hour before exposure to novel objects, galantamine (Gal; 3 mg/kg p.o.) or saline was administered to mice that had been previously treated with either saline or 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

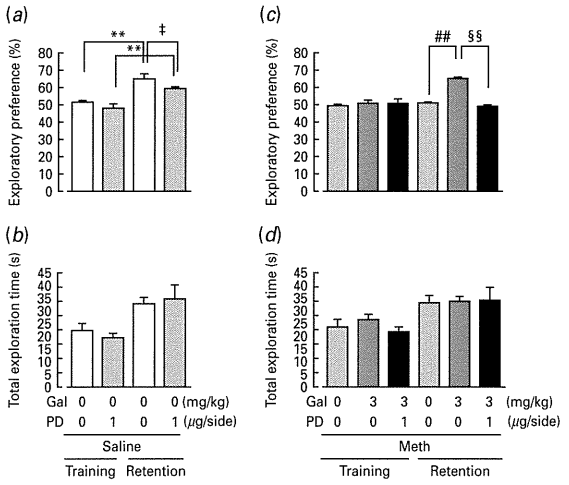


Fig. 8. Influence of an ERK inhibitor on the cognitive-improving effect of galantamine on methamphetamine (Meth)-treated mice: (*a*, *c*) exploratory preference and (*b*, *d*) total exploration time. One day after the cessation of repeated Meth (1 mg/kg s.c.) treatment for 7 d, mice underwent the novel 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.

Acknowledgements

This study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO05-27), by the Academic Frontier Project for Private Universities (2007–2011) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, by the International Research Project supported by the Meijo Asian Research Center (MARC), by a Grant-in-Aid for Exploratory Research from the Japan Society for the Promotion of Science, by a Grant-in-Aid for Scientific Research (B) from the Japan Society for the Promotion of Science, by a Grant-in-Aid on Priority Areas from the Japan Society for the Promotion of Science, by a Health and Labour Sciences Research Grant for Research on Regulatory Science of Pharmaceuticals and Medical Devices and Research on Risk of Chemical Substances from the Ministry of Health, Labour and Welfare of Japan, and by a grant from the Smoking Research Foundation.

Statement of Interest

None.

References

Cretzmeyer M, Sarrazin MV, Huber DL, Block RI, *et al.* (2003). Treatment of methamphetamine abuse: research findings and clinical directions. *Journal of Substance Abuse Treatment* **24**, 267–277.

Dias R, Robbins TW, Roberts AC (1996*a*). Dissociation in prefrontal cortex of affective and attentional shifts. *Nature* **380**, 69–72.

Dias R, Robbins TW, Roberts AC (1996*b*). Primate analogue of the Wisconsin card sorting test: effects of excitotoxic lesions of the prefrontal cortex in the marmoset. *Behavioral Neuroscience* **110**, 872–886.

Eisele JL, Bertrand S, Galzi JL, Devillers-Thiery A, *et al.* (1993). Chimaeric nicotinic-serotoninergic receptor combines distinct ligand binding and channel specificities. *Nature* **366**, 479–483.

Franklin KBJ, Paxinos G (1996). *The Mouse Brain in Stereotaxic Coordinates*. San Diego: Academic Press.

Friedman JI (2004). Cholinergic targets for cognitive enhancement in schizophrenia: focus on cholinesterase inhibitors and muscarinic agonists. *Psychopharmacology (Berlin)* **174**, 45–53.

Geerts H, Guillaumat PO, Grantham C, Bode W, *et al.* (2005). Brain levels and acetylcholinesterase inhibition with galantamine and donepezil in rats, mice and rabbits. *Brain Research* **1033**, 186–193.

Ito Y, Takuma K, Mizoguchi H, Nagai T, *et al.* (2007). A novel azaindolizinone derivative ZSET1446 [spiro[imidazo[1,2-a]pyridine-3,2-indan]-2(3H)-one] improves methamphetamine-induced impairment of recognition memory in mice by activating extracellular signal-regulated kinase 1/2. *Journal of Pharmacology and Experimental Therapeutics* **320**, 819–827.

Kalechstein AD, Newton TF, Green M (2003). Methamphetamine dependence is associated with neurocognitive impairment in the initial phases of abstinence. *Journal of Neuropsychiatry and Clinical Neurosciences* **15**, 215–220.

Kamei H, Nagai T, Nakano H, Togan Y, *et al.* (2006). Repeated methamphetamine treatment impairs recognition memory through a failure of novelty-induced ERK1/2 activation in the prefrontal cortex of mice. *Biological Psychiatry* **59**, 75–84.

Mizoguchi H, Yamada K, Mizuno M, Mizuno T, *et al.* (2004). Regulations of methamphetamine reward by extracellular signal-regulated kinase 1/2/ets-like gene-1 signaling pathway via the activation of dopamine receptors. *Molecular Pharmacology* **65**, 1293–1301.

Mouri A, Noda Y, Noda A, Nakamura T, *et al.* (2007). Involvement of a dysfunctional dopamine-D1/*N*-methyl-*d*-aspartate-NR1 and Ca^{2+}/calmodulin-dependent protein kinase II pathway in the impairment of latent learning in a model of schizophrenia induced by phencyclidine. *Molecular Pharmacology* **71**, 1598–1609.

Mouri A, Zou LB, Iwata N, Saido TC, *et al.* (2006). Inhibition of neprilysin by thiorphan (i.c.v.) causes an accumulation of amyloid beta and impairment of learning and memory. *Behavioral Brain Research* **168**, 83–91.

Nordahl TE, Salo R, Leamon M (2003). Neuropsychological effects of chronic methamphetamine use on neurotransmitters and cognition: a review. *Journal of Neuropsychiatry and Clinical Neurosciences* **15**, 317–325.

Paulus MP, Hozack NE, Zauscher BE, Frank L, *et al.* (2002). Behavioral and functional neuroimaging evidence for prefrontal dysfunction in methamphetamine-dependent subjects. *Neuropsychopharmacology* **26**, 53–63.

Rawson RA, Gonzales R, Brethen P (2002). Treatment of methamphetamine use disorders: an update. *Journal of Substance Abuse Treatment* **23**, 145–150.

Santos MD, Alkondon M, Aracava Y, Eisenberg HM, *et al.* (2002). The nicotinic allosteric potentiating ligand galantamine facilitates synaptic transmission in the mammalian central nervous system. *Molecular Pharmacology* **61**, 1222–1234.

Sato M, Chen CC, Akiyama K, Otsuki S (1983). Acute exacerbation of paranoid psychotic state after long-term abstinence in patients with previous methamphetamine psychosis. *Biological Psychiatry* **18**, 429–440.

Sawaguchi T, Goldman-Rakic PS (1991). D1 dopamine receptors in prefrontal cortex: involvement in working memory. *Science* **251**, 947–950.

Schilström B, Ivanov VB, Wiker C, Svensson TH (2007). Galantamine enhances dopaminergic neurotransmission in vivo via allosteric potentiation of nicotinic acetylcholine receptors. *Neuropsychopharmacology* **32**, 43–53.

Shintani F, Kanba S, Nakaki T, Nibuya M, *et al.* (1993). Interleukin-1b augments release of norepinephrine, dopamine, and serotonin in the rat anterior hypothalamus. *Journal of Neuroscience* **13**, 3574–3581.

Simon SL, Domier C, Carnell J, Brethen P, *et al.* (2000). Cognitive impairment in individuals currently using methamphetamine. *American Journal on Addictions* **9**, 222–231.

Srisurapanont M, Ali R, Marsden J, Sunga A, *et al.* (2003). Psychotic symptoms in methamphetamine psychotic in-patients. *International Journal of Neuropsychopharmacology* **6**, 347–352.

Valjent E, Corvol JC, Pages C, Besson MJ, *et al.* (2000). Involvement of the extracellular signal-regulated kinase cascade for cocaine-rewarding properties. *Journal of Neuroscience* **20**, 8701–8709.

