2.2. Drugs

Phencyclidine HCl (PCP) was synthesized by the authors according to the method of Maddox and colleagues (Maddox et al., 1965) and was checked for purity. Galantamine and risperidone were provided by Janssen Pharmaceutical K. K. (Tokyo, Japan). Mecamylamine hydrochloride, (−) scopolamine hydrobromide, *R*(+)-SKF 81297 hydrobromide and *R*(+)-SCH 23390 hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO, USA). All the doses were referred to the salt forms of the compounds. PCP was dissolved in 0.9% saline. Oral solution of risperidone and/or galantamine was freshly prepared by dissolving them in diluted tartaric acid solution (final pH 3.2). SKF 81297, SCH 23390, mecamylamine and scopolamine were dissolved in 0.9% saline.

2.3. Drug treatment

The doses of risperidone and galantamine used in the present study were determined in preliminary experiments. The doses of antagonists were referred to our previous publication (Wang et al., 2007a,b) and/or other related researches being done in the laboratory, and determined in preliminary experiments.

For the experiments that the drugs were given systemically, the mice were administered PCP (10 mg/kg per day s.c.) or saline once a day for 14 consecutive days (Noda et al., 1995). The saline- and PCP-treated mice were p.o. administered galantamine (0.05, 0.1 or 0.3 mg/kg) and risperidone (0.025, 0.05 or 0.1 mg/kg) 1 h before the training trial of the water-finding test, or immediately after baseline collections in microdialysis experiment. Mecamylamine (3 mg/kg) or scopolamine (0.1 mg/kg) was s.c. injected 20 min after the treatment with galantamine and/or risperidone, and SCH 23390 (0.02 mg/kg) was s.c. injected 30 min after the co-administration. All compounds were systemically administered at a volume of 0.1 ml/10 g body weight. Control mice received the same volume of saline or vehicle.

For mPFC-local microinjection, mice were anesthetized with diethyl ether and fixed on the stereotactic apparatus (Narishige, Tokyo, Japan) 20 min before the training trial of water-finding test. A L-shape injection cannula (27 gauge) with a bevel tip at the short end of it was clipped on a pincers and implanted into the mPFC (+0.3 mm mediolateral from the midpoint on the line linking the two rear canthi, −2.5 mm in depth). SKF 81297 at the dose of 0.15 μg/0.5 μL/mouse and SCH 23390 at the dose of 0.02 μg/0.5 μL/mouse were infused into the mPFC in 45 s using a Hamilton microsyringe connected to the cannula via a teflon tube, and the connection was held for another 45 s after the injection. Since the depth of the injection (−2.5 mm) was predetermined by the length of the short end of the L-shape injection cannula, a misinjection would be mainly resulted from a horizontal departure from the right position, and the misinjected brain structures would be the forces major of the corpus callosum (fmi) and the caudate putamen (CPu) partially surrounded by the fmi, as shown in the atlas of Franklin and Paxinos (1997). The fmi and CPu are easy to be discriminated from the PFC by the white color and outline of the fmi. After the behavioral experiments, the mice were decapitated, and the brains were taken out. The brains were transversely cut along the direction of the vertical insertion of the cannula to confirm the injection site, which was obvious by its dark red color and easy to be recognized, as shown in Fig. 2. Misinjected mice were excluded from subsequent data analysis.

2.4. Water-finding test

The protocol of Noda et al. (2001) was used for the study. The apparatus consisted of an open field [50 × 30 × 15 (H) cm] with an alcove [10 × 10 × 10 (H) cm] in the middle of one of the long walls of the enclosure. The floor of the open field was divided into 15 identical squares. The nozzle of a drinking bottle that was identical with those used in the home cages was inserted from the ceiling of the alcove.

The water-finding test consisted of two trials: a training trial (the 1st day) and a testing trial (the 2nd day). The training trial was started 3 days after the withdrawal of PCP treatment. Mice that had not been deprived of water were placed individually into and toward one corner of the open field of the apparatus. Each mouse was given 3 min to explore the environment. The mice that did not find the drinking nozzle during the 3-min exploratory period were omitted from the testing trial. The mice were immediately returned to their home cages after the training trial, and deprived of water for 24 h before the testing trial. In the testing trial, mice were again individually put into the apparatus. Finding latency was defined as the time from entering the alcove till drinking the water.

The field of the vision of mice is limited by their moving attitude, and it is extremely harder for them to casually find a short water nozzle located at the center of the ceiling than the things that are not at the center in the small alcove. In order to decrease the possibility of being found by chance in testing trials, the tip of the water nozzle inserted from the center of the ceiling was set further from the floor in the testing trial (7.5 cm) than in the training trial (6.5 cm).

