induced drug craving and psychosis in humans, these animal and human abnormalities induced by stimulants have been considered to share a common pathophysiology underlying the vulnerability to their recurrences (Ujike and Sato, 2004; Vanderschuren and Kalivas, 2000). Moreover, in remitted or stable schizophrenic patients, a hallucinatory-paranoid state reappeared or was exacerbated following a small and subpsychotomimetic dose of a psychostimulant for normal volunteers (Segal and Janowsky, 1978; Snyder, 1973). These results support the idea that, like the patients with stimulant-induced psychosis, a subpopulation of schizophrenic patients may be much more sensitive to psychotomimetic effects of stimulants than normal volunteers. Taken together, the behavioral sensitization may also be a useful model for the relapse vulnerability in schizophrenic patients. Therefore, the treatment that produces a lasting inhibition of the expression of the once established behavioral sensitization can be expected to possess a prophylactic efficacy on the recurrence of psychotic states of stimulant-induced psychosis and/or schizophrenia.

Recently, the repeated systemic administration with a dopamine D1 agonist (Li *et al*, 2000), an NMDA antagonist plus dopamine D2 receptor agonist (Li *et al*, 2000), serotonin-2 (5-hydroxytryptamine-2; 5-HT_2) antagonists (Davidson *et al*, 2002a), and a 5-HT_3 antagonist (King *et al*, 1998, 2000; Davidson *et al*, 2002b), during the withdrawal period after the repetitive injection of cocaine, amphetamine, or MAP has been reported to attenuate the expression of behavioral sensitization. Although the exact mechanisms underlying these attenuating effects are still unclear, several lines of evidence indicate that the cerebral serotonergic systems could be involved in the modification of the stimulant-induced long-lasting changes in the behavioral responses. Thus, (1) the unlimited self-administration of cocaine produced a sustained decrease in the extracellular 5-HT concentration in the nucleus accumbens during the withdrawal period (Parsons *et al*, 1995), (2) the enhanced synaptic levels of serotonin by administration of a 5-HT precursor L-tryptophan or of a 5-HT selective serotonin reuptake inhibitor (SSRI) fluoxetine (FLX) reduced the reinforcing effects of cocaine (Lyness, 1983; Carroll *et al*, 1990; Richardson and Roberts, 1991; Takamatsu *et al*, 2005), and (3) repeated MAP treatment has been shown to fail to cause behavioral sensitization in the mice lacking a 5-HT transporter with an excess of extracellular 5-HT contents (Shen *et al*, 2003). These data suggest that the decreased serotonergic tone may play an important role in the maintenance of sensitization elicited by the psychostimulant drugs and, in turn, increased cerebral serotonergic transmission could suppress the expression of the established sensitization.

To test the possible suppression by 5-HT agonists, we have studied the influences of repeated administration of typical SSRIs, FLX, and paroxetine (PRX), during withdrawal of the repetitive treatment with MAP, on the ability of a challenge dose of MAP to cause an augmented motor response in mice following a drug-free period after the SSRI injections. We have chosen these clinically available SSRIs because we have considered the future clinical applications of these drugs for the purpose of the prophylaxis against the relapses of stimulant-induced craving or psychotic state and/or of schizophrenia if they could reverse the established sensitization.

MATERIALS AND METHODS

Animals

The present animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University and were approved by the Animal Investigation Committee of the Institution. Male ddY mice (Clea Japan Inc., Japan) at ages ranging from postnatal days 50 to 56 weighing 32–42g were used. The animals were housed in groups of 4–5 per cage at $23.0 \pm 0.5^{\circ}C$ in a humidity-controlled room under a light-controlled (14-h/12-h light/dark cycle, lights on at 0600 hours) and had free access to food and water.

Chemicals

MAP hydrochloride was purchased from Dainippon Pharmaceutical Co., Ltd (Osaka, Japan), with official permission of the Tokyo Metropolitan Bureau of Public Health. FLX HCl and PRX maleate were purchased from TOCRIS (Avonmouth, UK). The other chemicals used were of ultrapure quality and were commercially available. Doses for the injections always refer to the free bases. Each drug was dissolved in saline (SAL) (0.9% NaCl) and subcutaneously (s.c.) injected in a volume of 0.005 ml/g body weight. The control mice were treated with SAL.

Drug Administration Schedule

Establishment and maintenance of behavioral sensitization. To confirm the establishment and maintenance of the behavioral sensitization under our experimental conditions, 16 mice were pretreated with 1.0 mg/kg of MAP (s.c.) (eight mice) or SAL (eight mice) once daily for 10 days. On days 1, 3, 7, and 10 of the pretreatment, these mice were placed into the movement measurement apparatus to count their spontaneous activities. On day 11 (12 animals) of withdrawal following pretreatment with MAP or SAL, the animals were injected with SAL and, on the next day, with a challenge dose of MAP (0.24 mg/kg, s.c.). The two experimental groups were

(1) MAP (1.0 mg/kg/day for 10 days) + MAP (0.24 mg/kg on day 12 of withdrawal) ($N=8$) and
(2) SAL (for 10 days) + MAP (0.24 mg/kg on day 12 of withdrawal) ($N=4$).

Treatment with SSRIs. In the experiments to study the effects of SSRIs on the MAP-induced behavioral sensitization, the mice pretreated with MAP or SAL for 10 days were repeatedly administered with FLX (10 mg/kg/day, s.c.) or PRX (8 mg/kg/day, s.c.) once daily from day 12 to 16 of pretreatment withdrawal. These animals received a challenge of MAP or SAL 13 days after the repeated treatment with FLX or PRX, respectively. Table 1 summarizes the 16 groups for the SSRI experiments.

Table 1 Schedules and Doses for Pretreatment and Challenge of MAP, FLX, PRX, and SAL and Cumulated Motor Activity after Challenge of MAP or SAL

Group (duration)	N	Pretreatment with MAP or SAL (10 days)	Withdrawal period I (11 days)	Pretreatment with SSRI or SAL (5 days)	Withdrawal period II (12 days)	Challenge	Cumulated motor activity (counts/60 min)
Fluoxetine							
1	8	SAL		SAL		SAL	1143±161
2	8	SAL		FLX		SAL	1581±300
3	8	MAP		SAL		SAL	1378±255
4	8	MAP		FLX		SAL	2682±344
5	8	SAL		SAL		MAP	2883±735
6	8	SAL		FLX		MAP	2488±800
7	8	MAP		SAL		MAP	8066±831
8	8	MAP		FLX		MAP	4689±725
Paroxetine							
1	10	SAL		SAL		SAL	1923±200
2	10	SAL		PRX		SAL	2016±657
3	10	MAP		SAL		SAL	1561±351
4	10	MAP		PRX		SAL	1822±399
5	10	SAL		SAL		MAP	3476±722
6	10	SAL		PRX		MAP	2353±620
7	10	MAP		SAL		MAP	7770±862
8	10	MAP		PRX		MAP	3843±776

The different dosing regimens for the eight groups in each experiment are summarized. Methamphetamine (MAP; 1.0 mg/kg, s.c.) or saline (SAL) was repeatedly injected during the pretreatment period. The pretreatment with FLX (fluoxetine; 10 mg/kg/day, s.c.), PRX (paroxetine; 8 mg/kg/day, s.c.), or SAL for 5 days was initiated from day 12 to 16 of withdrawal of the repeated MAP injection. The animals pretreated with MAP or SAL plus FLX, PRX, or SAL were challenged with MAP at the dose of 0.24 mg/kg or SAL (s.c.) on day 13 of withdrawal of FLX, PRX, or SAL treatment. Each cumulated motor activity is expressed as means with SEM of the data obtained from 8 to 10 determinations.

Behavioral Analyses

To evaluate the behavioral effects of MAP (1.0 or 0.24 mg/kg, s.c.), the spontaneous vertical and horizontal movements including locomotion, rearing, and head movements were quantified by automatically counting the number of heat changes in the multiple zones of the test cage by means of the heat sensor with a Supermex instrument (Muromachi-kikai Co. Ltd, Tokyo, Japan) (Masuo *et al*, 1995; Hara *et al*, 2001). The mice were placed into the acrylic test cage (24.5 × 17.5 × 12.5 cm) within a soundproof and illuminated wood box at an ambient temperature of 23.0±0.5°C. The Supermex consists of a monitor that was mounted above the test cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses. The body heat radiated by an animal was detected by the sensor head of the monitor, which contained paired infrared light ray pyroelectric detectors. Every behavioral analysis was always performed for 120–150 min before and for 60 min after the injection of the MAP or SAL.

Statistical Analyses

Results are usually reported as means with SEM of the data. For comparison between the two groups, statistical evaluations were made using the two-tailed Student's *t*-test. Statistical differences among more than three groups were estimated by a one-way analysis of variance (ANOVA; homogeneous variance) or the Kruskal–Wallis test (heterogeneous variance) followed by the Dunnett or Scheffé *post hoc* test. The significance level was set at $p<0.05$ for all comparisons.

RESULTS

Establishment and Maintenance of Behavioral Sensitization by Repeated MAP Treatment

As shown in Figure 1a, repeated treatment of ddY mice with MAP (1 mg/kg once daily for 10 days, s.c.) resulted in a progressive and significant enhancement of the ability of MAP to increase the amounts of motor activity for 60 min on the 7th ($p<0.05$ *vs* the 1st day) and 10th ($p<0.01$) day of the drug regimen. The enhanced motor responses to MAP were also observed 12 days after discontinuation of the repeated treatment with MAP (Figure 1b). Because these observations confirmed the establishment of the MAP-induced sensitization and were consistent with those in the previous sensitization experiments (Vanderschuren and Kalivas, 2000), we routinely applied this MAP treatment schedule to the present behavioral experiments.

Effects of FLX and PRX on the Established Behavioral Sensitization after Repeated MAP Treatment

In the experiments using MAP and SSRIs (see Figures 2 and 3), the long-lasting nature of behavioral sensitization

was further verified by the results that the mice pretreated with MAP (1 mg/kg once daily for 10 days, s.c.) exhibited augmented motor responses to a challenge dose of MAP on day 29 of withdrawal (SAL + SAL + MAP *vs* MAP + SAL + MAP in Figures 2 and 3).

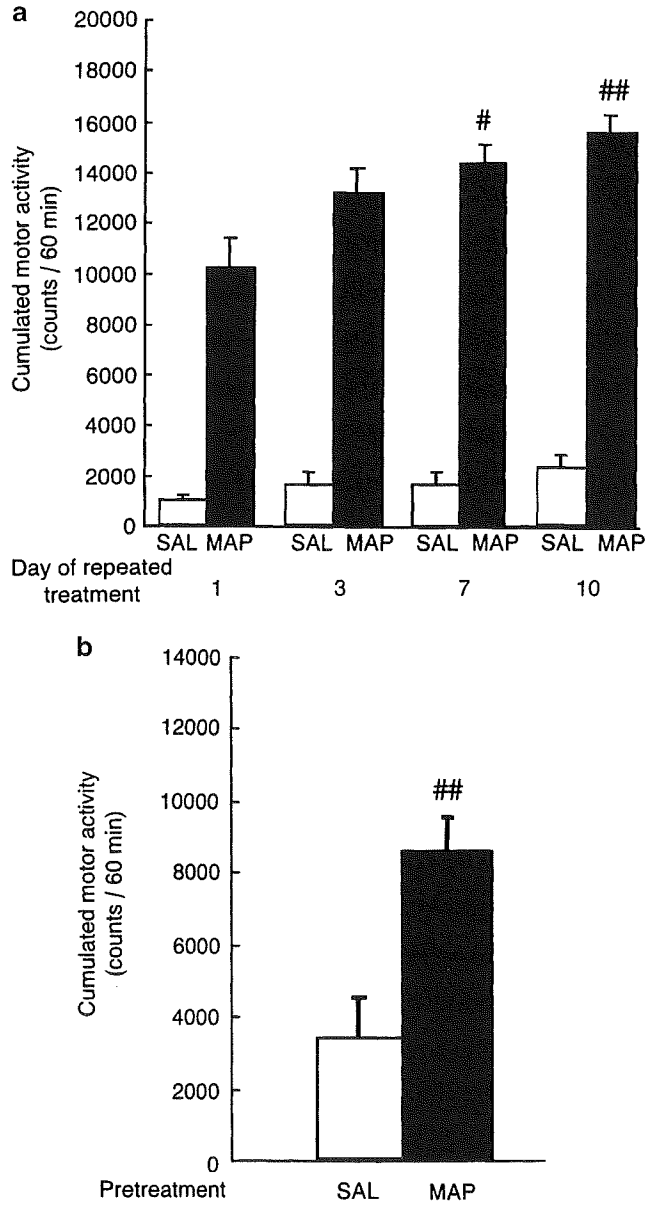

Figure 1 Changes in behavioral responses to MAP challenge during or after repeated MAP pretreatment. (a) Cumulated motor activity for 60 min following daily injection of MAP (1.0 mg/kg, s.c.) or SAL during repeated treatment for 10 days in mice. Each value is the mean with SEM of eight determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* values obtained on the first treatment day in the respective experimental group. Statistical analysis of the present data with a homogeneous variance (Bartlett test: MAP, $\chi^2=2.3480$, df = 3, $p=0.5033$; SAL, $\chi^2=5.3896$, df = 3, $p=0.1454$) was performed using a one-way ANOVA followed by the Dunnett *post hoc* test (SAL, $F(3, 28)=1.997$, $p=0.137$ (no statistically significant difference); MAP, $F(3, 28)=5.926$, $p<0.01$ ($p=0.0029$). (b) Cumulated motor activity for 60 min following a challenge dose of MAP (0.24 mg/kg, s.c.) on day 12 of withdrawal of the repeated treatment with MAP or SAL. Each value is the mean with SEM of 4–8 determinations. $^{\#\#}p<0.01$ *vs* the respective SAL-pretreated controls. Statistical analysis of the present data with a homogeneous variance ($F=0.604$, $p=0.3670$) was performed using the two-tailed Student's *t*-test ($t=-3.35$, $p<0.01$ ($p=0.0074$)).