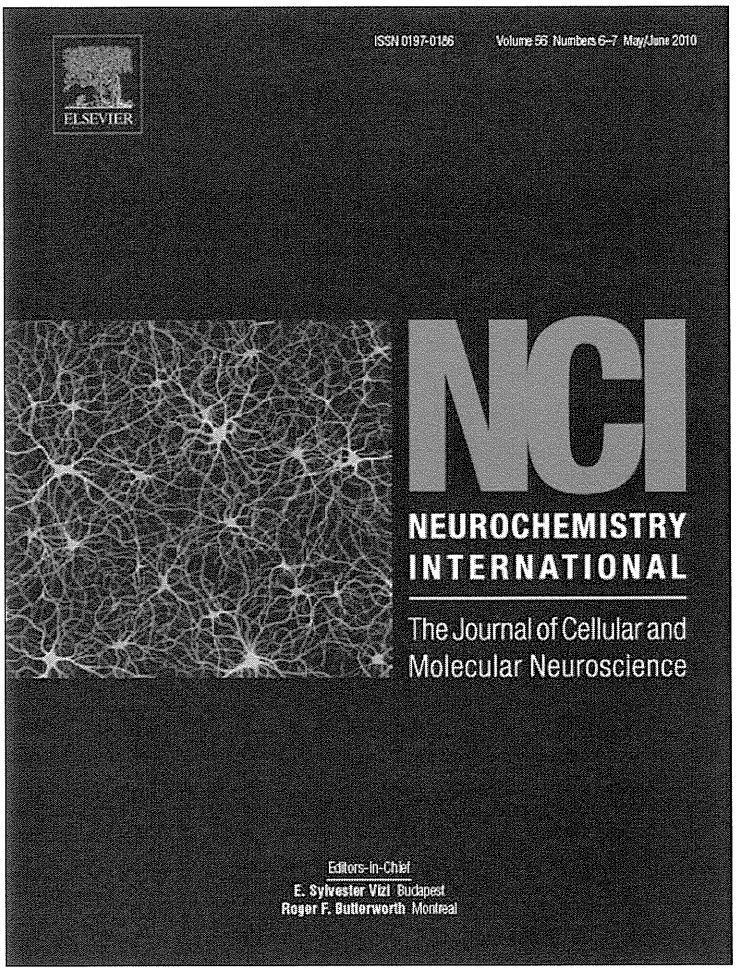
Wang D, Noda Y, Zhou Y, Mouri A, *et al.* (2007*a*). The allosteric potentiation of nicotinic acetylcholine receptors by galantamine ameliorates the cognitive dysfunction in beta $amyloid_{25\text{-}35}$ i.c.v.-injected mice: involvement of dopaminergic systems. *Neuropsychopharmacology* **32**, 1261–1271.

Wang D, Noda Y, Zhou Y, Nitta A, *et al.* (2007*b*). Synergistic effect of combined treatment with risperidone and galantamine on phencyclidine-induced impairment of latent visuospatial learning and memory: role of nAChR activation-dependent increase of dopamine D1 receptor-mediated neurotransmission. *Neuropharmacology* **53**, 379–389.

Winters BD, Saksida LM, Bussey TJ (2008). Object recognition memory: neurobiological mechanisms of encoding, consolidation and retrieval. *Neuroscience and Biobehavioral Reviews* **32**, 1055–1070.

Yui K, Ikemoto S, Goto K, Nishijima K, *et al.* (2002). Spontaneous recurrence of methamphetamine-induced paranoid-hallucinatory states in female subjects: susceptibility to psychotic states and implications for relapse of schizophrenia. *Pharmacopsychiatry* **35**, 62–71.

Zanassi P, Paolillo M, Feliciello A, Avvedimento EV, *et al.* (2001). cAMP-dependent protein kinase induces cAMP-response element-binding protein phosphorylation via an intracellular calcium release/ERK-dependent pathway in striatal neurons. *Journal of Biological Chemistry* **276**, 11487–11495.



Neurochemistry International 56 (2010) 736–739

Contents lists available at ScienceDirect

Neurochemistry International

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

Rapid communication

The expression of HMGA1a is increased in lymphoblastoid cell lines from schizophrenia patients

Tomomi Morikawa [a,1], Takayuki Manabe [a,1,*], Yoshihito Ito [b,e], Shinnosuke Yamada [b,c,d], Akira Yoshimi [b,c,d], Taku Nagai [d], Norio Ozaki [b,e], Akila Mayeda [a]

[a] Division of Gene Expression Mechanism, Institute for Comprehensive Medical Science, Fujita Health University, 1-98 Dengakugakubo, Kutsukake-cho, Toyoake, Aichi 470-1192, Japan
[b] Department of Psychiatry, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550, Japan
[c] Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya, Aichi 466-8550, Japan
[d] Division of Clinical Sciences and Neuropsychopharmacology, Meijo University Graduate School of Pharmaceutical Sciences, Nagoya, Aichi 468-8503, Japan
[e] CREST, Japan Science and Technology Agency, Tokyo, Japan

ARTICLE INFO

Article history:
Received 19 March 2010
Accepted 19 March 2010
Available online 27 March 2010

Keywords:
HMGA1a
Schizophrenia
Lymphoblastoid

ABSTRACT

The high-mobility group A protein 1a (HMGA1a) is a well-documented DNA-binding protein acting as an architectural transcription regulator. Recently, HMGA1a protein has been identified as a hypoxia-inducible RNA-binding *trans*-acting factor for aberrant splicing of presenilin-2 (PS2) pre-mRNA observed in the brains of sporadic Alzheimer's disease. Interestingly, this aberrant splicing of PS2 was also observed in the brains of bipolar disorder and schizophrenia. Many downstream genes under the control of HMGA1a could be associated with schizophrenia. On the other hand, many gene transcripts are aberrantly spliced in schizophrenia. Therefore, we examined the expression at the mRNA and protein levels of this DNA- and RNA-binding factor HMGA1a in the lymphoblastoid cell lines obtained from 16 schizophrenia patients with age-matched controls. We observed markedly higher HMGA1a mRNA and the increased HMGA1a protein in the nuclear fractions of schizophrenia patients. In contrast, there were no significant differences in the expression levels of HMGA1b, which is an alternatively spliced isoform of HMGA1a. The present study is the first to report a significant upregulation of HMGA1a in schizophrenia, suggesting its potential roles in both transcription and splicing of target genes linked with schizophrenia.

High-mobility group A protein 1a (HMGA1a) is a non-histone DNA-binding architectural transcription factor that regulates the expressions of many target genes, and thus serving as a central 'hubs' of nuclear functions (reviewed in Reeves, 2001). However, we have identified HMGA1a as an RNA-binding factor responsible for aberrant exon 5 skipping of presenilin-2 (PS2) pre-mRNA, which produces a deleterious protein variant PS2V in the brains of sporadic Alzheimer disease (AD) patients. (Sato et al., 1999, 2001; Higashide et al., 2004; Manabe et al., 2002, 2003, 2007b). HMGA1a expression and subsequent production of PS2V are stimulated by oxidative stresses such as hypoxia, aluminum and radicals in neuroblastoma cell lines (Sato et al., 1999, 2001; Manabe et al., 2003; Matsuzaki et al., 2004), which is mediated by the neuron-specific transcription factor N-Myc (Yanagita et al., 2005). PS2V protein increases amyloid-β (Sato et al., 2001) and causes conformational changes of tau proteins (Nishikawa et al., 2004) in neuroblastoma cell lines by impairing the signaling pathway of the unfolded-protein response in the endoplasmic reticulum (Sato et al., 2001), suggesting that HMGA1a-induced aberrant splicing of the PS2 pre-mRNA is involved in neurodegenerative disorders including sporadic AD. Most recently, the definitive mechanism of HMGA1a-induced exon skipping has been elucidated (Ohe and Mayeda, in press).

Schizophrenia is one of the mental disorders that chronically show various symptoms of delusion and illusion among others. Various risk factors are known to be associated with the onset of schizophrenia, which indicates complex cause of this serious disease. Nevertheless, a large number of reports support its close implications with the neural development abnormalities involved

Abbreviations: HMGA1a, high-mobility group A protein 1a; HMGA1b, high-mobility group A protein 1b; LCL, lymphoblastoid cell line; PS2V, presenilin-2 splice variant (lacking exon 5); MIF-1, melanocyte-stimulating hormone release inhibiting factor-1; SELEX, systematic evolution of ligands by exponential enrichment.