2.5. In vivo microdialysis

In vivo microdialysis was performed 3 days after the withdrawal of PCP treatment. One day before the microdialysis, mice were anesthetized with sodium pentobarbital and a guide cannula (MI-AG-6; Eicom Corp., Kyoto, Japan) was implanted into the mPFC (+1.9 mm anteroposterior, +1.0 mm mediolateral from the bregma, −1.5 mm dorsoventral from the skull, +15 degree angle from vertical) according to the atlas of Franklin and Paxinos (1997). One day after the operation, a dialysis probe (A-I-6-01; 1 mm membrane length; Eicom Corp.) was inserted through the guide cannula, and perfused with artificial cerebrospinal fluid (aCSF; 147 mM NaCl, 4 mM KCl, and 2.3 mM $CaCl_2$) at a flow rate of 1.2 μL/min (Shintani et al., 1993). The outflow fractions were collected every 10 min. Following the collection of 3 stable baseline fractions, galantamine and/or risperidone was p.o. administered to the mice, and dialysates were collected for 90 min after the drug administration. Dopamine levels in the dialysates were analyzed using an HPLC system equipped with an electrochemical detector (Nagai et al., 2004).

2.6. Statistical analysis

Statistical difference among the experimental groups was tested using a one-way analysis of variance (ANOVA) for behavioral experiments, and a two-way ANOVA for microdialysis. The modified Tukey test was adopted for multiple comparisons. *P* values less than 0.05 were accepted as significant.

3. Results

3.1. Individual effects of risperidone and galantamine on impairment of latent visuospatial learning and memory in PCP-treated mice

In the testing trial, PCP (10 mg/kg 14 days)-treated mice showed a deficit of latent visuospatial learning and memory after withdrawal from PCP treatment. The treatment with risperidone (0.1 mg/kg) and galantamine (0.3 mg/kg) ameliorated the deficit of latent visuospatial learning and memory in PCP-treated mice. Risperidone at the doses of 0.025 and 0.05 mg/kg, and galantamine at the doses of 0.05 and 0.1 mg/kg failed to ameliorate the impairment of latent visuospatial learning and memory in PCP-treated mice (Fig. 1a, $F_{(7,118)} = 3.323$, $p < 0.01$). Galantamine at the dose of 0.2 mg/kg showed tendency to ameliorate the deficit of latent visuospatial learning and memory, without reaching the level of statistical significant difference (data not shown).

The effects of risperidone (0.1 mg/kg) and galantamine (0.3 mg/kg) at their effective doses were antagonized by mPFC-local administration of SCH 23390 at the dose of 0.02 μg/0.5 μL/mouse, which did not significantly affect the performance in saline-treated mice (Fig. 1b, $F_{(6,83)} = 4.497$, $p < 0.01$). The mPFC-local injection site was shown in Fig. 2.