As shown in Figure 2, in the SAL-pretreated mice, repeated FLX administration failed to cause a significant change in the cumulated motor activity after a challenge of SAL (SAL + FLX + SAL) or MAP (SAL + FLX + MAP) on day 13 of FLX withdrawal as compared to the corresponding repetitive vehicle-treated animals (SAL + SAL + SAL and SAL + SAL + MAP). There was a trend toward, but not statistically significant, increase in the motor response to SAL challenge in the repeatedly MAP-pretreated FLX-injected mice (MAP + FLX + SAL) when compared to the repeatedly MAP-pretreated vehicle-injected mice (MAP + SAL + SAL) (Figure 2). However, in the MAP-pretreated behaviorally sensitized mice, repeated FLX injection led to significantly lower counts of spontaneous movements after a challenge dose of MAP (MAP + FLX + MAP) on day 13 of FLX withdrawal than the repeated vehicle injection (MAP + SAL + MAP) (Figure 2). No stereotyped behavior was observed after a MAP challenge in any of the experimental groups of animals. These results indicate that repeated FLX treatment may reduce the expression of the behavioral sensitization following a MAP challenge without apparent changes in the motor responses to SAL in the sensitized and the nonsensitized animals, and to MAP in nonsensitized mice.

Similarly, the repeated PRX administration inhibited the ability of a subsequent challenge of MAP to increase

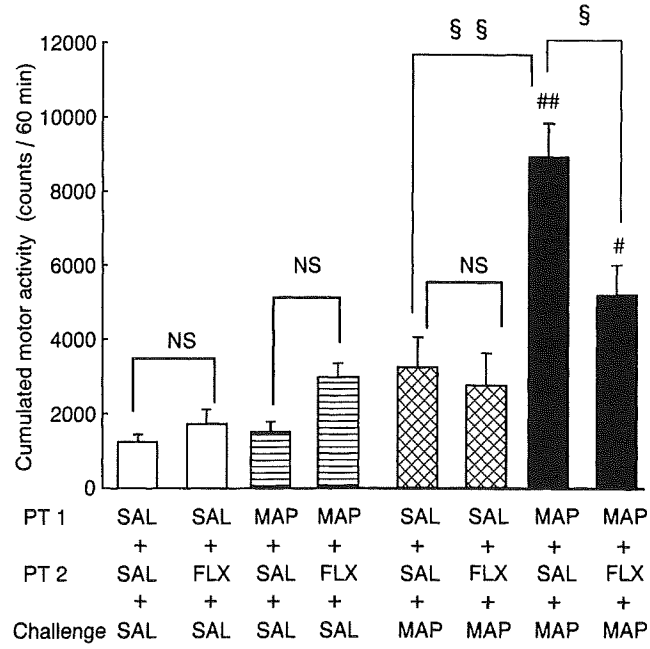

Figure 2 Effects of repeated injections of fluoxetine during withdrawal of MAP pretreatment on behavioral responses to MAP challenge. The detailed schedules of the drug administrations are shown in Table 1. The motor activity was automatically quantified and cumulated for 60 min following MAP challenge (0.24 mg/kg, s.c.) on days 29 and 13 of withdrawal of the repeated treatment with MAP (PT 1; pretreatment 1) and that with fluoxetine (PT 2; pretreatment 2), respectively. Each value is the mean with SEM of eight determinations. $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs* SAL-pretreated (for two times) and SAL-challenged animals (absolute controls). $^{\S}p<0.05$, $^{\S\S}p<0.01$ between the two groups linked with a solid line. NS: no statistically significant difference between the two groups linked with a solid line. Statistical analysis of the present data with a heterogeneous variance (Bartlett test: $\chi^2=28.5574$, df = 7, $p<0.01$ ($p=0.0002$)) was performed using the Kruskal–Wallis test ($p<0.0001$) followed by the Scheffé *post hoc* test.

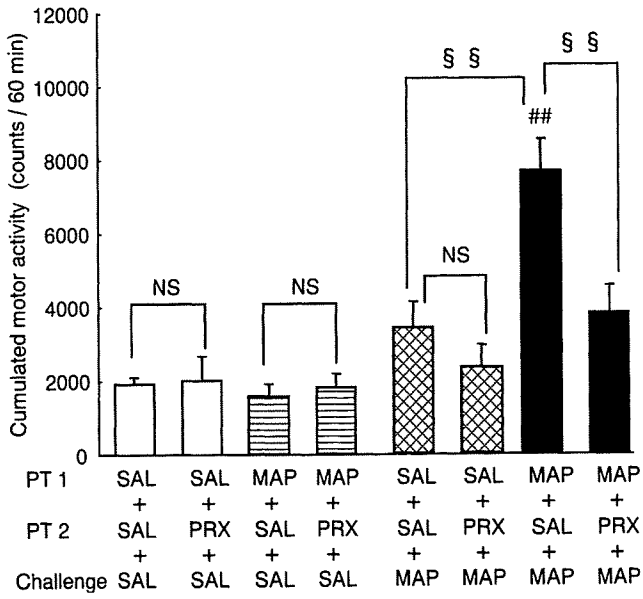

Figure 3 Effects of repeated injections of PRX during withdrawal of MAP pretreatment on behavioral responses to MAP challenge. The detailed schedules of the drug administrations are shown in Table 1. The motor activity was automatically quantified and cumulated for 60 min following MAP challenge (0.24 mg/kg, s.c.) on days 29 and 13 of withdrawal of the repeated treatment with MAP (PT 1; pretreatment 1) and that with PRX (PT 2; pretreatment 2), respectively. Each value is the mean with SEM of 10 determinations. $^{\#\#}p<0.01$ *vs* SAL-pretreated (for two times) and SAL-challenged animals (absolute controls). $^{\S\S}p<0.01$ between the two groups linked with a solid line. NS no statistically significant difference between the two groups linked with a solid line. Statistical analysis of the present data with a heterogeneous variance (Bartlett test: $\chi^2=22.2354$, df = 7, $p<0.01$ ($p=0.0023$)) was performed using the Kruskal–Wallis test ($p<0.0001$) followed by the Scheffé *post hoc* test.

the number of movements in the MAP-pretreated mice (MAP + PRX + MAP *vs* MAP + SAL + MAP) on day 13 of PRX withdrawal (Figure 3). This PRX regimen failed to change the behavioral response to SAL challenge in the SAL- and MAP-pretreated animals (SAL + PPX + SAL and MAP + PRX + SAL) and to a challenge dose of MAP in the SAL-pretreated mice (SAL + PRX + MAP *vs* SAL + SAL + MAP) (Figure 3). The MAP challenge produced no apparent stereotyped behavior in any of the experimental groups.

DISCUSSION

In the present study, we have verified that 10 daily administrations of MAP (1 mg/kg, s.c.) produced a progressive and enduring augmentation in the increased movements elicited by a subsequent challenge of MAP, that is, behavioral sensitization. Our obtained data first demonstrate that the repeated injection of FLX or PRX to behaviorally sensitized mice by MAP pretreatment attenuates the expression of the enhanced behavioral response to a challenge dose of MAP after a 13-day drug-free interval. This attenuation suggests that FLX and PRX may be able to reverse the established behavioral sensitization following an exposure to psychostimulants.

The nonspecific phenomena including the long-term sedation or accumulation of either SSRI or its active metabolites after repeated SSRI treatment could produce the attenuating effects of the SSRIs on the challenge MAP-induced movements. FLX and its active desmethyl metabolite, nor-fluoxetine, have indeed been reported to display long halflives ranging between 1 and 4 days and between 7 and 15 days, respectively, in humans (Hiemke and Hartter, 2000; Sills *et al*, 2000). However, the above presumptions are unlikely because (1) neither the repeated FLX nor PRX treatment diminished the basal amounts of movements (SAL-induced movements) in the SAL- and MAP-pretreated mice and the ability of a MAP challenge to increase significantly the movements in the SAL-pretreated mice (Figures 2 and 3), (2) a subchronic injection of FLX for 5 days potentiated the increasing effects of an acute amphetamine application on motor activity on days 1 and 2, but not on day 5, of withdrawal (Sills *et al*, 2000), and (3) repeated treatment with FLX or PRX for 27 days enhanced the psychomotor stimulatory effects of an alcohol challenge on the 28th day of the experiments (Goeldner *et al*, 2005). Moreover, no apparent stereotypy following a challenge dose of MAP in any experimental groups seems to deny the possibility that the apparent reduction in the MAP-induced movements (Figures 2 and 3) might reflect the diminished ambulation owing to the robust sensitization with increased frequencies of the stereotyped behavior in one location.

Both repeated FLX and PRX treatment by themselves tended to reduce, although nonsignificantly, the locomotor response to acute MAP administration. These tendencies are also likely to be associated with SSRI-induced attenuation of the expression of behavioral sensitization, because the repeated SAL treatment as repeated injection stress could augment the ability of amphetamines to induce abnormal behavior. This view seems to be supported by the previous observation (Antelman *et al*, 1980) indicating that repeated mild stress (tail pressure stress) resulted in an enhanced behavioral response to *d*-amphetamine.

Based upon the fact that the common selective and potent action between FLX and PRX is 5-HT uptake inhibition, it is more likely that the reduced expression of behavioral sensitization in the animals treated with these SSRIs after the establishment of the sensitization may be connected to an enhanced serotonergic tone in the brain. Although increased synaptic 5-HT has been reported to prevent the development of the stimulant-induced behavioral sensitization and craving (see Introduction), there has so far been no study to test the effects of 5-HT agonists on the sustainment of these behavioral changes. The elevated contents of the synaptic 5-HT by SSRIs (Felton *et al*, 2003) could reverse the stimulant-induced sensitization by compensating the plausible persistent decrease in the basal extracellular release of 5-HT in the nucleus accumbens, which has been suggested to play an important role in sustaining the sensitization (Parsons *et al*, 1995).

The SSRI-induced disruption of the sensitization could be mediated by the specific 5-HT receptor subtypes that interact with the ascending dopamine neurons projecting from the ventral tegmental area to the nucleus accumbens, because these neurons have been proved to participate in the development and expression of the long-lasting locomotor sensitization (Vanderschuren and Kalivas, 2000). In terms of this interaction, it is of interest to note that 5-HT_{1B} (Yan and Yan, 2001; Yan *et al*, 2004) and 5-HT_{2A}

(Auclair *et al*, 2004; Esposito, 2006) receptors in either of the two brain areas have been well known to be involved in the control of dopamine release from the nucleus accumbens. Recent studies have further suggested the modification of the meso-accumbens dopamine neurons by the 5-HT_{1A} (Andrews *et al*, 2005), 5-HT_{2C} (Esposito, 2006), and 5-HT_3 (De Deurwaerdere *et al*, 2005) receptors. The increased vulnerability to cocaine (Rocha *et al*, 1998) and amphetamine (Bronsert *et al*, 2001) in mice lacking the 5-HT_{1B} receptor favors the possible role of 5-HT_{1B} receptor stimulation in the reversal of the stimulant-induced locomotor sensitization. However, inhibition of the expression of the established behavioral sensitization was caused by the 5-HT_3 receptor antagonist, ondansetron, and some agents with the 5-HT_{2A} receptor antagonist property including clozapine, mianserin, and ketanserin (Davidson *et al*, 2002a, b). Activation of the 5-HT_{1A} receptor was reported to prevent the development of the behavioral sensitization to L-DOPA (L-3,4-dihydroxyphenylalanine) (Tomiyama *et al*, 2005), but has not yet been tested with respect to the established sensitization phenomenon. To clarify the 5-HT receptor subtypes critical for the reversal effects of SSRIs on the expression of the MAP sensitization, further investigation is needed to try to block the reversal effects using 5-HT_{1B}, 5-HT_{2A}, 5-HT_{1A}, 5-HT_{2C}, and 5-HT_3 antagonists.