* Corresponding author. Tel.: +81 562 93 9378; fax: +81 562 93 8834.
E-mail address: manabe@fujita-hu.ac.jp (T. Manabe).

[1] These authors contributed equally to this work.

0197-0186/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuint.2010.03.011

in oligodendrocyte development/myelination, neurotransmission of GABA and/or glutamate, and synapse plasticity (Rapoport et al., 2005; Makinodan et al., 2008, and references there in). Intriguingly, it was reported that PS2V expression is also observed in the brain cortex of schizophrenia patients (Smith et al., 2004). We thus postulate a potential linking of schizophrenia and HMGA1a expression, however, there are no reports to date regarding the HMGA1a expression status in schizophrenia.

Accumulating evidence indicates that the particular gene expression patterns in peripheral blood lymphocytes can often faithfully reflect the genetic phenotypes of many psychological and neurological diseases including schizophrenia (e.g., Vawter et al., 2006). Therefore, here we used immortalized lymphoblastoid cell lines (LCLs) derived from the peripheral blood lymphocytes from schizophrenia patients to quantitatively examine HMGA1a expression at the protein and mRNA levels.

1. Experimental procedures

1.1. LCL cultures and preparation of total RNA from nuclear extracts

A total of 16 batches of LCLs from the peripheral blood lymphocytes of schizophrenia patients and age-matched normal controls were immortalized by the widely used EB virus method. The LCLs were cultured in RPMI 1640 medium with 2 mM L-glutamine (Invitrogen) containing 10% fetal bovine serum (Gibco), 8 μg/ml tyrosine tartrate, and 50 μg/ml penicillin/streptomycin (Invitrogen) in a CO_2 incubator at 37 °C, and the medium was exchanged for fresh one at every 3 days. Nuclear fractions of the LCLs were prepared according to a previous report (Manabe et al., 2000). The protein concentrations in the extracts were quantified as previously described (Manabe et al., 2000), and aliquots of the protein extracts (50 μg of protein) were subjected to immunoblotting analyses. The method to extract total RNA was also described previously (Manabe et al., 2003).

1.2. Semi-quantitative reverse-transcription (RT)-PCR

First-strand cDNA was prepared by RT using Super Script III (Invitrogen) with aliquots of total RNA (5 μg) from the LCLs. The semi-quantitative RT-PCR experiments were carried out as described previously (Manabe et al., 2005, 2007a) with minor modifications. The primer sets (forward and reverse, respectively) used for amplification of HMGA1 mRNAs were as follows: Pan-HMGA1, 5′-AGCAAAAA-CAAGGGTGCTGCCAAGACCCGG-3′ and 5′-TGGTGTGCTGTGTAGTGTGGTGGTGAG-GGC-3′; HMGA1a-specific: 5′-CCTCCGGTGAGTCCCGGGACAGCGCTGGTAGGG-3′ and 5′-TGGTGTGCTGTGTAGTGTGGTGGTGAGGGC-3′; HMGA1b-specific: 5′-GGGGCAGGCCGCGCAAGCAGCCTCCGAAGG-3′ and 5′-TGGTGTGCTGTGTAGTGTGG-TGGTGAGGGC-3′; HMGA1a/1b-shared: 5′-GCAGGAAAAGGACGGCACTGAGAAG-CGGG-3′ and 5′-TGGTGTGCTGTGTAGTGTGGTGGTGAGGGC-3′. The determined conditions for each PCR amplification using Prime Star Max (Invitrogen) were as follows: pan-HMGA1 primer, 27 cycles of 95 °C for 10 s, 56 °C for 10 s and 72 °C for 20 s; HMGA1a-specific primer, 29 cycles of 95 °C for 10 s, 57 °C for 10 s and 72 °C for 20 s; HMGA1b-specific primer, 29 cycles of 95 °C for 10 s, 57 °C for 10 s and 72 °C for 20 s; HMGA1a/1b-shared primer, 28 cycles of 95 °C for 10 s, 56 °C for 10 s and 72 °C for 20 s.

1.3. Immunoblotting assay

Immunoblot analyses for HMGA1 were performed as described previously (Manabe et al., 2003; Okuda et al., 2006).

2. Results

The HMGA1 gene encodes two alternatively spliced isoforms, HMGA1a and HMGA1b proteins, which are identical except for an internal deletion of 11 amino acids in the latter protein (Fig. 1(A)). We thus examined the distinct mRNA levels of HMGA1a and HMGA1b in LCLs from the schizophrenia patients and age-matched normal controls. Importantly, the proliferative abilities of the LCLs from the schizophrenia patients and the controls hardly changed during these experiments (data not shown).

First, we used a primer set that amplified both HMGA1a and HMGA1b at the same time (Fig. 1(A)). The expressions of both isoforms were detected and showed somewhat increased mRNAs of both HMGA1 isoforms in the LCLs obtained from the schizophrenia patients (Fig. 1(B)). To verify these results quantitatively, we used specific 5′ primers that separately amplified HMGA1a and HMGA1b (Fig. 1(A)). Much higher expression of HMGA1a was observed in the LCLs obtained from the schizophrenia patients compared with those from the controls (Fig. 1(C)). Statistical analyses of the 16 samples revealed a significant increase of HMGA1a mRNA in the schizophrenia patients (Fig. 1(D)). However, no significant differences were observed between the schizophrenia patients and the controls using the HMGA1b-specific 5′primer (Fig. 1(C) and (D)).

Next, we examined the differences of the HMGA1 expression in the protein levels between the schizophrenia patients and the controls using immunoblotting assays with an anti-HMGA1 antibody. Increased levels of HMGA1a protein were observed in the nuclear fractions of the LCLs from the schizophrenia patients compared with those from the normal controls (Fig. 1(E)). Quantitative data for each of the 16 samples showed that the evident increase of HMGA1 proteins in the schizophrenia patients (Fig. 1(E)). HMGA1b protein showed apparent increase in the schizophrenia patients, however, the statistical analysis showed no significant differences among the LCL samples (Fig. 1(E) and (F)).

3. Discussion

Here we have demonstrated the potential upregulation of HMGA1a in patients of schizophrenia. HMGA1a is induced by oxidative stresses, such as hypoxia and aluminum, in several brain diseases such as Alzheimer's disease (Manabe et al., 2003, unpublished data). Since the pathophysiology of schizophrenia is considered to be a kind of neurodevelopmental abnormality, it is necessary to think about the oxidative stresses during the developmental stages and the term of growth. Indeed, serious obstetric complications with the potential for fetal hypoxia interact with schizophrenia candidate genes that are regulated by hypoxia, leading to increase the risk of schizophrenia (Nicodemus et al., 2008). These observations together with our present results well support the implication of HMGA1a upregulation in the pathology of schizophrenia.

Even though the particular gene expressions in LCLs reflect those in the real brain disorders, what we merely observe is the overall output of the gene expressions from the whole brain. For instance, MIF-1 (PLG), which is a tripeptide regulator in the central dopaminergic systems, downregulates hnRNP L, an RNA-binding splicing silencer analogous to HMGA1a, in the striatum and prefrontal cortex, but not in the nucleus accumbens even all these regions are known targets for the etiology of schizophrenia (Costain and Mishra, 2003). Therefore, pinpoint analyses targeted for the regions related to schizophrenia, such as the prefrontal cortex, nucleus basalis and cerebral limbic system, would be a future direction to elucidate the demanding cause of this disease.

Our previous study directly demonstrated that increased HMGA1a expression induces aberrant splicing of PS2 pre-mRNA to produce PS2V protein, which is one of the risk factors for sporadic Alzheimer's disease (Manabe et al., 2003, 2007b). It is notable that aberrant PS2V mRNA and protein expressions are also observed in the frontal cortex of schizophrenia patients (Smith et al., 2004). These results taken together are quite consistent with our present findings that HMGA1a mRNA and protein expressions were increased in LCLs from schizophrenia patients. We predict that the PS2V production in schizophrenia is mainly due to HMGA1a expression since our data revealed prominent expression of HMGA1a rather than HMGA1b. HMGA1b is capable of producing PS2V, however, it is much weaker than HMGA1a (Manabe et al., 2003). In contrast, HMGA1b has stronger oncogenic activity than that of HMGA1a (Reeves, 2001).