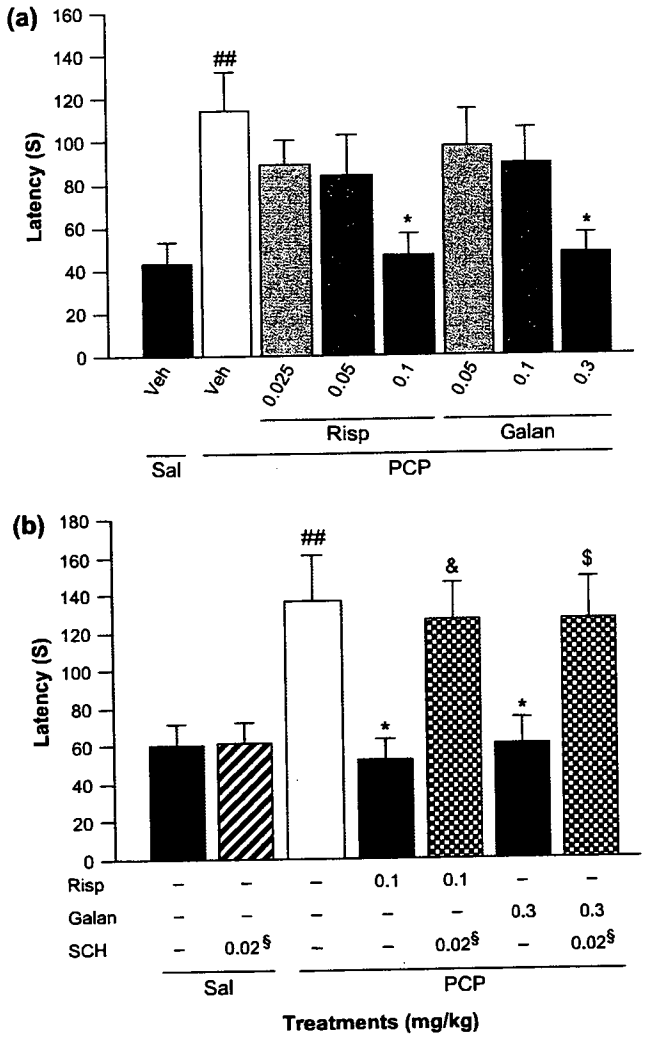

Fig. 1. Individual effects of risperidone and galantamine on impairment of latent visuospatial learning and memory in PCP-treated mice. PCP (10 mg/kg, s.c.) was injected for 14 days. Control groups were treated with same volume of saline (Sal). Galantamine (Galan, 0.05, 0.1 and 0.3 mg/kg, p.o.) and risperidone (Risp, 0.025, 0.05 and 0.1 mg/kg, p.o.) were administered 1 h before the training trial. The D_1 receptor antagonist SCH 23390 was s.c. injected at a dose of 0.02 mg/kg 30 min after the co-administration, or was mPFC-locally injected 15 min before the training trial. a. Effects of risperidone and galantamine on the finding latency in PCP-treated mice. b. Effects of risperidone and galantamine on the finding latency was antagonized by mPFC-local administration of SCH 23390. Results are expressed as means ± SEM, $n = 14–16$, and analyzed by a one-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/vehicle-treated group. $^{*}p < 0.05$, compared to PCP/vehicle-treated group. $^{\&}p < 0.05$, compared to PCP/risperidone-treated group. $^{\$}p < 0.05$, compared to PCP/galantamine-treated group. Veh: vehicle (dilute tartaric acid solution, pH 3.2). §mPFC-local administration of SCH 23390 at the dose of 0.02 μg/0.5 μL/mouse.

3.2. Synergistic effect of galantamine with risperidone on impairment of latent visuospatial learning and memory in PCP-treated mice

The co-administration of galantamine (0.05 mg/kg) and risperidone (0.025 mg/kg), both at their lowest doses, failed to ameliorate the impairment of latent visuospatial learning and memory in PCP-treated mice (data not shown). The co-administration of galantamine at the lowest dose of 0.05 mg/kg and risperidone at the sub-lowest dose of 0.05 mg/kg greatly ameliorated the impairment of latent visuospatial learning and memory in the PCP-treated mice, although they did not improve the latent visuospatial learning and memory in the saline-treated mice (Fig. 3, $F_{(5,95)} = 4.865$, $p < 0.01$). Since the individual treatment with risperidone and galantamine both at the dose of 0.05 mg/kg failed to improve the PCP-induced impairment of latent visuospatial learning and memory by themselves, but showed synergistic effect in the water-finding test, these doses of risperidone and galantamine were used in the subsequent experiments.

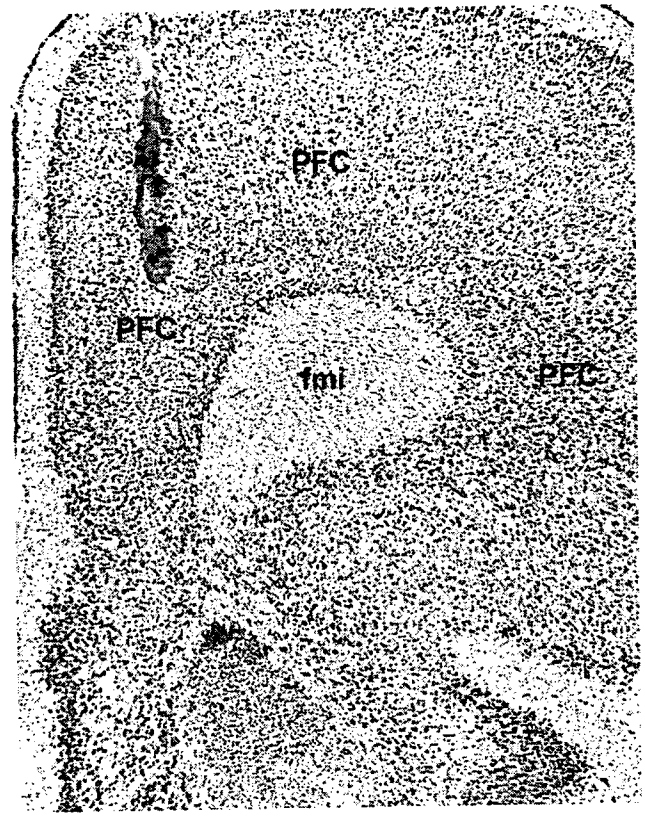

Fig. 2. Representative figure of mPFC-local injection site. PFC, prefrontal cortex. fmi, forces major of the corpus callosum.