Because the long-lasting nature of the behavioral sensitization has been considered to be associated with brain plasticity, the SSRIs used in this study could modulate the plastic changes underlying behavioral sensitization through their influences on the brain growth factors (Sodhi and Sanders-Bush, 2004) and hippocampal neurogenesis, which are related to the rearrangements or remodeling of the neuron circuits (Duman *et al*, 2001). This view is supported by the findings that (1) the repetitive administration of a psychostimulant, cocaine, has been shown to decrease neurogenesis in the adult rat hippocampus (Yamaguchi *et al*, 2004), (2) the single or repeated treatment with amphetamine, MAP, or cocaine has been found to alter the levels of mRNA or proteins in the brain-derived growth factor (Meredith *et al*, 2002; Grimm *et al*, 2003; Le Foll *et al*, 2005), and (3) stress causes the suppression of neurogenesis, debranching, and shortening of the dendrites in the adult rat hippocampal dentate gyrus, which have been documented to be reversed by repeated FLX (Malberg *et al*, 2000; Malberg and Duman, 2003; Kodama *et al*, 2004).

MAP-induced behavioral sensitization has been considered to be an animal model of MAP craving or psychosis, or paranoid schizophrenia (Ellinwood *et al*, 1973; Robinson and Becker, 1986; Ujike and Sato, 2004). The patients with these disorders often suffer from relapses for many years or a lifetime even after the long discontinuance of MAP and/or the continued treatment with antipsychotic drug. The markedly reduced expression of sensitization by a temporary treatment with FLX and PRX observed here suggests that the short-term treatment with these SSRIs might attenuate the relapse of the psychotic state associated with psychostimulants and/or schizophrenia. Therefore, it would be relevant for the development of an additional pharmacotherapy for MAP psychosis and/or a group of schizophrenia to test the ability of a subchronic regimen of FLX and PRX to mitigate or prevent the recurrence of the hallucinatory-paranoid state in these psychoses. However, before start of such a clinical test, careful considerations are required of the previous data indicating that SSRI augmentation of antipsychotics in the treatment of schizophrenia improved negative symptoms of schizophrenia and had no effect on positive symptoms (Silver and Shmugliakov, 1998; Silver, 2004), although the therapeutic target of the SSRIs is not the positive symptoms by themselves but the vulnerability to their relapse. It should also be noted that some cases were omitted from the clinical trials owing to the worsening of the positive symptoms (Silver and Shmugliakov, 1998; Poyurovsky *et al*, 1999).

In conclusion, the present study indicates that a 5 days treatment with SSRIs, FLX, and PRX, during the withdrawal period of chronic MAP treatment, may, at least in part, reverse the MAP-induced behavioral sensitization. It is proposed that these SSRIs could be clinically useful as prophylactic agents against the easy reactivation of serious psychotic states in patients with MAP craving or psychosis, and/or some schizophrenic patients.

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REFERENCES

Andrews CM, Kung HF, Lucki I (2005). The 5-HT_{1A} receptor modulates the effects of cocaine on extracellular serotonin and dopamine levels in the nucleus accumbens. *Eur J Pharmacol* **508**: 123–130.

Antelman SM, Eichler AJ, Black CA, Kocan D (1980). Interchangeability of stress and amphetamine in sensitization. *Science* **207**: 329–331.

Auclair A, Drouin C, Cotecchia S, Glowinski J, Tassin JP (2004). 5-HT_{2A} and alpha1b-adrenergic receptors entirely mediate dopamine release, locomotor response and behavioural sensitization to opiates and psychostimulants. *Eur J Neurosci* **20**: 3073–3084.

Bronsert MR, Mead AN, Hen R, Rocha BA (2001). Amphetamine-induced locomotor activation in 5-HT(1B) knockout mice: effects of injection route on acute and sensitized responses. *Behav Pharmacol* **12**: 549–555.

Carroll ME, Lac ST, Asencio M, Kragh R (1990). Intravenous cocaine self-administration in rats is reduced by dietary L-tryptophan. *Psychopharmacology (Berlin)* **100**: 293–300.

Davidson C, Lazarus C, Xiong X, Lee TH, Ellinwood EH (2002a). 5-HT_2 receptor antagonists given in the acute withdrawal from daily cocaine injections can reverse established sensitization. *Eur J Pharmacol* **453**: 255–263.

Davidson C, Lee TH, Xiong Z, Ellinwood EH (2002b). Ondansetron given in the acute withdrawal from a repeated cocaine sensitization dosing regimen reverses the expression of sensitization and inhibits self-administration. *Neuropsychopharmacology* **27**: 542–553.

De Deurwaerdere P, Moison D, Navailles S, Porras G, Spampinato U (2005). Regionally and functionally distinct serotonin3 receptors control *in vivo* dopamine outflow in the rat nucleus accumbens. *J Neurochem* **94**: 140–149.

Duman RS, Nakagawa S, Malberg J (2001). Regulation of adult neurogenesis by antidepressant treatment. *Neuropsychopharmacology* **25**: 836–844.

Ellinwood Jr EH, Sudilovsky A, Nelson LM (1973). Evolving behavior in the clinical and experimental amphetamine (model) psychosis. *Am J Psychiatry* **130**: 1088–1093.

Esposito E (2006). Serotonin–dopamine interaction as a focus of novel antidepressant drugs. *Curr Drug Targets* **7**: 177–185.

Felton TM, Kang TB, Hjorth S, Auerbach SB (2003). Effects of selective serotonin and serotonin/noradrenaline reuptake inhibitors on extracellular serotonin in rat diencephalon and frontal cortex. *Naunyn Schmiedebergs Arch Pharmacol* **367**: 297–305.

Goeldner FO, Pigatto G, Ribeiro AF, Machado HB, Boerngen-Lacerda R (2005). Influence of fluoxetine and paroxetine in behavioral sensitization induced by ethanol in mice. *Pharmacol Biochem Behav* **82**: 388–396.

Grimm JW, Lu L, Hayashi T, Hope BT, Su TP, Shaham Y (2003). Time-dependent increases in brain-derived neurotrophic factor protein levels within the mesolimbic dopamine system after withdrawal from cocaine: implications for incubation of cocaine craving. *J Neurosci* **23**: 742–747.

Hara J, Beuckmann CT, Nambu T, Willie JT, Chemelli RM, Sinton CM *et al* (2001). Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity. *Neuron* **30**: 345–354.

Hiemke C, Hartter S (2000). Pharmacokinetics of selective 5-HT reuptake inhibitors. *Pharmacol Ther* **85**: 11–28.

King GR, Xiong Z, Ellinwood Jr EH (1998). Blockade of the expression of sensitization and tolerance by ondansetron, a 5-HT_3 receptor antagonist, administered during withdrawal from intermittent and continuous cocaine. *Psychopharmacology (Berlin)* **135**: 263–269.

King GR, Xiong Z, Douglass S, Ellinwood EH (2000). Long-term blockade of the expression of cocaine sensitization by ondansetron, a 5-HT(3) receptor antagonist. *Eur J Pharmacol* **394**: 97–101.

Kodama M, Fujioka T, Duman RS (2004). Chronic olanzapine or fluoxetine administration increases cell proliferation in hippocampus and prefrontal cortex of adult rat. *Biol Psychiatry* **56**: 570–580.

Le Foll B, Diaz J, Sokoloff P (2005). A single cocaine exposure increases BDNF and D3 receptor expression: implications for drug-conditioning. *NeuroReport* **16**: 175–178.

Li Y, White FJ, Wolf ME (2000). Pharmacological reversal of behavioral and cellular indices of cocaine sensitization in the rat. *Psychopharmacology* **151**: 175–183.

Lyness WH (1983). Effect of L-tryptophan pretreatment on *d*-amphetamine self administration. *Subst Alcohol Actions Misuse* **4**: 305–312.

Malberg JE, Eisch AJ, Nestler EJ, Duman RS (2000). Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus. *J Neurosci* **20**: 9104–9110.

Malberg JE, Duman RS (2003). Cell proliferation in adult hippocampus is decreased by inescapable stress: reversal by fluoxetine treatment. *Neuropsychopharmacology* **28**: 1562–1571.

Masuo Y, Noguchi J, Morita S, Matsumoto Y (1995). Effects of intracerebroventricular administration of pituitary adenylate cyclase-activating polypeptide (PACAP) on the motor activity and reserpine-induced hypothermia in murines. *Brain Res* **700**: 219–226.

Meredith GE, Callen S, Scheuer DA (2002). Brain-derived neurotrophic factor expression is increased in the rat amygdala, piriform cortex and hypothalamus following repeated amphetamine administration. *Brain Res* **949**: 218–227.

Nishikawa T, Mataga N, Takashima M, Toru M (1983). Behavioral sensitization and relative hyperresponsiveness of striatal andlimbic dopaminergic neurons after repeated methamphetamine treatment. *Eur J Pharmacol* **88**: 195–203.

Parsons LH, Koob GF, Weiss F (1995). 5-HT dysfunction in the nucleus accumbens of rats during withdrawal after unlimited access to intravenous cocaine. *J Pharmacol Exp Ther* **274**: 1182–1191.

Poyurovsky M, Isakov V, Hromnikov S, Modai I, Rauchberger B, Schneidman M *et al* (1999). Fluvoxamine treatment of obsessive-compulsive symptoms in schizophrenic patients: an add-on open study. *Int Clin Psychopharmacol* **14**: 95–100.

Richardson NR, Roberts DC (1991). Fluoxetine pretreatment reduces breaking points on a progressive ratio schedule reinforced by intravenous cocaine self-administration in the rat. *Life Sci* **49**: 833–840.

Robinson TE, Becker JB (1986). Enduring changes in brain and behavior produced by chronic amphetamine administration: a review and evaluation of animal models of amphetamine psychosis. *Brain Res* **396**: 157–198.

Rocha BA, Scearce-Levie K, Lucas JJ, Hiroi N, Castanon N, Crabbe JC *et al* (1998). Increased vulnerability to cocaine in mice lacking the serotonin-1B receptor. *Nature* **393**: 175–178.

Segal DS, Janowsky DS (1978). Psychostimulant-induced behavioral effects: possible models of schizophrenia. In: Lipton MA, DiMascio A, Killam KF (eds). *Psychopharmacology: A Generation of Progress*. Raven Press: New York. pp 1113–1123.

Shen H, Hagino Y, Kobayashi H, Numachi Y, Yamamoto H, Yamamoto T *et al* (2003). Methamphetamine sensitization in dopamine (DAT) and/or 5-HT (SERT) transporter knockout mice. *The Society for Neuroscience 33rd Annual Meeting*, New Orleans, USA [2003/11/8-14].

Sills TL, Greenshaw AJ, Baker GB, Fletcher PJ (2000). Subchronic fluoxetine treatment induces a transient potentiation of amphetamine-induced hyperlocomotion: possible pharmacokinetic interaction. *Behav Pharmacol* **11**: 109–116.

Silver H, Shmugliakov N (1998). Augmentation with fluvoxamine but not maprotiline improves negative symptoms in treated schizophrenia: evidence for a specific serotonergic effect from a double-blind study. *J Clin Psychopharmacol* **18**: 208–211.

Silver H (2004). Selective 5-HT re-uptake inhibitor augmentation in the treatment of negative symptoms of schizophrenia. *Expert Opin Pharmacother* **5**: 2053–2058.

Snyder SH (1973). Amphetamine psychosis: a 'model' schizophrenia mediated by catecholamines. *Am J Psychiatry* **130**: 61–67.

Sodhi MS, Sanders-Bush E (2004). 5-HT and brain development. *Int Rev Neurobiol* **59**: 111–174.

Takamatsu Y, Yamamoto H, Hagino Y, Markou A, Ikeda K (2005). Fluoxetine as a potential pharmacotherapy for methamphetamine dependence. *Ann NY Acad Sci* (in press).

Tomiyama M, Kimura T, Maeda T, Kannari K, Matsunaga M, Baba M (2005). A serotonin 5-HT_{1A} receptor agonist prevents behavioral sensitization to L-DOPA in a rodent model of Parkinson's disease. *Neurosci Res* **52**: 185–194.

Ujike H, Sato M (2004). Clinical features of sensitization to methamphetamine observed in patients with methamphetamine dependence and psychosis. *Ann NY Acad Sci* **1025**: 279–287.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berlin)* **151**: 99–120.

Yamaguchi M, Suzuki T, Seki T, Namba T, Juan R, Arai H *et al* (2004). Repetitive cocaine administration decreases neurogenesis in adult rat hippocampus. *Ann NY Acad Sci* **1025**: 351–362.