HMGA1a was originally identified as a DNA-binding protein that acts as a versatile transcription regulator. Recently, we searched for high affinity DNA sequences of human HMGA1a by

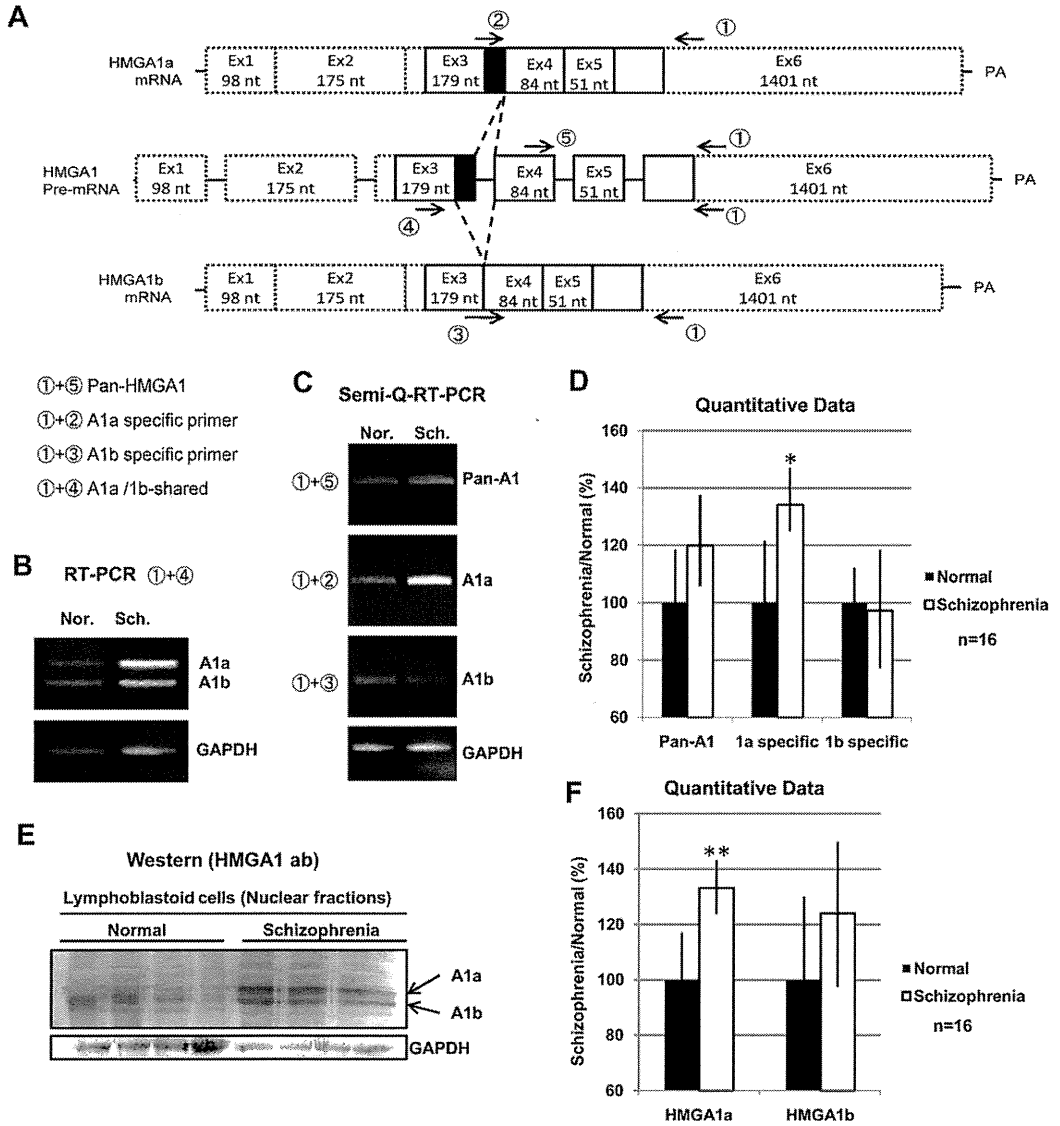


Fig. 1. Comparison of *HMGA1* gene expressions in LCLs from schizophrenia patients and age-matched controls. (A) Schematic representation of the mRNA structures of the HMGA1a and HMGA1b alternatively spliced isoforms. Arrows indicate the primer sets used in this study. The dotted-line square and solid-line square are the UTR and translated region, respectively. (B) RT-PCR assays of transcripts prepared from LCLs of the schizophrenia patients and normal controls. The A1a/1b-shared primer set (see (A)) was used to amplify both HMGA1a and HMGA1b at the same time. The amplification of GAPDH is shown as an internal control. (C) Semi-quantitative RT-PCR assays of pan-HMGA1, HMGA1a, and HMGA1b mRNAs in LCL samples obtained from the schizophrenia patients and normal controls. (D) Quantitative data for the results in (C) are shown for each of the 16 patients and normal controls. The diagram plots percentages of the individual signal strengths of the bands (mean ± SE) obtained from the schizophrenia patients relative to those obtained from the normal controls ($^{*}P < 0.05$). (E) Immunoblotting assay for HMGA1 protein expressions in the nuclear fractions of LCLs obtained from the schizophrenia patients and normal controls. GAPDH proteins were also detected as an internal control. (F) Quantitative data of the results in (E) are shown for each of the 16 patients and normal controls. The diagram plots percentages of the individual immunoreactivities (mean ± SE) obtained from the schizophrenia patients relative to those obtained from the normal controls ($^{**}P < 0.01$).

the SELEX procedure, and retrieved consensus sequences revealed a good match for most of the known promoters of human genes (Manabe et al., 2009). Intriguingly, many genes related to schizophrenia were extracted in the retrieved sequences, such as hnRNP K, hnRNP C, iNOS, ErbB2, estrogen receptor, TNF-alpha, IL-2, IL-4, IL-6, and elk-1 (Manabe et al., 2009). These findings indicate that the upregulated HMGA1a, as a DNA-binding protein, contributes for the transcriptional control of many genes linked with schizophrenia.

Acknowledgments

T. Manabe and T. Morikawa were supported by a Grant-in-Aid for Scientific Research on Innovative Areas (RNA regulation: area no. 3001). T. Manabe was partially supported by a Grant-in-Aid for Young Scientists (B) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a grant for the encouragement of research from the Takeda Science Foundation. This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, the Ministry of Health, Labor, and Welfare of Japan, MEXT ACADEMIC FRONTIER, and the Core Research for Evolutional Science and Technology (NO).

References

Costain, W.J., Mishra, R.K., 2003. PLG regulates hnRNP-L expression in the rat striatum and pre-frontal cortex: identification by ddPCR. Peptides 24, 137–146.
Higashide, S., Morikawa, K., Okumura, M., Kondo, S., Ogata, M., Murakami, T., Yamashita, A., Kanemoto, S., Manabe, T., Imaizumi, K., 2004. Identification of regulatory cis-acting elements for alternative splicing of presenilin 2 exon 5 under hypoxic stress conditions. J. Neurochem. 91, 1191–1198.

Makinodan, M., Tatsumi, K., Manabe, T., Yamauchi, T., Makinodan, E., Matsuyoshi, H., Shimoda, S., Noriyama, Y., Kishimoto, T., Wanaka, A., 2008. Maternal immune activation in mice delays myelination and axonal development in the hippocampus of the offspring. J. Neurosci. Res. 86, 2190–2200.

Manabe, T., Kitayama, T., Ogita, K., Yoneda, Y., 2000. Differential expression and phosphorylation of particular fos family members by kainate in nuclear and cytosolic fractions of murine hippocampus. Neuroscience 100, 453–463.

Manabe, T., Katayama, T., Sato, N., Kudo, T., Matsuzaki, S., Imaizumi, K., Tohyama, M., 2002. The cytosolic inclusion bodies that consist of splice variants that lack exon 5 of the presenilin-2 gene differ obviously from Hirano bodies observed in the brain from sporadic cases of Alzheimer's disease patients. Neurosci. Lett. 328, 198–200.

Manabe, T., Katayama, T., Sato, N., Gomi, F., Hitomi, J., Yanagita, T., Kudo, T., Honda, A., Mori, Y., Matsuzaki, S., Imaizumi, K., Mayeda, A., Tohyama, M., 2003. Induced HMGA1a expression causes aberrant splicing of Presenilin-2 pre-mRNA in sporadic Alzheimer's disease. Cell Death Differ. 10, 698–708.