The treatment with donepezil at the doses of 0.6 and 1.2 mg/kg ameliorated the impairment of latent visuospatial learning and memory in PCP-treated mice. However, the combined treatment with risperidone (0.05 mg/kg) and donepezil (0.3 mg/kg) did not show a synergistic effect on the cognitive impairment induced by PCP (Fig. 4, $F_{(6,100)} = 3.647$, $p < 0.01$).

3.3. Nicotinic, but not muscarinic, AChR is involved in the synergistic effect of galantamine with risperidone

We investigated whether nAChR or mAChR is involved in the synergism of galantamine with risperidone, by investigating whether mecamylamine (3 mg/kg) or scopolamine (0.1 mg/kg)

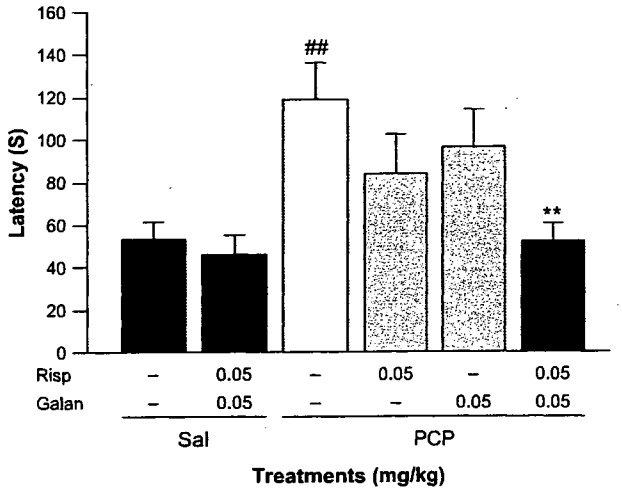

Fig. 3. Synergistic effect of galantamine with risperidone on impairment of latent visuospatial learning and memory in PCP-treated mice. PCP (10 mg/kg, s.c.) was injected for 14 days. Control groups were treated with same volume of saline (Sal). Galantamine (G, 0.05 mg/kg, p.o.) and risperidone (R, 0.05 mg/kg, p.o.) were administered 1 h before the training trial. Results are expressed as means ± SEM, $n = 12–17$, and analyzed by a one-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/vehicle-treated group. $^{**}p < 0.01$, compared to PCP/vehicle-treated group.

could block the behavioral effect of the co-administration. Mecamylamine (3 mg/kg) was s.c. injected to the mice 20 min after the co-administration of galantamine (0.05 mg/kg) and risperidone (0.05 mg/kg). Forty minutes after the injection, mecamylamine blocked the effects of the co-administration on the performance in the PCP-treated mice in the water-finding test

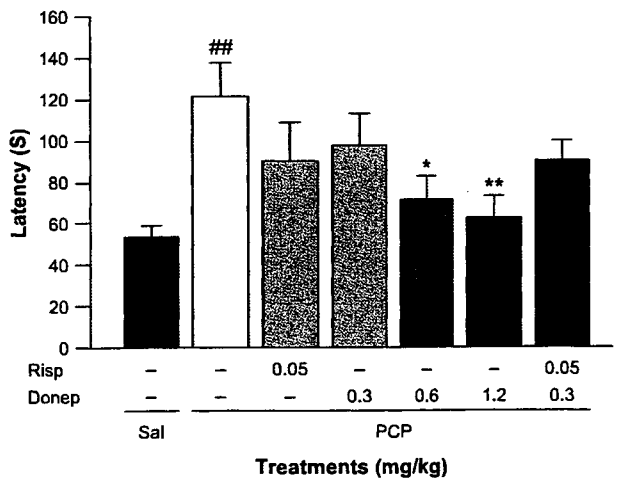

Fig. 4. Combined treatment with donepezil and risperidone did not show synergistic effect on latent visuospatial learning and memory in PCP-treated mice. PCP (10 mg/kg, s.c.) was injected for 14 days. Control groups were treated with same volume of saline (Sal). Donepezil (Donep, 0.3, 0.6 and 1.2 mg/kg, p.o.) and risperidone (Risp, 0.05 mg/kg, p.o.) were administered 1 h before the training trial. Results are expressed as means ± SEM, $n = 12–17$, and analyzed by a one-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/vehicle-treated group. $^{*}p < 0.05$, $^{**}p < 0.01$, compared to PCP/vehicle-treated group.

at the dose (3 mg/kg) that did not inhibit latent visuospatial learning and memory in saline-treated mice (Fig. 5a), $F_{(4,57)} = 6.203$ ($p < 0.01$).