Yan QS, Yan SE (2001). Activation of 5-HT(1B/1D) receptors in the mesolimbic dopamine system increases dopamine release from the nucleus accumbens: a microdialysis study. *Eur J Pharmacol* **418**: 55–64.

Yan QS, Zheng SZ, Yan SE (2004). Involvement of 5-HT_{1B} receptors within the ventral tegmental area in regulation of mesolimbic dopaminergic neuronal activity via GABA mechanisms: a study with dual-probe microdialysis. *Brain Res* **1021**: 82–91.

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ARTICLE

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Effects of schizophrenomimetics on the expression of the *CCN1* (*CYR 61*) gene encoding a matricellular protein in the infant and adult neocortex of the mouse and rat

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Abstract

The acute systemic administration of a schizophrenomimetic phencyclidine [5 or 10 mg/kg, subcutaneously (s.c.)] markedly up-regulated the neocortical expression of the *CCN1* gene encoding a secreted extracellular matrix-associated protein at postnatal day 56, but not at postnatal day 8, after 60 min in the mouse and rat. The development-dependent nature of the up-regulation between postnatal days 8 and 56 seems to be similar to that of the adult type phencyclidine-induced abnormal behaviours, which have been considered to be models of schizophrenic symptoms. In the young adult rat, 5, 10, and 20 mg/kg phencyclidine (given s.c.) induced an increase in the *CCN1* gene transcripts in a dose-related and bell-shaped manner with a maximum at the dose of 10 mg/kg, 60 min post-injection. Other schizophrenomimetics, dizocilpine (1 mg/kg) and methamphetamine (4.8 mg/kg), also caused a prominent up-regulation of the neocortical expression of the *CCN1* gene in adult rats. These results indicate that the *CCN1* gene or protein could be implicated in a molecular cascade associated with the age-dependent onset of schizophrenia that usually occurs after puberty.

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Key words: *CCN1* gene expression, dizocilpine, methamphetamine, neocortex phencyclidine, postnatal development.

Introduction

It has been well established that phencyclidine [1(1-phenylcyclohexyl)-piperidine; PCP] and other *N*-methyl-D-aspartate (NMDA) receptor antagonists cause schizophrenia-like psychosis consisting of both positive and negative symptoms (Javitt and Zukin, 1991). This psychotomimetic action may be explained by the plausibility that NMDA antagonists disturb the molecular and cellular equipment in an information-processing system or neuron circuit that is involved in the control of certain essential mental functions and specifically malfunctioned in schizophrenia (Sato et al., 1997). Although the exact causative mechanism for schizophrenia is still unclear, the pathophysiological changes in the information-processing system might be associated with the development-dependent nature of schizophrenia and NMDA antagonist-induced psychosis (Sato et al., 1997). Thus, the onset of schizophrenia typically occurs after adolescence (APA, 2000). A chemically PCP-related and non-competitive antagonist for the NMDA receptor, ketamine, has been observed to often produce psychotic symptoms in adults, but not in children (Reich and Silvay, 1989; White et al., 1982). In experimental animals, substantial changes have been reported in phencyclidine- or dizocilpine hydrogen maleate [(+)-MK-801; (+)-5-methyl-10, 11-dihydroxy-5H-dibenzo-(a,d) cyclohepten-5,10-imine hydrogen maleate] induced abnormal behaviour as a model of schizophrenic symptoms during postnatal development (Sato et al., 1997; Scalzo and Burge, 1994). Moreover, repeated treatment with PCP during the adult, but not infant, period augments the ability of a subsequent

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challenge of either of the NMDA antagonists to elicit abnormal behaviour (Scalzo and Holson, 1992).

These developmental features suggest that the schizophrenia-related information-processing system might reach maturity around adolescence in humans or during a critical period of the postnatal development in experimental animals, and could not play a pivotal role in the regulation of mental functions or behaviours before these climacteric periods. The hypothetical human system and its animal homologue should contain molecules that are responsive to NMDA antagonists only after adolescence and the critical period for the animal model of schizophrenia. To explore the candidates for the schizophrenia-associated and developmentally regulated molecules we have compared, in mice and rats, the effects of PCP on gene expression in the neocortex between the infant and young adult period using a DNA microarray and RT–PCR technique. The neocortex was chosen for this series of experiments because we previously observed the most marked postnatal changes before and after the critical period in PCP-induced abnormal activities as revealed by the *c-fos* gene in the cortical area of the rat (Sato et al., 1997).

Materials and methods

Animals and chemicals

The present animal experiments were performed in strict accordance with the guidelines of the Tokyo Medical and Dental University, and were approved by the Animal Investigation Committee of the university. Male C57BL mice (C57BL/N strain, Clea Japan Inc., Tokyo, Japan) at postnatal day (PD) 8 (3.5–5.0 g) and PD 56 (22–27 g) and male Wistar albino rats (ST strain, Clea Japan) at PD 8 (16–20 g) and PD 56 (200–230 g) were used. The animals were housed at 22.0 ± 2 °C in a humidity-controlled room under a 12-h light/dark cycle and had free access to food and water. PCP hydrochloride was kindly synthesized and donated by Yamanouchi Pharmaceutical Co. Ltd (Tsukuba, Japan). All other chemicals were of ultrapure quality and commercially available. PCP hydrochloride and dizocilpine hydrogen maleate were dissolved in physiological saline for subcutaneous (s.c.) injection. Doses always refer to the free bases.

Screening procedures of the candidates for developmentally regulated and PCP-responsive molecules

Gene expression analysis using a DNA microarray system [Mouse Gene Expression Microarray (GEM) 1, version 1.0 (Incyte Genomics, Palo Alto, CA, USA)], was performed with mice as the first step to screen the developmentally regulated and PCP-responsive genes despite the fact that our series of animal experiments concerning schizophrenomimetics and development had been done using rats (Kajii et al., 2003; Sato et al., 1997). Mice were used because (1) no ready-made DNA array system for rat tissues was available at the starting point of the present study, and (2) the acute PCP-treated mouse is also considered to be a pharmacological model of schizophrenia. From the developmental point of view, the validity of the mouse experiments appears to be supported by our observations that the systemic administration of PCP caused different behavioural changes between infant (PD 8) and young adult (PD 56) mice as seen in rats (Sato et al., 1997). Based upon the results of the DNA microarray, we picked up the genes markedly altered by PCP at PD 56 in the neocortex, and further selected the transcript that represented the most prominent response to the acute PCP injection at PD 56 with the minimal change at PD 8. Using a semi-quantitative RT–PCR technique, we subsequently studied the effects of PCP on the expression of the selected gene transcript in the neocortex of the rat at PD 56 and/or PD 8 to compare the present findings to our previous biochemical and behavioural data obtained from PCP-treated rats. The difference between the basal and PCP-induced expression of the target transcript in the adult rat was also verified by Northern blot analysis. The doses of 5 and 10 mg/kg were chosen for the screening procedures of the developmentally regulated PCP-responsive genes, because in studies PCP has generally been injected at doses ranging from 5 to 10 mg/kg owing to its acute psychotomimetic action in rodents (Tanii et al., 1994; Toth and Lajtha, 1986).

Tissue and total RNA preparation

Mice and rats (PD 8 or PD 56) were killed by cervical dislocation 1 h after s.c. administration of PCP, dizocilpine or saline. The neocortex (the dorsal part of the cerebral cortex divided along the rhinal fissure) was rapidly dissected out in the cold, frozen in liquid nitrogen, and stored at −80 °C until use.

The total RNA was prepared from the mouse and rat neocortex using an RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany). The RNA quality was verified by gel electrophoresis (Agilent Bioanalyzer; Agilent Technologies, Palo Alto, CA, USA).

DNA microarray

For the DNA array analysis, equal amounts of the total RNA of the individual mice from every experimental group (10 saline-treated and 10 PCP-treated infant mice, and 6 saline- and 6 PCP-treated adult mice) were pooled. The poly(A) RNA isolated from the pooled total RNA was reverse-transcribed with 5-Cy3-labelled (the saline-treated sample) or Cy5-labelled (the PCP-treated sample) random 9-mers (Operon Technologies Inc., Alameda, CA, USA). The labelled cDNA was applied to the DNA microarray [Mouse GEM 1, version 1.0 (Incyte Genomics); Yue et al., 2001] and was hybridized at 60 °C for 6.5 h. After washing, the microarray was scanned on a microarray scanner (GenePix™ scanner, Axon Instruments, Foster City, CA) with 10 μm resolution. The image was analysed using specialized Incyte GEMtools™ software (Incyte Pharmaceuticals). The elements were determined by a gridding and region detection algorithm. The area surrounding each element image was used to calculate the local background and then subtracted from the total element signal. The background subtracted element signals were used to calculate the Cy3:C5 ratios. The average of the resulting total Cy3 and Cy5 signals gives the ratio. The expression of a gene on a specific spot was considered relevant if the signal intensity was greater than twice the respective background.

Semi-quantitative RT–PCR

For the semi-quantitative RT–PCR, reverse transcription was performed with random hexamer primers (Superscript II; Invitrogen, Carlsbad, CA, USA) using 0.2 μg of neocortical total RNA from an individual rat, and the resulting cDNA was suspended in 10 vol. TE buffer (pH 8.0). The cDNA samples were aliquoted and stored at −80 °C.

Semi-quantitative measurements of the target RNA levels in individual samples were achieved using the LightCycler Fast Start DNA Master SYBR Green I system (Roche Diagnostics, Mannheim, Germany) (Castello et al., 2002). A 5-μl aliquot of the cDNA suspension was added to a master mixture containing the dNTP mix, *Taq* DNA polymerase, SYBR Green I dye, 3 mM $MgCl_2$, and 0.5 μM of each primer in a capillary. Expression of the 307-bp segment in rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was determined as an internal standard for each sample because the *GAPDH* gene has been reported to be a housekeeping gene that is constantly expressed in neural tissues (Thellin et al., 1999). In each assay, standard curves were generated from four increasing amounts of the pooled neocortical cDNA templates of equal volumes of the individual samples. The primer sequences are as follows: *CCN1*, 5′-AGTGCCGCCTGGTGAAAGAGA-3′ and 5′-CACGCAGGAGCCGCAGTATTT-3′; GAPDH, 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCCACC-ACCCTGTTGCTGTA-3′. All the cDNA templates were amplified according to the LightCycler protocol. The FastStart polymerase was activated and the cDNAs were denatured by pre-incubation for 10 min at 95 °C. Subsequently, the templates were amplified for 40 cycles of denaturation for 15 s at 95 °C, annealing of the primers at 65 °C for 5 s, and extension at 72 °C for 10 s. The fluorescent intensity data were obtained during each extension phase. After the amplification procedures, melting curve analysis was done by heating the product to 95 °C at the rate of 20 °C/s, cooling it to 65 °C at 20 °C/s, and then slowly heating it to 95 °C at 0.1 °C/s. The resultant melting curve was used to determine the specificity of the PCR product. The relative amounts of *CCN1* and GAPDH transcripts of each sample were automatically calculated using the respective standard curves of LightCycler analysis software version 3.5. The ratios of the relative amounts of the *CCN1* mRNAs to those of the GAPDH mRNAs were used for the statistical comparisons as the normalized values of the *CCN1* mRNA expression.

Northern blot analysis

The total RNA of rat brain tissues was isolated using an RNeasy Midi Kit (Qiagen). For Northern blot analysis, 15 μg of the total RNA from each sample was separated by electrophoresis through an agarose-formaldehyde gel [6.3% formaldehyde, 1% agarose, 1× 3-[*N*-morpholino]propanesulfonic acid (MOPS) buffer (0.4 M MOPS, 0.1 M sodium acetate, 0.01 M EDTA; pH 7.0)] and transferred by capillary elution onto a positively charged nylon membrane (Hybond N+, Amersham Bioscience, Freiburg, Germany). The total RNA was then fixed to the membrane by ultraviolet irradiation for 1 min. After prehybridization at 42 °C for 30 min in a sealed bag containing the hybridization buffer (ULTRAhyb; Ambion, Austin, TX, USA), the blotted filter was subsequently hybridized with ^{32}P-labelled cDNA probes corresponding to the 1140 bases (nucleotides 186–1325, GenBank accession no. NM031327) of the rat *CCN1* cDNA or the 609 bases (nucleotides 331–939, GenBank accession no. X02231) of the rat GAPDH cDNA at 42 °C for 16 h in the hybridization solution (Ultrahyb, Ambion). The filters were washed under high stringency conditions in 0.2× standard saline-sodium citrate (SSC)

buffer containing 0.1% (w/v) SDS (sodium dodecyl sulphate) at 68 °C for 60 min, air dried and exposed to a radio-sensitive imaging plate (Fuji Image Plate, Fujifilm Co. Ltd, Tokyo, Japan) using a scanning laser photometer. The visualized blots were quantified using a densitometric image analyser (Fuji BAS 2000 Image Analyzer).