Manabe, T., Tatsumi, K., Inoue, M., Matsuyoshi, H., Makinodan, M., Yokoyama, S., Wanaka, A., 2005. L3/Lhx8 is involved in the determination of cholinergic or GABAergic cell fate. J. Neurochem. 94, 723–730.

Manabe, T., Tatsumi, K., Inoue, M., Makinodan, M., Yamauchi, T., Makinodan, E., Yokoyama, S., Sakumura, R., Wanaka, A., 2007a. L3/Lhx8 is a pivotal factor for cholinergic differentiation of murine embryonic stem cells. Cell Death Differ. 14, 1080–1085.

Manabe, T., Ohe, K., Katayama, T., Matsuzaki, S., Yanagita, T., Okuda, H., Bando, Y., Imaizumi, K., Reeves, R., Tohyama, M., Mayeda, A., 2007b. HMGA1a: sequence-specific RNA-binding factor causing sporadic Alzheimer's disease-linked exon skipping of presenilin-2 pre-mRNA. Genes Cells 12, 1179–1191.

Manabe, T., Katayama, T., Tohyama, M., 2009. HMGA1a recognition candidate DNA sequences in humans. PLoS One 4, e8004.

Matsuzaki, S., Manabe, T., Katayama, T., Nishikawa, A., Yanagita, T., Okuda, H., Yasuda, Y., Miyata, S., Meshitsuka, S., Tohyama, M., 2004. Metals accelerate production of the aberrant splicing isoform of the presenilin-2. J. Neurochem. 88, 1345–1351.

Nicodemus, K.K., Marenco, S., Batten, A.J., Vakkalanka, R., Egan, M.F., Straub, R.E., Weinberger, D.R., 2008. Serious obstetric complications interact with hypoxia-regulated/vascular-expression genes to influence schizophrenia risk. Mol. Psychiatry 13, 873–877.

Nishikawa, A., Manabe, T., Katayama, T., Kudo, T., Matsuzaki, S., Yanagita, T., Okuda, H., Bando, Y., Tohyama, M., 2004. Novel function of PS2V: change in conformation of tau proteins. Biochem. Biophys. Res. Commun. 318, 435–438.

Ohe, K., Mayeda, A., in press. HMGA1a trapping of U1 snRNP at an authetic 5′ splice site induces aberrant exon skipping in spordadic Alzheimer's disease. Mol. Cell. Biol. doi:10.1128/MCB.00114-10.

Okuda, H., Manabe, T., Yanagita, T., Matsuzaki, S., Bando, Y., Katayama, T., Wanaka, A., Tohyama, M., 2006. Novel interaction between HMGA1a and StIP1 in murine terminally differentiated retina. Mol. Cell. Neurosci. 33, 81–87.

Rapoport, J.L., Addington, A.M., Frangou, S., Psych, M.R., 2005. The neurodevelopmental model of schizophrenia: update 2005. Mol. Psychiatry 10, 434–449.

Reeves, R., 2001. Molecular biology of HMGA proteins: hubs of nuclear funcrion. Gene 277, 63–81.

Sato, N., Hori, O., Yamaguchi, A., Lambert, J.C., Chartier-Harlin, M.C., Robinson, P.A., Delacourte, A., Schmidt, A.M., Furuyama, T., Imaizumi, K., Tohyama, M., Takagi, T., 1999. A novel presenilin-2 splice variant in human Alzheimer's disease brain tissue. J. Neurochem. 72, 2498–2505.

Sato, N., Imaizumi, K., Manabe, T., Taniguchi, M., Hitomi, J., Katayama, T., Yoneda, T., Morihara, T., Yasuda, Y., Takagi, T., Kudo, T., Tsuda, T., Itoyama, Y., Makifuchi, T., Fraser, P.E., St George-Hyslop, P., Tohyama, M., 2001. Increased production of beta-amyloid and vulnerability to endoplasmic reticulum stress by an aberrant spliced form of presenilin 2. J. Biol. Chem. 276, 2108–2114.

Smith, M.J., Sharples, R.A., Evin, G., McLean, C.A., Dean, B., Pavey, G., Fantino, E., Cotton, R.G., Imaizumi, K., Masters, C.L., Cappai, R., Culvenor, J.G., 2004. Expression of truncated presenilin 2 splice variant in Alzheimer's disease, bipolar disorder, and schizophrenia brain cortex. Mol. Brain Res. 127, 128–135.

Vawter, M.P., Atz, M.E., Rollins, B.L., Cooper-Casey, K.M., Shao, L., Byerley, W.F., 2006. Genome scans and gene expression microarrays converge to identify gene regulatory loci relevant in schizophrenia. Human Genet. 119, 558–570.

Yanagita, T., Manabe, T., Okuda, H., Matsuzaki, S., Bando, Y., Katayama, T., Tohyama, M., 2005. Possible involvement of the expression and phosphorylation of N-Myc in the induction of HMGA1a by hypoxia in the human neuroblastoma cell line. Neurosci. Lett. 374, 47–52.

Journal of Human Genetics (2010) 55, 133–136

www.nature.com/jhg

ORIGINAL ARTICLE

Association study of *ubiquitin-specific peptidase 46* (*USP46*) with bipolar disorder and schizophrenia in a Japanese population

Itaru Kushima[1,12], Branko Aleksic[1,12], Yoshihito Ito[1,12], Yukako Nakamura[1,12], Kazuhiko Nakamura[2], Norio Mori[2], Mitsuru Kikuchi[3], Toshiya Inada[4], Hiroshi Kunugi[5], Shinichiro Nanko[6], Tadafumi Kato[7], Takeo Yoshikawa[8], Hiroshi Ujike[9], Michio Suzuki[10], Nakao Iwata[11,12] and Norio Ozaki[1,12]

Recently, *ubiquitin-specific peptidase 46* (*Usp46*) has been identified as a quantitative trait gene responsible for immobility in the tail suspension test and forced swimming test in mice. Mice with 3-bp deletion in *Usp46* exhibited loss of 'behavioral despair' under inescapable stresses in addition to abnormalities in circadian behavioral rhythms and the GABAergic system. Considering the face and construct validity as an animal model for bipolar disorder, we explored an association of *USP46* and bipolar disorder in a Japanese population. We also examined an association of *USP46* and schizophrenia. We found nominal evidence for an association of rs12646800 and schizophrenia. This association was not significant after correction for multiple testing. No significant association was detected for bipolar disorder. In conclusion, our data argue against the presence of any strong genetic susceptibility factors for bipolar disorder or schizophrenia in the region *USP46*.
Journal of Human Genetics (2010) **55,** 133–136; doi:10.1038/jhg.2009.139; published online 29 January 2010

Keywords: association study; bipolar disorder; schizophrenia; ubiquitin–proteasome system; *USP46*

INTRODUCTION

Biological studies have shown that the ubiquitin–proteasome system, which is highly conserved from yeast to man as the principal means of targeting cytosolic proteins for degradation, has an important role in neuronal function, such as synaptic formation, transmission and plasticity.[1–3] Genetic studies also have implicated the ubiquitin–proteasome system in a range of neuropsychiatric diseases, such as Parkinson's disease,[4] autism spectrum disorders,[5,6] mental retardation,[7–9] bipolar disorder[10,11] and schizophrenia.[11,12]

More recently, quantitative trait locus studies in mice have revealed that *ubiquitin-specific peptidase 46* (*Usp46*) is responsible for negligible immobility in the tail suspension test and forced swimming test, the experimental paradigms for assessing antidepressant activity and depression-like behavior.[13] Usp46 is one of approximately a hundred deubiquitinating enzymes. Protein deubiquitination by deubiquitinating enzymes can either antagonize or facilitate substrate presentation to the proteasome.[2] Deubiquitinating enzymes have also been associated with neurogenetic disorders, including Parkinson's disease, spinocerebellar ataxia. In the aforementioned study,[13] mice with 3-bp deletion in the exon region of *Usp46* exhibit loss of 'behavioral despair' under short-term, inescapable stresses of being suspended by their tail (tail suspension test) or being forced to swim in a water-filled cylinder (forced swimming test). 'Behavioral despair' was a characteristic immobile posture adopted by animals under stresses. Abnormalities in circadian behavioral rhythms and the GABAergic system, both of which are observed in bipolar disorder,[14] were also reported in the mice.[13] Furthermore, the *USP46* locus (4q12) corresponds to the linkage regions for bipolar disorder.[15] Considering the face and construct validity as an animal model for bipolar disorder and the findings of the linkage study, we explored an association of *USP46* with bipolar disorder in a Japanese population.