Scopolamine (0.1 mg/kg) was s.c. injected to the mice 20 min after the co-administration. The performance in saline-treated mice was impaired by scopolamine at the relatively low dose of 0.1 mg/kg (Fig. 5b). However, scopolamine at this dose failed

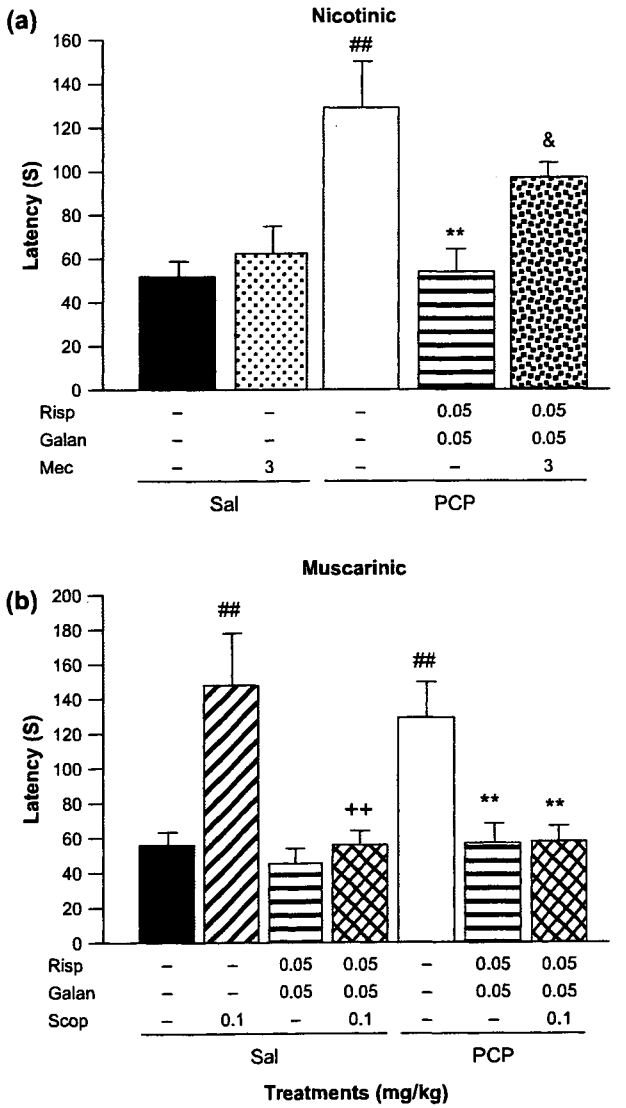

Fig. 5. Nicotinic, but not muscarinic, AChRs are critical for the synergism of galantamine with risperidone: PCP (10 mg/kg, s.c.) was injected for 14 days. Control groups were treated with same volume of saline (Sal). Galantamine (Galan, 0.05 mg/kg, p.o.) and risperidone (Risp, 0.05 mg/kg, p.o.) were administered 1 h before the training trial, and mecamylamine (Mec, 3 mg/kg, s.c.) or scopolamine (Scop, 0.1 mg/kg, s.c.) was injected 20 min after the co-administration. a. Synergistic effect of galantamine with risperidone on finding latency was antagonized by mecamylamine. b. Synergistic effect of galantamine with risperidone on finding latency was not antagonized by scopolamine. Results are expressed as means ± SEM, $n = 10–12$, and analyzed by a one-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#\#}p < 0.01$, compared to Sal/vehicle-treated group. $^{++}p < 0.01$, compared to Sal/vehicle/Scop-treated group. $^{**}p < 0.01$, compared to PCP/vehicle-treated group. $^{\&}p < 0.05$, compared to PCP/Risp/Galan-treated group.

to antagonize the synergistic effects of the co-administration of galantamine (0.05 mg/kg) and risperidone (0.05 mg/kg) in the PCP-treated mice (Fig. 5b), $F_{(6,77)} = 6.226$ ($p < 0.01$). In order to understand it well, we also investigated the effects of the co-administration followed by the injection of scopolamine (0.1 mg/kg) in saline-treated mice. The treatment with scopolamine at the dose of 0.1 mg/kg also failed to antagonize the effect of the co-administration of these two drugs on the performance in saline-treated mice (Fig. 5b). In other words, the impairing effect of scopolamine at the relatively low dose of 0.1 mg/kg was compensated by the co-administration of galantamine (0.05 mg/kg) and risperidone (0.05 mg/kg) in saline-treated mice.