The specificity of the present Northern blot analysis of the *CCN1* and GAPDH mRNAs was ensured by the following observations: (1) no hybridization of each sense strand probe was detected, and (2) a single band for *CCN1* or GAPDH mRNA was obtained by the hybridization of either specific antisense probe. Ratios of the optical densities of the bands of *CCN1* mRNAs to those of the respective GAPDH mRNAs in the neocortical tissues of the PCP- or saline-injected 56-d-old adult rats were calculated for statistical comparison between the two experimental groups.

We confirmed the excellent linearity between the concentration of mRNA and its signal intensity in the autoradiogram (data not shown). Optical densities were standardized to the peak optical densities in the pooled neocortical tissues of the PCP-injected 56-d-old adult rats and expressed as the ratio relative to the maximum value. The standardization was done on autoradiograms of filters that were hybridized in the same reaction mixture and exposed to the same plate.

Statistics

For comparison between the two groups, the statistical significance of the data was evaluated using the two-tailed Student's (homogeneous variance) or Cochran–Cox (heterogeneous variance) t test. Statistical differences among more than three groups were estimated by a one-way analysis of variance (ANOVA) or Kruskal–Wallis test followed by the Scheffé post-hoc test. The developmental differences in the effects of PCP were examined by a two-way ANOVA followed by the Scheffé post-hoc test.

Results

Detection of CCN1 *mRNA as a developmentally regulated PCP-responsive transcript by a DNA microarray method and RT–PCR analysis*

From the DNA microarray data of the expressed signal intensities of the 8374 clones following PCP or saline treatment in infant and adult mice, 'the up-regulation index' or 'the down-regulation index' for each gene was calculated as the ratio of the signal after the PCP treatment to that after the saline treatment with a plus value or the ratio of the signal after the saline treatment to that after the PCP treatment with a minus value, respectively. We selected genes that showed in adult animals 'the up-regulation indices' and 'the down-regulation indices' more than the absolute value of 1.5 (Table 1), and then identified the gene transcript that represented the largest absolute value of either index with little alteration by PCP at PD 8.

The filtering procedure for the candidate gene using the mouse DNA array system indicated that the acute systemic administration of PCP (10 mg/kg s.c.) produced a marked and minimal up-regulation of connective tissue growth factor/cysteine-rich 61/nephroblastoma overexpressed [*CCN1* = cysteine-rich protein 61 (*CYR61*)] mRNA expression in the 56- and 8-d-old mice, respectively (Table 1). These contrasting responses to PCP between the two developmental stages were semi-quantitatively verified by the real-time RT–PCR method in rats. As shown in Figure 1(b), the PCP treatment (5 mg/kg s.c.) increased the ratios of the levels of *CCN1* mRNA to those of GAPDH by four times compared to the saline-treated controls in adult rats [mean ± S.E.M. of *CCN1*:GAPDH signal-intensity ratios (% of control animals): saline 100 ± 11%, PCP 398 ± 11**, ** $p < 0.01$ vs. saline-treated controls], but only by 1.4 times in the infant rats (saline 100 ± 7%, PCP 140 ± 10, $p > 0.05$ vs. saline-treated controls) [postnatal days × PCP treatment effect, $F(1, 16) = 40.813$, $p < 0.0001$].

In Northern blot analysis of the total RNA fraction prepared from the adult rat neocortex, a specific cDNA probe for the *CCN1* or GAPDH transcripts detected a single band of 2.4 kb (Figure 2a) or 2.0 kb, respectively. The size of the band of the *CCN1* transcripts coincided with that previously reported (Albrecht et al., 2000). Quantification of the signal intensity of the bands showed that PCP injection (10 mg/kg s.c.) caused a marked increase in *CCN1* mRNA expression [mean ± S.E.M. of *CCN1*:GAPDH signal-intensity ratios (% of control animals): saline 100 ± 1%, PCP 317 ± 4%**, ** $p < 0.01$ vs. saline-treated controls] in the adult rat neocortex (Figure 2b). These observations agreed with both the results of the cDNA microarray and real-time RT–PCR assay. Thus, we routinely quantified the *CCN1* and GAPDH mRNAs in the present experiments by real time RT–PCR assay using the primer sets indicated in the Materials and methods section.

Effects of the acute systemic injection of PCP at various doses on expression of CCN1 *transcripts in the neocortex of adult rats*

Acute subcutaneous administration of 5, 10 and 20 mg/kg PCP produced a dose-dependent increase

Table 1. Screening of developmentally regulated PCP-responsive transcripts in the mouse neocortex by DNA microarray

		Adult (signal intensity)			Infant (signal intensity)		
Criteria	Gene	Sal	PCP	PCP/Sal	Sal	PCP	PCP/Sal
Up-regulation index: PCP/Sal $\geqslant 1.5$	Connective tissue growth factor/cysteine-rich 61/ nephromablastoma overexpressed (CCN1 = cysteine-rich protein 61)	358	798	2.2	372	390	1.0
	ESTs, moderately similar to protein phosphatase 2C alpha isoforms (*Mus musculus*)	629	1265	2.0	313	763	2.4
	Transthyretin	2014	3561	1.8	186	192	1.0
	ESTs	3574	6018	1.7	3879	5851	1.5
	Haemoglobin, beta adult major chain	2034	3076	1.5	1805	2274	1.3
Down-regulation index Sal/PCP $\leqslant -1.5$	Leukotriene A4 hydrolase	1191	748	−1.6	444	498	−1.1
	Transferrin receptor	2145	1328	−1.6	1829	1325	−1.4
	ESTs, weakly similar to NTC-3_Mouse neurogenic locus Notch 3 protein (*Mus musculus*)	692	463	−1.5	672	477	−1.4
	GATA-binding protein 2	1781	1176	−1.5	1227	983	−1.2
	Cathepsin E	1824	1194	−1.5	1653	1189	−1.4

Sal, Saline; PCP, phencyclidine; ESTs, expressed sequence tag.
The neocortical expression of the 8374 clomes was analysed by the DNA microarray system [Mouse Gene Expression Microarray 1, version 1 (Incyte Genomics)] in the young adult (PD 56) and infant (PD 8) mice 60 min after acute s.c. administration of PCP (10 mg/kg) or saline. The up-regulation index or the down-regulation index were calculated as the ratio of the relative expressed signal intensity after the PCP treatment to that after the saline treatment with a plus sign and as the ratio of the relative expressed signal intensity after the saline treatment to that after the PCP treatment with a minus sign, respectively. We selected the genes that showed in the adult animals 'the up-regulation indices' and 'the down-regulation indices' more than the absolute value of 1.5, and then identified the gene transcript that represented the largest absolute value of either index with little alteration by PCP at PD 8.

in neocortical levels of the *CCN1* transcripts up to 10 mg/kg. However, 20 mg/kg PCP up-regulated *CCN1* gene expression, but induced lower *CCN1*: GAPDH mRNA ratios than 10 mg/kg PCP in the neocortex (Figure 3). These observations suggest that a bell-shaped dose-related augmentation of the neocortical *CCN1* gene expression might be caused by the systemic injection of PCP at doses ranging from 5 to 20 mg/kg.

Effects of acute systemic injection of various psychotomimetic drugs on expression of CCN1 *transcripts in the neocortex of adult rats*

Because *CCN1* has been detected as a novel candidate for a schizophrenic symptoms-related gene in the present study, we also assessed the effects of different psychotomimetic drugs including PCP (7.5 mg/kg s.c.), dizocilpine (1 mg/kg s.c.) and methamphetamine (4.8 mg/kg s.c.) on neocortical *CCN1* mRNA expression at doses that elicit robust abnormal behaviours such as hyperlocomotion and stereotypy. These drug treatments markedly up-regulated the relative amounts of the *CCN1* transcripts in the adult rat neocortex (Figure 4) (% of control animals: saline $100 \pm 7\%$, PCP 452 ± 5**, dizocilpine $396 \pm 9\%$**, methamphetamine $564 \pm 9\%$**, ** $p < 0.01$ vs. saline-treated controls).

Discussion

Using a DNA microarray technique, we revealed that *CCN1* is a developmentally regulated and

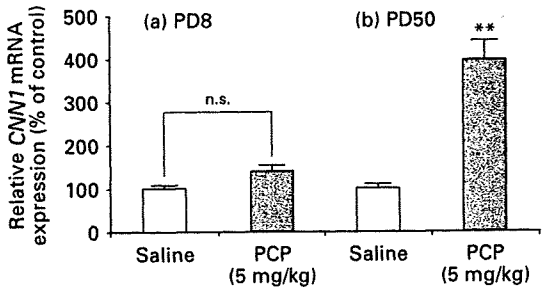

Figure 1. Detection of *CCN1* mRNA as a developmentally regulated PCP-responsive transcript by RT–PCR analysis. Differential effects of PCP on *CCN1* gene expression in the neocortex of developing rats were found. Marked and minimal increases in *CCN1* mRNA expression following an acute administration of PCP (5 mg/kg s.c.) were seen in the neocortex of (b) the adult (PD 56) and (a) the infant (PD 8) rat, respectively. Results are means with S.E.M. of data (*CCN1*:GAPDH mRNA ratio) 60 min after acute systemic injection of PCP (5 mg/kg), or saline, and are expressed as a percentage of the values of the respective saline-treated 56-d-old controls. The data were obtained by the real-time RT–PCR method and are expressed as a percentage of the values of the respective saline-treated controls. ** $p<0.01$ vs. respective saline-treated controls. n.s., No significant difference ($n=5$).

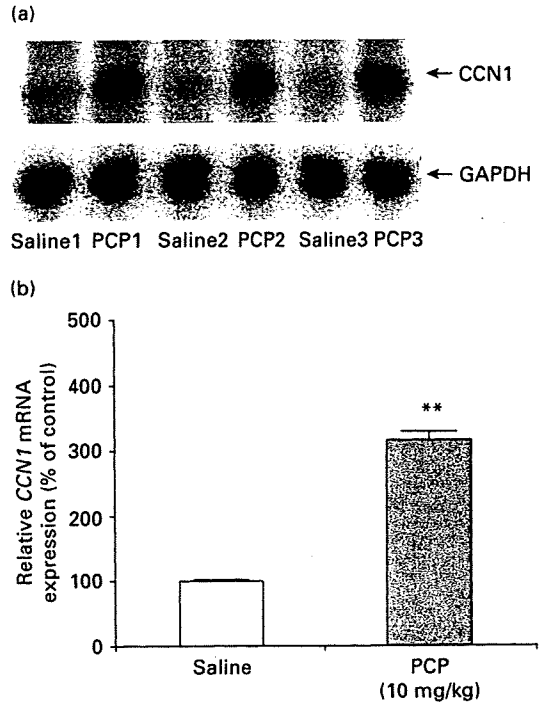

Figure 2. Northern blot analysis of the effects of acute systemic injection of PCP on expression of *CCN1* transcripts in the neocortex of adult rats. (a) Northern blot analyses of total RNAs (15 μg/lane) from the neocortex of the adult rat treated with saline or PCP. (a) The top bands show hybridization with a selective probe to the *CCN1* mRNA against the sequence of the nucleotides 186–1325 recognized a single band in each lane containing the neocortical total RNAs prepared from the adult rats treated with PCP (PCP: 10 mg/kg s.c.) or saline. The bottom bands indicate the results of the Northern hybridization with the specific ^{32}P-labelled antisense probe to the GAPDH mRNA. (b) Results are means with S.E.M. of data (*CCN1*:GAPDH mRNA ratio) 60 min after the acute systemic injection of PCP (10 mg/kg s.c.), or saline, and are expressed as a percentage of the values of the saline-treated 56-d-old controls. ** $p<0.01$ vs. saline-treated controls ($n=3$).

PCP-inducible gene in the rat neocortex in that the schizophrenomimetic PCP causes a slight and prominent up-regulation of neocortical *CCN1* gene expression in the infant and young adult rat, respectively. Moreover, the amounts of *CCN1* transcripts are increased in the neocortex of the adult animals following acute systemic administration of dizocilpine and methamphetamine that are capable of eliciting schizophrenia-like symptoms.

This is the first report to show the profound influence of psychotomimetics on brain mRNA expression of the *CCN1* gene. *CCN1* was originally identified in both mouse 3T3 fibroblasts (O'Brien et al., 1990) and human umbilical vein endothelial cells (Kolesnikova and Lau, 1998), and *CCN1* mRNAs are rapidly and transiently induced by serum and serum growth factors. Relatively low levels of basal expression of the neocortical *CCN1* gene agreed with those found in previous studies by other research groups (Albrecht et al., 2000; Chung and Ahn, 1998). Further, marked responses as early as 1 h after psychotomimetic injection appear to fit with the immediate early gene property of *CCN1* (Rachfal and Brigstock, 2005).