In addition, recent results from a genome-wide association study[16] and a population-based epidemiological study[17] provided evidence that schizophrenia and bipolar disorder share some common genetic causes. Therefore, we also examined an association between *USP46* and schizophrenia. It should be noted that abnormalities in circadian

[1]Department of Psychiatry, Graduate School of Medicine, Nagoya University, Aichi, Japan; [2]Department of Psychiatry and Neurology, Hamamatsu University School of Medicine, Hamamatsu, Japan; [3]Department of Psychiatry and Neurobiology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan; [4]Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, Japan; [5]National Center of Neurology and Psychiatry, Mental Disorder Research, National Institute of Neuroscience, Tokyo, Japan; [6]Department of Psychiatry, Teikyo University of Medicine, Tokyo, Japan; [7]Laboratory for Molecular Dynamics of Mental Disorders, RIKEN Brain Science Institute, Saitama, Japan; [8]Laboratory for Molecular Psychiatry, RIKEN Brain Science Institute, Saitama, Japan; [9]Department of Neuropsychiatry, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Science, Okayama, Japan; [10]Department of Neuropsychiatry, University of Toyama, Toyama, Japan; [11]Department of Psychiatry, Graduate School of Medicine, Fujita Health University, Aichi, Japan and [12]CREST, Japan Science and Technology Agency, Tokyo, Japan
Correspondence: Dr I Kushima, Department of Psychiatry, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan.
E-mail: kushima.itaru@a.mbox.nagoya-u.ac.jp
Received 28 September 2009; revised 25 November 2009; accepted 20 December 2009; published online 29 January 2010

rhythms and the GABAergic system[18] have been reported in schizophrenia as well.

MATERIALS AND METHODS

Subjects

For the bipolar disorder study, 867 cases (mean age, 50.7 ± 14.2 years) and 895 age- and gender-matched controls (49.9 ± 13.5 years) were used. This sample panel was the same as used in the Collaborative Study of Mood Disorder consortium study.[19] Seven laboratories (National Institute of Neuroscience, two laboratories of RIKEN Brain Science Institute, Kohnodai Hospital, Teikyo University, Okayama University and Fujita Health University) provided case and control samples. The proportion of cases with each disorder was 67.5, 31.9 and 0.6% for bipolar I disorder, bipolar II disorder and schizoaffective disorder, respectively. For the schizophrenia study, 715 cases (47.5 ± 14.0 years) and age- and gender-matched 711 controls (46.7 ± 13.1 years) were used. Controls used in the bipolar disorder or schizophrenia studies were independent. All subjects were of Japanese descent. Consensus diagnosis of bipolar disorder or schizophrenia was made according to the criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, by at least two experienced psychiatrists, on the basis of unstructured interviews, available medical records, and information from hospital staff and relatives. Controls were psychiatrically screened using an unstructured interview to exclude subjects with brain disorders or psychotic disorders. This study was approved by the Ethics Committees of all participating institutes. All participants provided written informed consent.

Tagging SNP selection, SNP genotyping and quality control

To test for genetic association, the gene-based approach was implemented. This method implies inclusion of both gene and gene-adjacent regions in the association study.[20] Therefore, the screened region was extended 10 kb upstream of the annotated transcription start site and downstream at the end of the last *USP46* exon. Consulting the HapMap database (release #24, population: Japanese in Tokyo), tagging single-nucleotide polymorphisms (SNPs) were selected to capture common SNPs (minor allele frequency >5%) in the predefined *USP46* locus. Given the linkage disequilibrium structure, seven tagging SNPs were selected, capturing all 30 common SNPs in the *USP46* locus at correlation coefficient (r^2)=1.

Genomic DNA was extracted from leukocytes by using the standard method. SNP genotyping was performed using the TaqMan system (Applied Biosystems, Foster City, CA, USA). PCR was performed using ABI 7900HT Fast Real-time PCR system and fluorescent signals were analyzed using SDS v2.2.1 software (Applied Biosystems).

For quality control, first, deviation from the Hardy–Weinberg equilibrium was checked in controls. Second, we excluded samples with call rates <100% from analyses.

Statistical analyses

The χ^2-test was used to compare the allele or genotype frequencies between cases and controls. Deviation from the Hardy–Weinberg equilibrium was also tested by the χ^2-test. Haplotype frequencies were estimated in a two- and three-marker sliding window manner by expectation maximization algorithm. Log likelihood ratio tests were performed for global *P*-values with COCAPHASE program in the UNPHASED v2.403 program (http://www.mrc-bsu.cam.ac.uk/personal/frank/software/unphased/).[21] All *P*-values reported were two-tailed. Statistical significance was defined as $P<0.05$. Power calculation was conducted with CaTS software (http://www.sph.umich.edu/csg/abecasis/CaTS/download.html).

RESULTS

All tagging SNPs were in Hardy–Weinberg equilibrium in controls. After excluding samples with call rates <100%, 845 cases and 869 controls in the bipolar disorder study, and 699 cases and 701 controls in the schizophrenia study remained for subsequent analyses. Assuming a multiplicative genetic model and a disease prevalence of 1%, power calculations showed that our sample had sufficient power (>80%) to detect gene-wide significant associations with genotype-relative risk values of 1.21–1.58 (minor allele frequency, 0.036–0.496) and 1.23–1.58 (minor allele frequency, 0.044–0.486) in bipolar disorder and schizophrenia, respectively. The linkage disequilibrium structures around *USP46* locus in 1570 control samples (869 controls in the bipolar disorder study+701 controls in the schizophrenia study) are shown in Table 1 and are highly similar to those of the JPT HapMap samples, ensuring that our genotyping were conducted correctly. The results of analyses are shown in Tables 2 and 3. We found nominal evidence for an association of rs12646800 with schizophrenia (allelic $P=0.04$, genotypic $P=0.01$). However, this association was not significant after Bonferroni correction. In bipolar disorder, no significant association was detected in allele-/genotype-/haplotype-wise analyses.

DISCUSSION

Although we could not detect evidence of a strong association of the *USP46* locus with bipolar disorder or schizophrenia in a Japanese population, these results could be interpreted in several ways. First, the results could indicate that there is no relevance of the *USP46* locus to these psychiatric disorders. Second, although this study was based on the common disease–common variant model, the genetic architecture of psychiatric disorders might be closer to the multiple rare variant model, making detection of causal variants difficult. Concerning this, on the basis of the epidemiological data and evolution theory, Uher[22] recently argues that severe mental illnesses, including schizophrenia that confer strong reproductive disadvantage, are likely to have a large and pleiotropic contribution from rare variants of recent origin. Third, it is possible that we overestimated the effect size of disease-related variants; that is, this study might be underpowered to detect variants

Table 1 Linkage disequilibrium analysis of *USP46*

SNP	*rs346005*	*rs10034164*	*rs2244291*	*rs12646800*	*rs6554557*	*rs17675844*	*rs10517263*
rs346005		1.00	1.00	1.00	1.00	0.99	1.00
rs10034164	0.14		1.00	1.00	0.99	1.00	1.00
rs2244291	0.43	0.06		1.00	0.98	0.99	1.00
rs12646800	0.04	0.01	0.02		1.00	1.00	1.00
rs6554557	0.14	0.97	0.06	0.01		1.00	1.00
rs17675844	0.10	0.02	0.23	0.00	0.02		1.00
rs10517263	0.09	0.62	0.04	0.00	0.61	0.01	

Abbreviations: SNP, single-nucleotide polymorphism; *USP46*, *ubiquitin-specific peptidase 46*.
Values shown above the diagonal are D′ and values shown below are r^2. Data of 1570 controls (control in bipolar disorder analysis, *N*=869; controls in schizophrenia analysis, *N*=701) were used for the calculation.