3.4. The synergistic effect of galantamine with risperidone is mediated by dopamine D_1 receptors

Since the mesocortical dopaminergic neurotransmission through D_1 receptors is thought to be involved in the cognitive symptoms of schizophrenia (Abi-Dargham, 2004; Albert et al., 2002; Fink-Jensen, 2000; Kolb, 1990), we investigated whether the synergistic cognitive effect of galantamine with risperidone is mediated by D_1 receptors.

The effects of the co-administration of galantamine and risperidone on the performance in the PCP-treated mice were abolished by systemic administration of a D_1 receptor antagonist, SCH 23390, at the dose of 0.02 μg/kg that did not significantly impair the latent visuospatial learning and memory in saline-treated mice (Fig. 6a), $F_{(4,75)} = 5.660$ ($p < 0.01$).

The mesocortical dopaminergic system that correlated with the cognitive symptoms projects to the PFC. In order to know whether the synergistic effect of galantamine with risperidone on the impairment of latent visuospatial learning and memory is mediated by the D_1 receptors in the mPFC, the strategy of mPFC-local microinjection of the dopamine D_1 receptor agonist SKF 81297 and the antagonist SCH 23390 was used in the present study. SKF 81297 (0.15 μg/0.5 μL/mouse) and SCH 23390 (0.02 μg/0.5 μL/mouse) were injected into the mPFC of the mice 15 min before the training trial. The microinjection of the D_1 receptor agonist SKF 81297 ameliorated the impairment of latent visuospatial learning and memory in PCP-treated mice (Fig. 6b). In contrast to SKF 81297, the dopamine D_1 receptor antagonist SCH 23390 blocked the effect of the co-administration of the drugs on the latent visuospatial learning and memory ($p < 0.05$) in the PCP-treated mice at the dose (0.02 μg/0.5 μL/mouse) that did not significantly impair the latent visuospatial learning and memory in saline-treated mice (Fig. 6b), $F_{(5,83)} = 4.292$ ($p < 0.01$).

3.5. Performance in mice in training trial of water-finding test

In the training trial, the statistical differences of the discovering latency and ambulation counts in mice were not found among the groups treated with saline or other compounds. All of the treatments used in the study at the present doses did not significantly affect the exploratory activity in the mice in the training trial (Table 1), $F_{(20,272)} = 0.628$ (for discovering