The altered expression of the neocortical *CCN1* gene by PCP may be attributed to the potent antagonist property of the drug against the NMDA receptor. This assumption is supported by the fact that a selective non-competitive antagonist of the glutamate receptor, dizocilpine, causes a similar up-regulation of *CCN1* expression in the neocortex. Alternatively, the increasing effects of the three schizophrenomimetics on *CCN1* transcripts may be mediated by the cortical dopaminergic systems because these drugs have the common feature of overactivating dopaminergic transmission in the neocortex. Thus, PCP and dizocilpine have been reported to augment extracellular dopamine release and dopamine turnover by blocking the NMDA receptor in the medial frontal

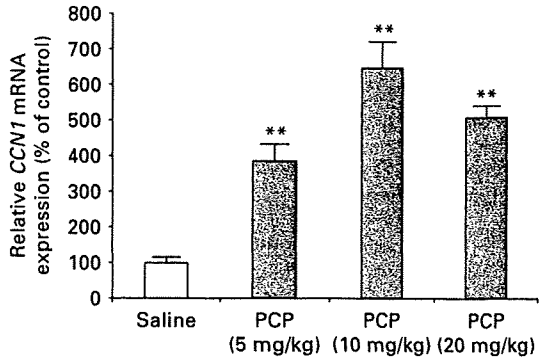

Figure 3. Effects of the acute systemic injection of PCP at various doses on expression of *CCN1* transcripts in the neocortex of the adult rats as revealed by quantitative RT–PCR analysis. Results are means with S.E.M. of data (*CCN1*:GAPDH mRNA ratio) 60 min after the acute systemic injection of different doses of PCP (5, 10, and 20 mg/kg s.c.) or saline and are expressed as a percentage of the values of the respective saline-treated 56-d-old controls. ** $p<0.01$ vs. saline-treated controls ($n=5$).

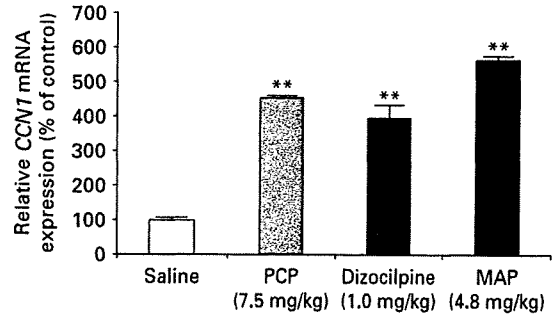

Figure 4. Effects of the acute systemic injection of various schizophrenomimetic drugs on expression of *CCN1* transcripts in the neocortex of the adult rats as revealed by qua ntitative RT–PCR analysis. Results are means with S.E.M. of data (*CCN1*:GAPDH mRNA ratio) 60 min after the acute systemic injection of PCP (7.5 mg/kg s.c., $n=10$), dizocilpine (1 mg/kg s.c., $n=6$), methamphetamine (4.8 mg/kg s.c., $n=10$) or saline ($n=10$) and are expressed as a percentage of the values of the respective saline-treated 56-d-old controls. ** $p<0.01$ vs. saline-treated controls ($n=6$–10).

cortex (Deutch et al., 1987; Kashiwa et al., 1995; Nishijima et al., 1994, 1996; Umino et al., 1998). Moreover, methamphetamine and other amphetamines have been well documented as elevating synaptic dopamine levels in the cortical and subcortical brain areas by facilitating dopamine release and inhibiting dopamine reuptake (see Nishijima et al., 1994). It cannot be excluded that NMDA receptor blocking by PCP and dizocilpine and the elevation of dopamine signals by methamphetamine independently elicit neocortical *CCN1* induction. Another immediate early gene, c-*fos*, has indeed been found to be up-regulated in a dopamine-dependent and a dopamine-independent NMDA receptor-mediated manner in the different regions of the neocortex (Sato et al., 1997; Umino et al., 1995). The stimulation of the muscarinic acetylcholine receptor has been found to enhance *CCN1* gene expression in the brain (Albrecht et al., 2000; Chung and Ahn, 1998). However, low or no substantial affinities of PCP, dizocilpine or methamphetamine for the cholinergic receptor (Javitt and Zukin, 1991) suggest that this mechanism does not seem to underlie the up-regulation of the cortical *CCN1* gene by these psychoactive agents in the present study.

Based upon the above pharmacological properties of *CCN1* induction, we can postulate that the marked developmental changes in the magnitudes of the PCP-induced increase in *CCN1* mRNA could be attributed to the neuroanatomical and functional development of the NMDA receptor subunits (Watanabe et al., 1992) and/or the cortical dopmainergic systems (Perez-Navarro et al., 1993). Whatever the neurotransmitter systems, the distinct responses to PCP between the infant and adult period indicate the postnatal maturation of PCP-responsive information processing in the brain. This maturation may be represented by the late developing nature of an adult type of PCP-induced acute behavioural changes (Sato et al., 1997) and long-lasting behavioural sensitization (Scalzo and Holson, 1992) that have been accepted as models of schizophrenia. Therefore, the PCP-responsive information-processing system might be disturbed in NMDA antagonist-induced schizophrenia-like psychosis and schizophrenia, and *CCN1* could be a member of a molecular cascade in the developmentally regulated system(s).

The functional consequences and the pathophysiological significance of the schizophrenomimetic-induced changes in cortical *CCN1* expression are still unclear. As the up-regulation of *CCN1* gene expression by acetylcholine agonists and various growth factors has been observed in neurons in the brain tissues and immortalized neuronal hippocampal cell line H19-7 (Albrecht et al., 2000; Chung and Ahn, 1998, Rachfal and Brigstock, 2005), it is probable that the schzophrenomimetic induction of *CCN1* transcripts may occur in the neurons and/or glial cells included in a specific neural system(s). The *CCN1* gene is a member of the CCN family comprising CCN1–6

and has been shown to encode a modular, secretory and heparin-binding protein that includes structural domains common to the extracellular matrix proteins (Rachfal and Brigstock, 2005). Evidence has now been accumulated indicating that *CCN1* binds to $\alpha_6\beta_1$, $\alpha_v\beta_2$, $\alpha_{IIb}\beta_3$, $\alpha_M\beta_2$ and $\alpha_v\beta_5$ integrins in a variety of cell types and may participate in the regulation of diverse cell functions such as mitogenesis, adhesion, extracellular matrix production, migration and proliferation (Kireeva et al., 1996; Rachfal and Brigstock, 2005). These phenomena, together with the known functions of brain integrins in neuronal differentiation, migration, neurite guidance and long-term potentiation and the role of *CCN1* during neuronal differentiation (Rachfal and Brigstock, 2005), suggest that up-regulation of *CCN1* could be involved in the possible synaptic rearrangements following the use of psychotomimetics which have been considered to lead to enduring changes in behavioural or psychological responses to these drugs or stress (see Vanderschuren and Kalivas, 2000, for a review). From this point of view, it is of interest to note that another secreted extracellular matrix-associated protein, the tissue type plasminogen activator, has been shown to be up-regulated by PCP and methamphetamine (Hashimoto et al., 1998) and is required for the development of a long-lasting drug-craving behaviour (Nagai et al., 2004). It is also possible that overproduction of *CCN1* transcripts might be connected to brain cell damage caused by NMDA antagonists (Olney et al., 1989) and amphetamines (McCann and Ricaurte, 2004), because *CCN1*-mediated neuronal cell death has been found in H19-7 immortalized hippocampal cells (Kim et al., 2003).

In conclusion, the present findings demonstrate that *CCN1* gene expression is markedly up-regulated by schizophrenomimetics, PCP, dizocilpine and methamphetamine, in the adult rat neocortex while PCP causes no significant change in the levels of neocortical *CCN1* gene transcripts in the infant rat. The schizophrenomimetic-inducible nature and the development-dependent up-regulation across the critical period for the adult type of PCP-induced abnormal behaviours that is thought to be a model of schizophrenia suggest that the secreted extracellular matrix-associated protein *CCN1* is a candidate molecule that could be involved in the molecular basis of the vulnerability or pathophysiology of schizophrenia.

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Statement of Interest

None.

References

Albrecht C, von Der Kammer H, Mayhaus M, Klaudiny J, Schweizer M, Nitsch RM (2000). Muscarinic acetylcholine receptors induce the expression of the immediate early growth regulatory gene CYR61. *Journal of Biological Chemistry 275*, 28929–28936.

APA (2000). Schizophrenia and other psychotic disorders. In: *Diagnostic and Statistical Manual of Mental Disorders* (4th edn) (pp. 297–343). Washington, DC: American Psychiatric Association.

Castello R, Estelles A, Vazquez C, Falco C, Espana F, Almenar SM, Fuster C, Aznar J (2002). Quantitative real-time reverse transcription-PCR assay for urokinase plasminogen activator, plasminogen activator inhibitor type 1, and tissue metalloproteinase inhibitor type 1 gene expressions in primary breast cancer. *Clinical Chemistry 48*, 1288–1295.

Chung KC, Ahn YS (1998). Expression of immediate early gene cyr61 during the differentiation of immortalized embryonic hippocampal neuronal cells. *Neuroscience Letters 255*, 155–158.

Deutch AY, Tam SY, Freeman AS, Bowers Jr. MB, Roth RH (1987). Mesolimbic and mesocortical dopamine activation induced by phencyclidine: contrasting pattern to striatal response. *European Journal of Pharmacology 134*, 257–264.

Hashimoto T, Kajii Y, Nishikawa T (1998). Psychotomimetic-induction of tissue plasminogen activator mRNA in corticostriatal neurons in rat brain. *European Journal of Pharmacology 10*, 3387–3399.

Javitt DC, Zukin SR (1991). Recent advances in the phencyclidine model of schizophrenia. *American Journal of Psychiatry 148*, 1301–1308.

Kajii Y, Muraoka S, Hiraoka S, Fujiyama K, Umino A, Nishikawa T (2003). A developmentally regulated and psychostimulant-inducible novel rat gene mrt1 encoding PDZ-PX proteins isolated in the neocortex. *Molecular Psychiatry 8*, 434–444.

Kashiwa A, Nishikawa T, Nishijima K, Umino A, Takahashi K (1995). Dizocilpine (MK-801) elicits a tetrodotoxin-sensitive increase in extracellular release of dopamine in rat medial frontal cortex. *Neurochemistry International 26*, 269–279.

Kim KH, Min YK, Baik JH, Lau LF, Chaqour B, Chung KC (2003). Expression of angiogenic factor Cyr61 during neuronal cell death via the activation of c-Jun N-terminal kinase and serum response factor. *Journal of Biological Chemistry 278*, 13847–13854.

Kireeva ML, MO FE, Yang GP, Lau LF (1996). Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. *Molecular and Cellular Biology 16*, 1326–1334.

Kolesnikova TV, Lau LF (1998). Human CYR61-mediated enhancement of bFGF-induced DNA synthesis in human umbilical vein endothelial cells. *Oncogene 16*, 747–754.

McCann UD, Ricaurte GA (2004). Amphetamine neurotoxicity: accomplishments and remaining challenges. *Neuroscience and Biobehavioral Reviews 27*, 821–826.

Nagai T, Yamada K, Yoshimura M, Ishikawa K, Miyamoto Y, Hashimoto K, Noda Y, Nitta A, Nabeshima T (2004). The tissue plasminogen activator-plasmin system participates in the rewarding effect of morphine by regulating dopamine release. *Proceedings of the National Academy of Sciences USA 101*, 3650–3655.

Nishijima K, Kashiwa A, Hashimoto A, Iwama H, Umino A, Nishikawa T (1996). Differential effects of phencyclidine and methamphetamine on dopamine metabolism in rat frontal cortex and striatum as revealed by in vivo dialysis. *Synapse 22*, 304–312.

Nishijima K, Kashiwa A, Nishikawa T (1994). Preferential stimulation of extracellular release of dopamine in rat frontal cortex to striatum following competitive inhibition of the N-methyl-D-aspartate receptor. *Journal of Neurochemistry 63*, 375–378.

O'Brien TP, Yang GP, Sanders L, Lau LF (1990). Expression of cyr61, a growth factor-inducible immediate-early gene. *Molecular and Cellular Biology 10*, 3569–3577.

Olney JW, Labruyere J, Price MT (1989). Pathological changes induced in cerebrocortical neurons by phencyclidine and related drugs. *Science 244*, 1360–1362.

Perez-Navarro E, Alberch J, Marsal J (1993). Postnatal development of functional dopamine, opioid and tachykinin receptors that regulate acetylcholine release from rat neostriatal slices. Effect of 6-hydroxydopamine lesion. *International Journal of Developmental Neuroscience 11*, 701–708.

Rachfal AW, Brigstock DR (2005). Structural and functional properties of CCN proteins. *Vitamins and Hormones 70*, 69–103.

Reich DL, Silvay G (1989). Ketamine: an update on the first twenty-five years of clinical experience. *Canadian Journal of Anaesthesia 36*, 186–197.