Table 2 Allele-/genotype-/haplotype-wise analyses in bipolar disorder

		Genotype counts						Single SNP		Haplotype-wise[a]	
		Case (N=845)			Control (N=869)						
dbSNP	*M/m*	*M/M*	*M/m*	*m/m*	*M/M*	*M/m*	*m/m*	*Allele -wise*	*Genotype -wise*	*2-window*	*3-window*
rs346005	A/C	213	427	205	215	432	222	0.61	0.83		
										0.26	
rs10034164	A/G	632	197	16	674	179	16	0.22	0.39		0.30
										0.34	
rs2244291	A/G	419	363	63	413	378	78	0.25	0.45		0.34
										0.35	
rs12646800	G/A	769	72	4	807	61	1	0.10	0.19		0.42
										0.25	
rs6554557	T/G	630	199	16	669	183	17	0.30	0.46		0.30
										0.37	
rs17675844	T/G	713	128	4	716	147	6	0.25	0.50		1.00
										1.00	
rs10517263	G/C	709	130	6	739	122	8	0.62	0.66		

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.
[a]Sliding window analysis, rare haplotype threshold 10%.

Table 3 Allele-/genotype-/haplotype-wise analyses in schizophrenia

		Genotype counts						Single SNP		Haplotype-wise[a]	
		Case (N=699)			Control (N=701)						
dbSNP	*M/m*	*M/M*	*M/m*	*m/m*	*M/M*	*M/m*	*m/m*	*Allele -wise*	*Genotype -wise*	*2-window*	*3-window*
rs346005	A/C	169	342	188	170	342	189	1.00	1.00		
										0.95	
rs10034164	A/G	526	160	13	533	155	13	0.76	0.94		0.93
										0.92	
rs2244291	A/G	346	278	75	336	293	72	0.75	0.74		0.84
										0.55	
rs12646800	G/A	661	36	2	640	61	0	0.04	0.01		0.82
										0.79	
rs6554557	T/G	527	157	15	533	156	12	0.67	0.83		0.89
										0.75	
rs17675844	T/G	579	115	5	571	122	8	0.41	0.62		1.00
										1.00	
rs10517263	G/C	590	101	8	590	106	5	0.93	0.67		

Abbreviations: M, major allele; m, minor allele; SNP, single-nucleotide polymorphism.
[a]Sliding window analysis, rare haplotype threshold 10%.

with small effect. For example, the range of odds ratios was 1.15–1.24 in seven markers, which were recently reported to show genome-wide significant association with schizophrenia.[23] Although the association between rs12646800 and schizophrenia was not significant after Bonferroni correction in our study, this correction may be too stringent because of the presence of linkage disequilibrium. For this reason, we checked whether there was an association of *USP46* with schizophrenia in a recent genome-wide association study. In the genome-wide association study by Need *et al.*[24], *USP46* locus included one SNP nominally associated with schizophrenia in a Caucasian population (rs2244291; allelic P=0.027). Although rs2244291 is not associated with schizophrenia in our study (allelic P=0.75, genotypic P=0.74) and rs12646800 is not polymorphic in HapMap Caucasian samples, it should be noted that rs2244291 is only ~200 bp away from rs12646800. This might point to the relevance of this region within *USP46* to risk for schizophrenia.

In addition, we searched two databases for further evidence of association of *USP46* with bipolar disorder or schizophrenia. First, we referred to the Stanley Medical Research Institute Online Genomics Database (https://www.stanleygenomics.org/) to examine the differences in USP46 expression in post-mortem brains.[25] Although we did not find a significant difference in USP46 expression between patients with schizophrenia and controls in a combined analysis of the results from 16 studies, we detected evidence of a trend for association in USP46 expression change between patients with bipolar disorder and controls in a combined analysis of the results from 18 studies (P=0.089), with USP46 expression in bipolar disorder reduced. Second, we referred to the Database of Genomic Variants[26] in search

of copy number variations with functional implication at *USP46* locus. Although we could not find copy number variations in this locus, it cannot be ruled out that unknown copy number variations located in this locus have an important role in the etiology of psychiatric disorders.

In conclusion, our data argue against the presence of any strong genetic susceptibility factors for bipolar disorder or schizophrenia in the region *USP46*. However, considering the limitations of this genetic association study and supportive evidence from various datasets, expansion of samples or resequencing strategy would be required for a more conclusive result.

ACKNOWLEDGEMENTS

We sincerely thank the patients and healthy controls for their participation in this study. We also thank Mrs R Ishihara and Mrs J Tsuda for their technical assistance. This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan, the Ministry of Health of Japan, Labor and Welfare, Grant-in-Aid for Scientific Research on Pathomechanisms of Brain Disorders from the Ministry of Education, Culture, Sports, Science and Technology of Japan, MEXT ACADEMIC FRONTIER and the Core Research for Evolutional Science and Technology. Lastly, we thank the Stanley Medical Research Institute for expression data of post-mortem brains.