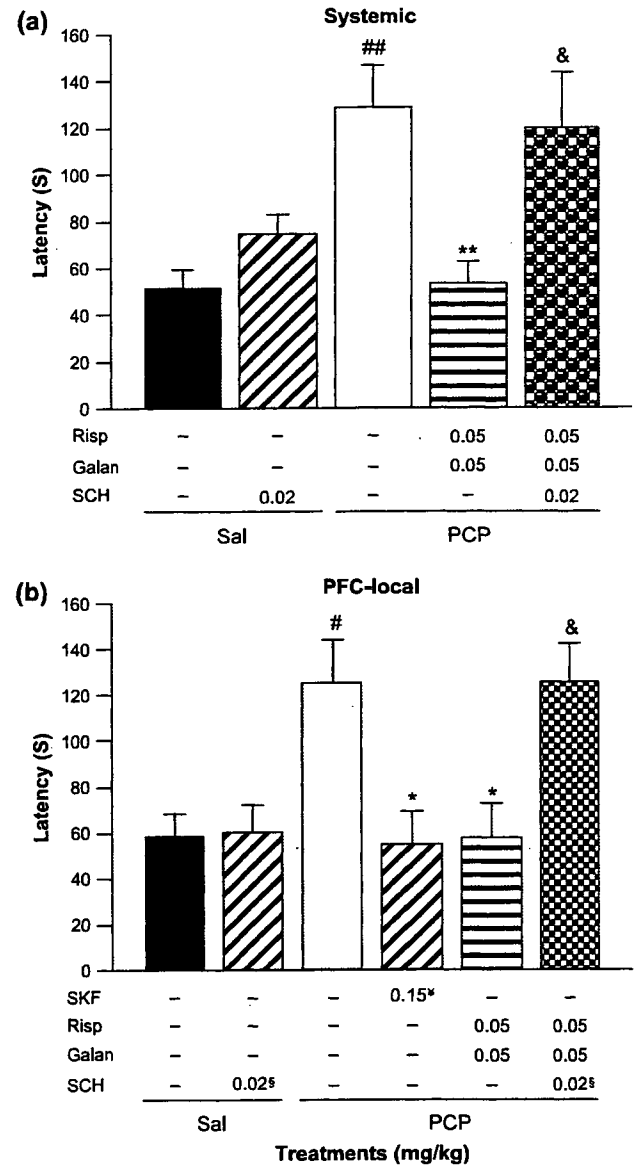

Fig. 6. Synergistic effect of galantamine with risperidone is mediated by D_1 receptors. PCP (10 mg/kg, s.c.) was injected for 14 days. Control groups were treated with same volume of saline (Sal). Galantamine (Galan, 0.05 mg/kg, p.o.) and risperidone (Risp, 0.05 mg/kg, p.o.) were administered 1 h before the training trial. The Dopamine D_1 receptor agonist SKF 81297 was mPFC-locally injected 15 min before the training trial. The D_1 receptor antagonist SCH 23390 was s.c. injected at a dose of 0.02 mg/kg 30 min after the co-administration, or was mPFC-locally injected 15 min before the training trial. a. Synergistic effect of galantamine with risperidone on finding latency was blocked by systemic administration of SCH 23390. b. Synergistic effect of galantamine with risperidone on finding latency was blocked by mPFC-local administration of SCH 23390. Results are expressed as means ± SEM, $n = 11–17$, and analyzed by a one-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, compared to Sal/vehicle-treated group, $^{*}p < 0.05$, $^{**}p < 0.01$, compared to PCP/vehicle-treated group, $^{\&}p < 0.05$, compared to PCP/Risp/Galan-treated group. ¥mPFC-local administration of SKF 81297 at a dose of 0.15 μg/0.5 μL/mouse. §mPFC-local administration of SCH 23390 at a dose of 0.02 μg/0.5 μL/mouse.

Table 1
Discovering latency and ambulation counts in mice in training trial of the water-finding test

Treatments (mg/kg)		DL (s)	Amb (counts)
Saline control		52.0 ± 9.78	25.4 ± 9.78
Saline/Risp (0.05) + Galan (0.05)		53.9 ± 8.17	35.2 ± 6.65
Saline/SCH (0.02)		55.1 ± 6.55	21.4 ± 3.27
Saline/Mec (3)		52.0 ± 6.29	30.4 ± 6.98
Saline/Scop (0.1)		60.6 ± 12.6	38.5 ± 5.69
Saline/Risp (0.05) + Galan (0.05)/Scop (0.1)		67.5 ± 10.9	36.9 ± 5.25
PCP control		45.5 ± 6.97	28.0 ± 4.21
PCP/Risp	(0.025)	47.1 ± 7.80	35.1 ± 6.58
	(0.05)	55.3 ± 6.98	26.9 ± 3.92
	(0.1)	60.6 ± 12.2	35.7 ± 7.36
PCP/Galan	(0.05)	55.3 ± 6.74	31.0 ± 3.37
	(0.1)	45.5 ± 6.97	26.5 ± 4.30
	(0.3)	47.1 ± 7.80	24.9 ± 3.62
PCP/Donep	(0.3)	53.2 ± 6.41	26.9 ± 3.38
	(0.6)	50.6 ± 7.17	27.3 ± 4.12
	(1.2)	51.9 ± 6.47	25.9 ± 3.39
PCP/Risp (0.05) + Galan (0.05)		56.3 ± 6.61	32.2 ± 3.05
PCP/Risp (0.05) + Donep (0.3)		53.9 ± 6.83	28.9 ± 3.26
PCP/Risp (0.05) + Galan (0.05)/SCH (0.02)		53.4 ± 8.96	25.4 ± 3.52
PCP/Risp (0.05) + Galan (0.05)/Mec (3)		60.0 ± 12.6	33.0 ± 4.63
PCP/Risp (0.05) + Galan (0.05)/Scop (0.1)		58.2 ± 11.0	36.7 ± 6.51

PCP, phencyclidine (10 mg/kg, s.c. injected for 14 d); Risp, risperidone; Galan, galantamine; Donep, donepezil; SCH, SCH 23390; Mec, mecamylamine; Scop, scopolamine; DL, the latency to discover the water nozzle for the first time after being put into the apparatus, the discovery of the water nozzle is defined as exploring (approaching, sniffing or touching) it with their noses or mouths, which is a part of their environmental exploratory activity and a behavioral characteristic of mice when they newly find an immobile object in the environment; Amb: the ambulation counts from start till discovering the water nozzle. Values are means ± SEM, $n = 10$–17; statistically analyzed by one-way ANOVA. Significant differences were not found among the groups.