Sato D, Umino A, Kaneda K, Takigawa M, Nishikawa T (1997). Developmental changes in distribution patterns of phencyclidine-induced c-Fos in rat forebrain. *Neuroscience Letters 239*, 21–24.

Scalzo FM, Burge LJ (1994). The role of NMDA and sigma systems in the behavioral effects of phencyclidine in preweanling rats. *Neurotoxicology 15*, 191–200.

Scalzo FM, Holson RR (1992). The ontogeny of behavioral sensitization to phencyclidine. *Neurotoxicology and Teratology 14*, 7–14.

Tanii Y, Nishikawa T, Hashimoto A, Takahashi K (1994). Stereoselective antagonism by enantiomers of alanine and serine of phencyclidine-induced hyperactivity, stereotypy and ataxia in the rat. *Journal of Pharmacology and Experimental Therapeutics 269*, 1040–1048.

Thellin O, Zorzi W, Lakaye B, De Borman B, Coumans B, Hennen G, Grisar T, Igout A, Heinen E (1999). Housekeeping genes as internal standards: use and limits. *Journal of Biotechnology 75*, 291–295.

Toth E, Lajtha A (1986). Antagonism of phencyclidine-induced hyperactivity by glycine in mice. *Neurochemical Research 11*, 393–400.

Umino A, Nishikawa T, Takahashi K (1995). Methamphetamine-induced nuclear c-Fos in rat brain regions. *Neurochemistry International 26*, 85–90.

Umino A, Takahashi K, Nishikawa T (1998). Characterization of the phencyclidine-induced increase in prefrontal cortical dopamine metabolism in the rat. *British Journal of Pharmacology 124*, 377–385.

Vanderschuren LJ, Kalivas PW (2000). Alterations in dopaminergic and glutamatergic transmission in the induction and expression of behavioral sensitization: a critical review of preclinical studies. *Psychopharmacology (Berlin) 151*, 99–120.

Watanabe M, Inoue Y, Sakimura K, Mishina M (1992). Developmental changes in distribution of NMDA receptor channel subunit mRNAs. *Neuroreport 3*, 1138–1140.

White PF, Way WL, Trevor AJ (1982). Ketamine – its pharmacology and therapeutic uses. *Anesthesiology 56*, 119–136.

Yue H, Eastman PS, Wang BB, Minor J, Doctolero MH, Nuttall RL, Stack R, Becker JW, Montgomery JR, Vainer M, Johnston R (2001). An evaluation of the performance of cDNA microarrays for detecting changes in global mRNA expression. *Nucleic Acids Research 29*, E41-1.

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Selective increase in the extracellular D-serine contents by D-cycloserine in the rat medial frontal cortex

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Abstract

A partial agonist of the *N*-methyl-D-aspartate (NMDA) receptor, D-cycloserine, acting at its glycine modulatory site, ameliorates the neuropsychiatric symptoms that are mimicked by NMDA antagonists and include cognitive disturbances, antipsychotic-resistant schizophrenic symptoms and cerebellar ataxia. To obtain a further insight into the mechanisms of the therapeutic efficacies of D-cycloserine, we investigated the effects of the systemic administration of D-cycloserine on the extracellular contents of an endogenous NMDA co-agonist, D-serine, in the medial frontal cortex of the rat using an in vivo dialysis technique. An acute intraperitoneal injection of D-cycloserine (50 and 100 mg/kg) caused an increase in extracellular concentrations of D-serine without significant effects on those of L-serine, glycine, L-glutamate, L-aspartate, L-glutamine, L-asparagine, L-alanine, L-threonin and taurine in the medial frontal cortex. The selective increase in the extracellular D-serine contents may, at least partially, be associated with the facilitating effects of D-cycloserine on the NMDA receptor functions in addition to its direct stimulation of the NMDA receptor glycine site.

Keywords: D-Cycloserine; Glycine; In vivo microdialysis; Medial frontal cortex; D-Serine; L-Serine

1. Introduction

D-Cycloserine has been clinically used as an anti-tuberculosis drug since the 1960s (Heifets, 1994). In the 1980s, this compound was found to facilitate *N*-methyl-D-aspartate (NMDA) receptor functions by acting at the glycine modulatory site of the NMDA receptor in a partial agonist manner (Danysz and Parsons, 1998). The stimulating effects of D-cycloserine on the NMDA receptor has been proposed for the treatment of various neuropsychiatric symptoms that are mimicked by the use of drugs possessing the potent blocking actions of the NMDA receptor and include memory and cognitive disturbances, schizophrenic symptoms, fear and cerebellar ataxia (Coyle et al., 2002; Danysz and Parsons, 1998; Guastella et al., 2007; Javitt, 2004; Nishikawa, 2005; Davis et al., 2006), Indeed, recent clinical studies have reported that D-cycloserine ameliorates the antipsychotic-resistant symptoms of schizophrenia (Coyle et al., 2002), cognitive dysfunctions in Alzheimer's disease (Butterfield and Pocernich, 2003), post-traumatic stress disorders (Heresco-Levy et al., 2002), and anxiety disorders (Guastella et al., 2006; Ressler et al., 2004; Hofmann et al., 2006), and the ataxic movements in patients with spinocerebellar degeneration (Ogawa et al., 2003).

The structural relationships between D-cycloserine and D-serine suggest that D-cycloserine could potentiate the NMDA receptor-mediated transmission by not only direct stimulation of the NMDA glycine site, but also influencing the metabolism of endogenous D-serine that has been considered to be an intrinsic co-agonist for the NMDA receptor. However, there has been no study on this possibility. To obtain a further insight into the mechanisms of the therapeutic efficacies of D-cycloserine, we studied the effects of D-cycloserine on the extracellular contents of D-serine in the medial frontal cortex of freely moving rats using an in vivo microdialysis technique.

2. Materials and methods

The present animal experiments were performed in strict accordance with the guidance of the Tokyo Medical and Dental University and were approved by

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the Animal Investigation Committee of the Institution. Male Wistar rats (ST strain, Clea Japan, Inc., Japan) at postnatal days 56, weighing 200–230 g, were used. The animals were housed at 23.0 ± 0.5 °C in a humidity-controlled room under a 12 h light/dark cycle and had free access to food and water. D-Cycloserine was purchased from Sigma–Aldrich, Co., Ltd. (St. Louis, USA) and dissolved in physiological saline for intraperitoneal (i.p.) injection. The control animals received only the vehicle. Other all chemicals were of ultrapure quality and commercially available.

The in vivo microdialysis was performed as previously reported with some modifications (Tanii et al., 1990; Nishijima et al., 1996; Hashimoto et al., 1995). The rats were anesthetized with pentobarbital (40 mg/kg, intraperitoneally) and mounted on a stereotaxic frame. A straight-shaped cellulose dialysis tube (3.0 mm in length, 0.16 mm internal diameter, molecular weight cutoff 50,000, EICOM Co., Ltd., Japan) was then implanted into the medial prefrontal cortex (mPFC: A +3.2 mm, V +5.2 mm, L −0.6 mm) according to the atlas of Paxinos and Watson (2005). Two days after surgery, the dialysis probe was perfused with Ringer solution (NaCl, 147 mM; KCl, 4 mM; $CaCl_2$, 1.3 mM; pH 7.3) at the flow rate of 2 μl/min in a freely moving rat. After stabilizing for at least 80 min, the dialysate samples were collected every 20 min. The consecutive three samples immediately before the D-cycloserine injection were used to determine the basal release of each amino acid, and then the Ringer solution was perfused. After termination of the experiments, the location of the dialysis probe was macroscopically verified in each case on 150-μm-thick serial coronal slices.

The collected samples were stored at −80 °C until derivatization following the addition of D-homocysteic acid (D-HCA) as the internal standard. For quantification of the amino acids by HPLC with fluorometric detection, an aliquot of each sample was derivatized with *N-tert*-butyloxycarbonyl-L-cysteine and *o*-phthaldialdehyde (OPA) for 2 min at room temperature. The derivatized sample was immediately applied to the HPLC system and then analyzed on a 4 μm (particle size) Nova-Pak C18 column (300 mm × 3.9 mm) (Waters, Japan). The column was operated at the constant flow rate of 1.0 ml/min at 35 °C. Mobile phase A was 0.1 M acetate buffer (pH 6.0) containing 10% acetonitrile and mobile phase B was the acetate buffer containing 20% acetonitrile. The separation of the amino acid derivatives was performed with a linear gradient from mobile phase A to B in 53 min. The fluorescent amino acid derivatives were detected using a Waters 2475 Multi λ fluorescence detector spectroflurometer (Waters Co., Ltd., Japan). The excitation and emission wavelengths were 344 and 443 nm, respectively.

The average concentration of each substance during the period preceding the drug treatment (three measurements were performed every 20 min) was used as the baseline control value (=100%). The baseline extracellular levels of each amino acid were spelled out in absolute concentrations in Table 1 that are not corrected for recovery from the microdialysis probe. The individual data are expressed as percentages of this baseline period. The means with S.E.M. of the results obtained from 4–8 animals were calculated using the corresponding periods. The areas under the curves (AUC) of the concentration versus time plots for the dialysate amino acids at 0–160 min post-injection were calculated and used as the overall measures of the treatment effects (Hjörth and Sharp, 1991; Matthews et al., 1990).

Statistical comparisons were performed among the control and D-cycloserine-treated groups on the AUC data of the various quantified amino acids and on the percentages of the respective baseline values at each time point. For comparison between the two groups, the statistical significance of the data was evaluated using the two-tailed Student's (homogeneous variance) or Aspin–Welch *t*-test (heterogeneous variance). Statistical differences among more than three groups were estimated by the Kruskal–Wallis test followed by a multiple comparison method, the Steel test or the Steel–Dwass' test, instead of ANOVA (analysis of variance) method, because the variance of the data was not homogeneous among the present experimental groups.

3. Results

As shown in Fig. 1, the acute intraperitoneal injection of 50 (△ and ▲) and 100 (□ and ■) mg/kg of D-cycloserine caused a relatively rapid and lasting increase in the extracellular contents of D-serine in the medial frontal cortex from 40 min to at least 160 min post-injection as compared to the saline administration (○). D-Cycloserine at the dose of 100 mg/kg (□ and ■) tended to produce a greater increase in the cortical extracellular D-serine contents than at the doses of 50 mg/kg (△ and ▲) (Fig. 1A), although the differences in the increasing effects between the two dosages were not statistically significant. In contrast to the influence on D-serine, we observed no significant change in the extracellular contents of L-serine (Fig. 1B) and glycine (Fig. 1C) in the medial frontal cortex after the systemic administration of 50 (△) and 100 (□) mg/kg of D-cycloserine. The intraperitoneal D-cycloserine injection at 100 mg/kg also failed to affect the cortical extracellular concentrations of L-glutamate, L-aspartate, L-glutamine, L-asparagine, L-threonin, L-arginine, L-alanine and taurine (data not shown).

4. Discussion

The present study is the first to demonstrate that D-cycloserine, given systemically, produces an elevation of the frontal dialysate levels of D-serine without significant effects on

Table 1
Baseline extracellular concentrations of D-serine and all other amino acids measured in the medial frontal cortex of the rat before the systemic administration of saline, 50 or 100 mg/kg of D-cycloserine

Treatment	Amino acid concentrations (μM)		
	D-Serine	L-Serine	Glycine
Saline	1.17 ± 0.13 (8)	6.30 ± 0.54 (8)	7.59 ± 0.91 (8)
D-Cycloserine (50 mg/kg)	1.10 ± 0.07 (4)	6.42 ± 1.00 (4)	6.23 ± 0.92 (4)
D-Cycloserine (100 mg/kg)	1.05 ± 0.17 (8)	7.42 ± 0.83 (8)	8.50 ± 1.36 (8)

Treatment	Amino acid concentrations (μM)							
	L-Aspartate	L-Aspargine	L-Glutamate	L-Glutamine	L-Threonine	L-Arginine	L-Alanine	Taurine
Saline	0.71 ± 0.10 (7)	1.37 ± 0.25 (7)	0.78 ± 0.19 (7)	36.11 ± 3.32 (7)	8.10 ± 1.18 (7)	3.16 ± 0.24 (6)	8.42 ± 1.68 (7)	2.77 ± 0.28 (7)
D-Cycloserine (100 mg/kg)	0.72 ± 0.06 (8)	1.62 ± 0.26 (8)	0.81 ± 0.19 (8)	36.54 ± 2.56 (8)	8.36 ± 1.33 (8)	3.27 ± 0.47 (8)	10.82 ± 2.04 (8)	2.94 ± 0.43 (8)

The average concentrations of each amino acid in the perfusates from the rat medial frontal cortex during the period preceding the treatment with saline, 50 or 100 mg/kg of D-cycloserine (three measurements were performed every 20 min) was used as the baseline value (=100%). The concentrations are not corrected for recovery from the microdialysis probe. The results represent the means with S.E.M. of the data obtained from 4–8 rats. The number of animals is shown in parentheses.