1 Hegde, A. N. & DiAntonio, A. Ubiquitin and the synapse. *Nat. Rev. Neurosci.* **3,** 854–861 (2002).
2 Tai, H. C. & Schuman, E. M. Ubiquitin, the proteasome and protein degradation in neuronal function and dysfunction. *Nat. Rev. Neurosci.* **9,** 826–838 (2008).
3 Yi, J. J. & Ehlers, M. D. Ubiquitin and protein turnover in synapse function. *Neuron* **47,** 629–632 (2005).
4 Kitada, T., Asakawa, S., Hattori, N., Matsumine, H., Yamamura, Y., Minoshima, S. *et al.* Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. *Nature* **392,** 605–608 (1998).
5 Glessner, J. T., Wang, K., Cai, G., Korvatska, O., Kim, C. E., Wood, S. *et al.* Autism genome-wide copy number variation reveals ubiquitin and neuronal genes. *Nature* **459,** 569–573 (2009).
6 Bucan, M., Abrahams, B. S., Wang, K., Glessner, J. T., Herman, E. I., Sonnenblick, L. I. *et al.* Genome-wide analyses of exonic copy number variants in a family-based study point to novel autism susceptibility genes. *PLoS Genet.* **5,** e1000536 (2009).
7 Froyen, G., Corbett, M., Vandewalle, J., Jarvela, I., Lawrence, O., Meldrum, C. *et al.* Submicroscopic duplications of the hydroxysteroid dehydrogenase HSD17B10 and the E3 ubiquitin ligase HUWE1 are associated with mental retardation. *Am. J. Hum. Genet.* **82,** 432–443 (2008).
8 Tarpey, P. S., Raymond, F. L., O'Meara, S., Edkins, S., Teague, J., Butler, A. *et al.* Mutations in CUL4B, which encodes a ubiquitin E3 ligase subunit, cause an X-linked mental retardation syndrome associated with aggressive outbursts, seizures, relative macrocephaly, central obesity, hypogonadism, pes cavus, and tremor. *Am. J. Hum. Genet.* **80,** 345–352 (2007).
9 Field, M., Tarpey, P. S., Smith, R., Edkins, S., O'Meara, S., Stevens, C. *et al.* Mutations in the BRWD3 gene cause X-linked mental retardation associated with macrocephaly. *Am. J. Hum. Genet.* **81,** 367–374 (2007).
10 Ryan, M. M., Lockstone, H. E., Huffaker, S. J., Wayland, M. T., Webster, M. J. & Bahn, S. Gene expression analysis of bipolar disorder reveals downregulation of the ubiquitin cycle and alterations in synaptic genes. *Mol. Psychiatry* **11,** 965–978 (2006).
11 Bousman, C. A., Chana, G., Glatt, S. J., Chandler, S. D., Lucero, G. R., Tatro, E. *et al.* Preliminary evidence of ubiquitin proteasome system dysregulation in schizophrenia and bipolar disorder: Convergent pathway analysis findings from two independent samples. *Am. J. Med. Genet. B Neuropsychiatr Genet.* (2009). (e-pub ahead of print).
12 Chen, X., Wang, X., Sun, C., Chen, Q., O'Neill, F. A., Walsh, D. *et al.* FBXL21 association with schizophrenia in Irish family and case-control samples. *Am. J. Med. Genet. B Neuropsychiatr Genet.* **147B,** 1231–1237 (2008).
13 Tomida, S., Mamiya, T., Sakamaki, H., Miura, M., Aosaki, T., Masuda, M. *et al.* Usp46 is a quantitative trait gene regulating mouse immobile behavior in the tail suspension and forced swimming tests. *Nat. Genet.* **41,** 688–695 (2009).
14 Brambilla, P., Perez, J., Barale, F., Schettini, G. & Soares, J. C. GABAergic dysfunction in mood disorders. *Mol. Psychiatry* **8,** 721–737, 715 (2003).
15 Lambert, D., Middle, F., Hamshere, M. L., Segurado, R., Raybould, R., Corvin, A. *et al.* Stage 2 of the Wellcome Trust UK-Irish bipolar affective disorder sibling-pair genome screen: evidence for linkage on chromosomes 6q16-q21, 4q12-q21, 9p21, 10p14-p12 and 18q22. *Mol. Psychiatry* **10,** 831–841 (2005).
16 Purcell, S. M., Wray, N. R., Stone, J. L., Visscher, P. M., O'Donovan, M. C., Sullivan, P. F. *et al.* Common polygenic variation contributes to risk of schizophrenia and bipolar disorder. *Nature* **460,** 748–752 (2009).
17 Lichtenstein, P., Yip, B. H., Bjork, C., Pawitan, Y., Cannon, T. D., Sullivan, P. F. *et al.* Common genetic determinants of schizophrenia and bipolar disorder in Swedish families: a population-based study. *Lancet* **373,** 234–239 (2009).
18 Lewis, D. A., Hashimoto, T. & Volk, D. W. Cortical inhibitory neurons and schizophrenia. *Nat. Rev. Neurosci.* **6,** 312–324 (2005).
19 Iwayama, Y., Hattori, E., Maekawa, M., Yamada, K., Toyota, T., Ohnishi, T. *et al.* Association analyses between brain-expressed fatty-acid binding protein (FABP) genes and schizophrenia and bipolar disorder. *Am. J. Med. Genet. B Neuropsychiatr Genet.* (2009). (e-pub ahead of print).
20 Neale, B. M. & Sham, P. C. The future of association studies: gene-based analysis and replication. *Am. J. Hum. Genet.* **75,** 353–362 (2004).
21 Dudbridge, F. Pedigree disequilibrium tests for multilocus haplotypes. *Genet. Epidemiol.* **25,** 115–121 (2003).
22 Uher, R. The role of genetic variation in the causation of mental illness: an evolution-informed framework. *Mol. Psychiatry* **14,** 1072–1082 (2009).
23 Stefansson, H., Ophoff, R. A., Steinberg, S., Andreassen, O. A., Cichon, S., Rujescu, D. *et al.* Common variants conferring risk of schizophrenia. *Nature* **460,** 744–747 (2009).
24 Need, A. C., Ge, D., Weale, M. E., Maia, J., Feng, S., Heinzen, E. L. *et al.* A genome-wide investigation of SNPs and CNVs in schizophrenia. *PLoS Genet.* **5,** e1000373 (2009).
25 Higgs, B. W., Elashoff, M., Richman, S. & Barci, B. An online database for brain disease research. *BMC Genomics* **7,** 70 (2006).
26 Iafrate, A. J., Feuk, L., Rivera, M. N., Listewnik, M. L., Donahoe, P. K., Qi, Y. *et al.* Detection of large-scale variation in the human genome. *Nat. Genet.* **36,** 949–951 (2004).

RESEARCH ARTICLE

AMERICAN JOURNAL OF
medical genetics
Neuropsychiatric Genetics

Association Study of Bromodomain-Containing 1 Gene With Schizophrenia in Japanese Population

Itaru Kushima,[1,2]* Branko Aleksic,[1,2] Masashi Ikeda,[2,3,4] Yoshio Yamanouchi,[2,3] Yoko Kinoshita,[2,3] Yoshihito Ito,[1,2] Yukako Nakamura,[1,2] Toshiya Inada,[5] Nakao Iwata,[2,3] and Norio Ozaki[1,2]

[1]Department of Psychiatry, Graduate School of Medicine, Nagoya University, Aichi, Japan

[2]CREST, Japan Science and Technology Agency, Saitama, Japan

[3]Department of Psychiatry, Graduate School of Medicine, Fujita Health University, Aichi, Japan

[4]Department of Psychological Medicine, School of Medicine, Cardiff University, Health Park, Cardiff, UK

[5]Seiwa Hospital, Institute of Neuropsychiatry, Tokyo, Japan

Received 24 March 2009; Accepted 28 September 2009

Chromosome 22q13 region has been implicated in schizophrenia in several linkage studies. Genes within this locus are therefore promising genetic and biologic candidate genes for schizophrenia if they are expressed in the brain or predicted to have some role in brain development. A recent study reported that bromodomain-containing 1 gene (*BRD1*), located in 22q13, showed an association with schizophrenia in a Scottish population. Except for being a putative regulator of transcription, the precise function of *BRD1* is not clear; however, expression analysis of *BRD1* mRNA revealed widespread expression in mammalian brains. In our study, we explored the association of *BRD1* with schizophrenia in a Japanese population (626 cases and 770 controls). In this association analysis, we first examined 10 directly genotyped single-nucleotide polymorphisms (SNPs) and 20 imputed SNPs. Second, we compared the *BRD1* mRNA levels between cases and controls using lymphoblastoid cell lines (LCLs) derived from 29 cases and 30 controls. Although the SNP (rs138880) that previously has been associated with schizophrenia showed the same trend in the Japanese population, no significant association was detected between *BRD1* and schizophrenia in our study. Similarly, no significant differences in *BRD1* mRNA levels in LCLs were detected. Taken together, we could not strongly show that common SNPs in the *BRD1* gene account for a substantial proportion of the genetic risk for schizophrenia in the Japanese population. © 2009 Wiley-Liss, Inc.

Key words: association analysis; imputation; gene expression analysis; meta-analysis

How to Cite this Article:
Kushima I, Aleksic B, Ikeda M, Yamanouchi Y, Kinoshita Y, Ito Y, Nakamura Y, Inada T, Iwata N, Ozaki N. 2010. Association Study of Bromodomain-Containing 1 Gene With Schizophrenia in Japanese Population.
Am J Med Genet Part B 153B:786–791.

INTRODUCTION

Schizophrenia is a severe, debilitating disorder characterized by delusional beliefs, hallucinations, disordered speech, and deficits in emotional and social behavior. It is strongly familial, and heritability is around 80% based on twin studies [Sullivan et al., 2003]. However, the pattern of inheritance is complex, with most studies suggesting an interaction of multiple genes. There are now several positional candidate regions all over the genome that have been shown to be related to schizophrenia in genetic studies [Badner and Gershon, 2002; Williams et al., 2003].

One promising region is chromosome 22q. Initial evidence for linkage to chromosome 22q came from three markers spanning ~23 cM in the 22q13.1 region in the Maryland family sample [Pulver et al., 1994]. Additional interest in 22q13 came from a genome scan of catatonic schizophrenia pedigrees, which showed suggestive evidence for linkage ($P = 1.8 \times 10^{-3}$; non-parametric logarithms of the odds [LOD] score 1.85) on 22q13 [Stober et al., 2000]. Furthermore, a multicenter linkage study that evaluated 10 microsatellite markers spanning 22q in 779 schizophrenia pedigrees

Additional Supporting Information may be found in the online version of this article.
Grant sponsor: Ministry of Education, Culture, Sports, Science and Technology of Japan; Grant sponsor: Ministry of Health of Japan, Labor and Welfare; Grant sponsor: MEXT ACADEMIC FRONTEIER; Grant sponsor: Japan Health Sciences Foundation; Grant sponsor: Core Research for Evolutional Science and Technology.
Itaru Kushima and Branko Aleksic contributed equally to this work.
*Correspondence to:
Dr. Itaru Kushima, Department of Psychiatry, Nagoya University Graduate School of Medicine, 65 Tsurumai-Cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. E-mail: kushima.itaru@a.mbox.nagoya-u.ac.jp
Published online 11 November 2009 in Wiley InterScience (www.interscience.wiley.com)
DOI 10.1002/ajmg.b.31048