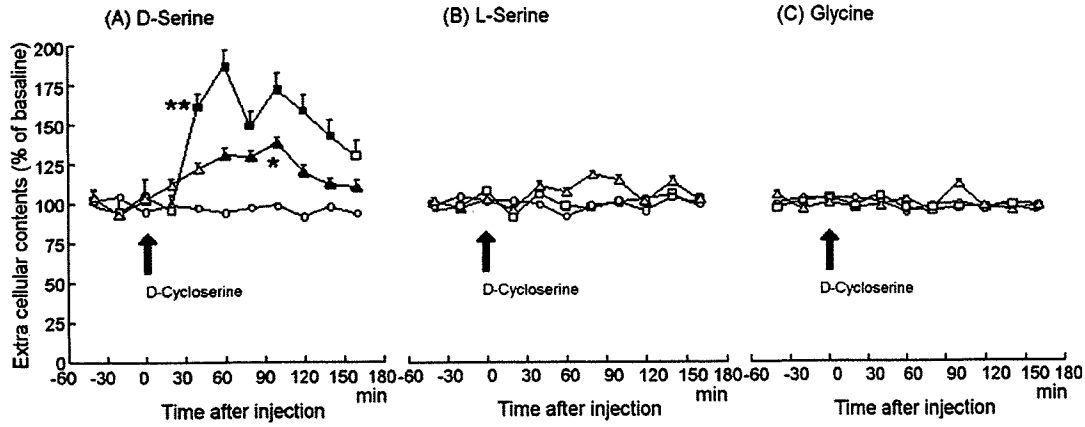

Fig. 1. Effect of the systemic administration of D-cycloserine on the basal extracellular releases of D-serine (A), L-serine (B) and glycine (C) in the medial frontal cortex of the rat. D-Cycloserine (50 and 100 mg/kg) or saline was intraperitoneally administered at the time indicated by the arrow. Results are means with S.E.M. of data obtained from 4 to 8 animals and expressed as percentages of the basal levels. The area under the curve (AUC) is calculated for an individual by adding the areas under the graph of the concentration of the respective amino acid between each pair of every 20 min consecutive observation from 20 to 160 min of the treatment. Statistical comparisons were carried out among the saline (○)- and D-cycloserine (△ (50 mg/kg) and □ (100 mg/kg))-treated groups on the AUC data and on the percentages of the respective baseline values at each time point, using the by the Kruskal–Wallis test followed by the Steel test or the Steel–Dwass' test. $*P < 0.05$, $**P < 0.01$ for the AUC data as compared to the saline-injected control animals (the Steel test). The filled symbols (▲ and ■) indicate the statistically significant differences in the time point data at the $P < 0.05$ or 0.01 as compared to the saline-treated controls (the Steel test). Neither of the data suggested the significant changes between the increasing effects of the two doses of D-cycloserine on the extracellular D-serine contents (the Steel–Dwass' test).

those of the other determined amino acids, i.e., L-serine, glycine, L-glutamate, L-aspartate, L-glutamine, L-asparagine, L-alanine, L-threonin and taurine. The selective effects seem to deny the possibility that the alterations in the D-serine contents are due to a non-specific phenomenon. These alteration profiles of the amino acids by D-cycloserine in the medial frontal extracellular fluid differ from those in the cortical tissue homogenate previously reported by Baran et al. (1995).

D-Cycloserine is likely to increase the extracellular D-serine contents by modification of its metabolism such as the facilitation of the D-serine release processes, the inhibition of the D-serine reuptake into the frontal neuronal or glial cells, and the upregulation of the D-serine synthesis or the attenuated D-serine degradation. No evidence for the direct conversion of D-cycloserine to D-serine has been reported, but this hypothesis cannot totally be excluded.

The inhibitory effects of D-cycloserine on the proton/amino acid transporter 1 (PAT1) (Metzner et al., 2005) and 2 (PAT2) (Chen et al., 2003), which are able to take up D-serine and are expressed in the brain, might contribute to the changes in the extracellular D-serine contents in the frontal cortex. Because the enhanced levels of extracellular L-serine was shown to produce the release of D-serine by the antiport mechanism between the two amino acids through a certain carrier (Ribeiro et al., 2002), the possible exchange between the extracellular D-cycloserine and the intracellular D-serine by such a carrier could result in the augmented outflow of D-serine. However, D-cycloserine failed to augment the release of D-serine in the brain homogenate preparation in which the exchanging transport between the serine stereoisomers occurs (Ribeiro et al., 2002).

It is also plausible that the attenuation by D-cycloserine of the activity of D-amino acid oxidase (Frecking and Hoeprich, 1966), that can decompose D-serine (Hashimoto et al., 1993), could elevate the tissue and extracellular levels of D-serine. In contrast, D-cycloserine does not seem to influence the activity of the D-serine synthesizing and degrading enzyme, serine racemase (Dunlop and Neidle, 2005). No significant change in the extracellular L-serine contents argues against the view that D-cycloserine secondarily increases the release of D-serine by escalating the brain levels of the D-serine precursor, L-serine (Nishikawa, 2005; Wolosker et al., 2002).

Whatever the mechanisms, our present data indicate that D-cycloserine may facilitate the NMDA receptor functions by the elevation of the extracellular contents of D-serine, in addition to the direct stimulation of the NMDA glycine site. The additional potentiating effects of D-cycloserine on the NMDA receptor functions could contribute to the therapeutic efficacies of D-cycloserine and allow us to use clinical doses of D-cycloserine lower than we expected for the treatment of neuropsychiatric disorders with the NMDA receptor dysfunction.

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References

Baran, H., Gramer, M., Loscher, W., 1995. Alterations in plasma and brain amino acids after administration of the glycine/NMDA receptor partial agonist, D-cycloserine, to mice and rats. Eur. J. Pharmacol. 273, 197–201.

Butterfield, D.A., Pocernich, C.B., 2003. The glutamatergic system and Alzheimer's disease: therapeutic implications. CNS Drugs 17, 641–652.

Chen, Z., Kennedy, D.J., Wake, K.A., Zhuang, L., Ganapathy, V., Thwaites, D.T., 2003. Structure, tissue expression pattern, and function of the amino acid transporter rat PAT2. Biochem. Biophys. Res. Commun. 304, 747–754.

Coyle, J.T., Tsai, G., Goff, D.C., 2002. Ionotropic glutamate receptors as therapeutic targets in schizophrenia. Curr. Drug Targets CNS Neurol. Disord. 1, 183–189.

Danysz, W., Parsons, A.C., 1998. Glycine and *N*-methyl-D-aspartate receptors: physiological significance and possible therapeutic applications. Pharmacol. Rev. 50, 597–664.

Davis, M., Ressler, K., Rothbaum, B.O., Richardson, R., 2006. Effects of D-cycloserine on extinction: translation from preclinical to clinical work. Biol. Psychiat. 60, 369–375.

Dunlop, D.S., Neidle, A., 2005. Regulation of serine racemase activity by amino acids. Brain Res. Mol. Brain Res. 133, 208–214.

Frecking, M.G., Hoeprich, P.D., 1966. Effect of cycloserines on D-amino acid oxidase. Arch. Biochem. Biophys. 115, 108–111.

Guastella, A.J., Dadds, M.R., Lovibond, P.F., Mitchell, P., Richardson, R., 2007. A randomized controlled trial of the effect of D-cycloserine on exposure therapy for spider fear. J. Psychiat. Res. 41, 466–471.

Hashimoto, A., Nishikawa, T., Konno, R., Niwa, A., Yasumura, Y., Oka, T., Takahashi, K., 1993. Free D-serine, D-aspartate and D-alanine in central nervous system and serum in mutant mice lacking D-amino acid oxidase. Neurosci. Lett. 152, 33–36.

Hashimoto, A., Oka, T., Nishikawa, T., 1995. Extracellular concentration of endogenous free D-serine in the rat brain as revealed by in vivo dialysis. Neuroscience 66, 635–643.

Heifets, L.B., 1994. Antimycobacterial drugs. Semin. Respir. Infect. 9, 84–103.

Heresco-Levy, U., Kremer, I., Javitt, D.C., Goichman, R., Reshef, A., Blanaru, M., Cohen, T., 2002. Pilot-controlled trial of D-cycloserine for the treatment of post-traumatic stress disorder. Int. J. Neuropsychopharmacol. 5, 301–307.

Hjörth, S., Sharp, T., 1991. Effect of the 5-HT1A receptor agonist 8-OH-DPAT on the release of 5-HT in dorsal and median raphe-innervated rat brain regions as measured by in vivo microdialysis. Life Sci. 48, 1779–1786.

Hofmann, S.G., Meuret, A.E., Smits, J.A., Simon, N.M., Pollack, M.H., Eisenmenger, K., Shiekh, M., Otto, M.W., 2006. Augmentation of exposure therapy with D-cycloserine for social anxiety disorder. Arch. Gen. Psychiat. 63, 298–304.

Javitt, D.C., 2004. Glutamate as a therapeutic target in psychiatric disorders. Mol. Psychiat. 9 (984–997), 979.

Matthews, J.N.S., Altman, D.G., Campbell, M.J., Royston, P., 1990. Analysis of serial measurements in medical research. Br. Med. J. 300, 230–235.

Metzner, L., Kottra, G., Neubert, K., Daniel, H., Brandsch, M., 2005. Serotonin, L-tryptophan, and tryptamine are effective inhibitors of the amino acid transport system PAT1. FASEB J. 19, 1468–1473.

Nishijima, K., Kashiwa, A., Hashimoto, A., Iwama, H., Umino, A., Nishikawa, T., 1996. Differential effects of phencyclidine and methamphetamine on dopamine metabolism in rat medial frontal cortex and striatum as revealed by in vivo dialysis. Synapse 22, 304–312.

Nishikawa, T., 2005. Metabolism and functional roles of endogenous D-serine in mammalian brains. Biol. Pharm. Bull. 28, 1561–1565.

Ogawa, M., Shigeto, H., Yamamoto, T., Oya, Y., Wada, K., Nishikawa, T., Kawai, M., 2003. D-Cycloserine for the treatment of ataxia in spinocerebellar degeneration. J. Neurol. Sci. 210, 53–56.

Paxinos, G., Watson, C., 2005. The Rat Brain in Stereotaxic Coordinates, 5th ed. Elsevier Academic Press, Amsterdam.

Ressler, K.J., Rothbaum, B.O., Tannenbaum, L., Anderson, P., Graap, K., Zimand, E., Hodges, L., Davis, M., 2004. Cognitive enhancers as adjuncts to psychotherapy: use of D-cycloserine in phobic individuals to facilitate extinction of fear. Arch. Gen. Psychiat. 61, 1136–1144.

Ribeiro, C.S., Reis, M., Panizzutti, R., de Miranda, J., Wolosker, H., 2002. Glial transport of the neuromodulator D-serine. Brain Res. 929, 202–209.

Tanii, Y., Nishikawa, T., Umino, A., Takahashi, K., 1990. Phencyclidine increases extracellular dopamine metabolites in rat medial frontal cortex as measured by in vivo dialysis. Neurosci. Lett. 112, 318–323.

Wolosker, H., Panizzutti, R., de Miranda, J., 2002. Neurobiology through the looking-glass: D-serine as a new glial-derived transmitter. Neurochem. Int. 41, 327–332.

European Neuropsychopharmacology (2010) 20, 176–186

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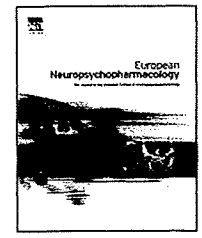

The development- and phencyclidine-regulated induction of synapse-associated protein-97 gene in the rat neocortex

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Abstract

Using the RNA arbitrarily-primed PCR and the competitive RT-PCR, we have isolated the neocortical transcripts that are upregulated and unchanged in the adult and infant rats, respectively, after a systemic injection of an N-methyl-D-aspartate (NMDA) receptor antagonist phencyclidine (PCP), and found them identical to the synapse-associated protein-97 (SAP97) gene mRNAs. The upregulation of the SAP97 transcripts in the adult neocortex after the acute PCP injection was mimicked by another NMDA antagonist, dizocilpine, but not by the indirect dopamine agonists, methamphetamine and cocaine, a selective D1 receptor antagonist SCH23390, a D2 receptor-preferring antagonist haloperidol and a GABAergic anesthetic pentobarbital. Moreover, the pretreatment with a typical antipsychotic haloperidol failed to antagonize the increased neocortical SAP97 gene expression by PCP. These findings suggest that SAP97 might be involved in the molecular basis of the development-dependent onset of the non-dopaminergic symptoms seen in schizophrenia and the schizophrenia-like psychosis induced by NMDA receptor blocking.

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