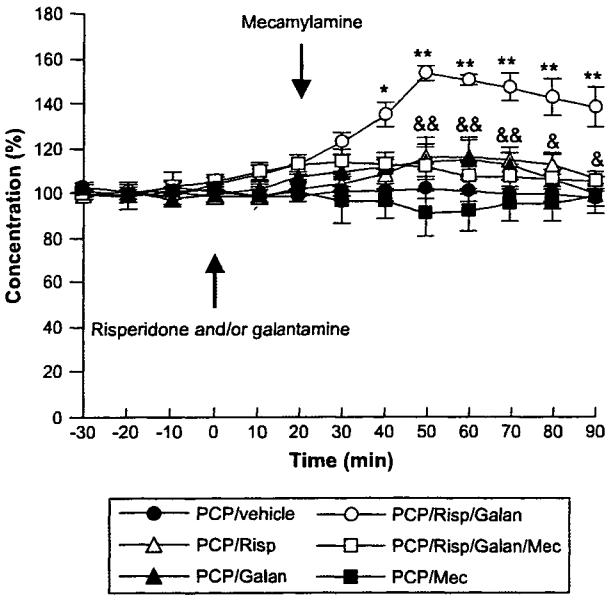

Fig. 7. Synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in mPFC of PCP-treated mice. PCP (10 mg/kg, s.c.) was injected for 14 days. Galantamine (Galan, ▲, 0.05 mg/kg, p.o.) and risperidone (Risp, △, 0.05 mg/kg, p.o.) were administered individually or together (○, p.o.). The control group was given equivalent amount of vehicle (●). Mecamylamine (Mec, 3 mg/kg, s.c.) was injected 20 min after the co-administration (□), or injected individually as a control group (■). Results are expressed as means ± SEM, $n = 4$–5, and analyzed by a two-way ANOVA, followed by the modified Tukey test for multiple comparisons $^{*}p < 0.05$, $^{**}p < 0.01$, compared between PCP/vehicle-treated and PCP/Risp/Galan-treated groups. $^{\&}p < 0.05$, $^{\&\&}p < 0.01$, compared between PCP/Risp/Galan-treated and PCP/Risp/Galan/Mec-treated groups.

latency), $p = 0.890$, $F_{(20,272)} = 0.958$ (for ambulation counts), $p = 0.514$.

3.6. Synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in mPFC of PCP-treated mice

It has been reported that the extracellular dopamine concentration decreased in the PFC of repeated PCP-treated mice (Jentsch et al., 1998a,b; Jentsch and Roth, 1999). The change in the basal level was mirrored in the dopaminergic response to potassium stimulation at high concentration, which is observed in another study in our laboratory, in which the release of dopamine in the mPFC of PCP-treated (10 mg/kg, 14 days) mice was insensitive to the potassium stimulation (data not shown).

The extracellular concentration of dopamine was not significantly increased in the mPFC of the PCP-treated mice by the individual treatment with galantamine (0.05 mg/kg) or risperidone (0.05 mg/kg) at their non-effective doses. In contrast to the individual treatments, the co-administration of galantamine (0.05 mg/kg) and risperidone (0.05 mg/kg) significantly increased extracellular concentration of dopamine in the mPFC (Fig. 7), $F_{group(5,357)} = 25.093$ ($p < 0.01$), $F_{time(12,357)} = 3.635$ ($p < 0.01$).

3.7. Nicotinic AChR antagonist, mecamylamine, blocked synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in mPFC of PCP-treated mice

It has been reported by Ichikawa et al. (2002) that risperidone increases cortical acetylcholine release, which in turn promotes dopamine release. In the present study, the increasing effect of the co-administration of galantamine and risperidone on extracellular concentration of dopamine was antagonized by the nAChR antagonist mecamylamine (3 mg/kg, s.c. injected 20 min after the co-administration) at the dose that did not significantly change the extracellular concentration of dopamine in the mPFC (Fig. 7). These findings indicated the mechanism how the dopamine release was increased by the co-administration of galantamine and risperidone, but not by their individual treatments.

3.8. Effects of co-administration of risperidone and galantamine on extracellular concentration of dopamine in mPFC of saline-treated mice

The individual treatment with risperidone (0.05 mg/kg) or galantamine (0.05 mg/kg) did not significantly affect the extracellular concentration of dopamine in the mPFC of

saline-treated mice (Fig. 8). However, the co-administration of risperidone (0.05 mg/kg) and galantamine (0.05 mg/kg) increased the extracellular concentration of dopamine in the mPFC of saline-treated mice 40 and 50 min after the co-administration (Fig. 6), $F_{group(3,151)} = 9.742$ ($p < 0.01$), $F_{time(12,151)} = 2.490$ ($p < 0.01$).

4. Discussion

PCP induces psychomimetic state in human and behavioral changes in animals that closely resemble schizophrenia (Javitt and Zukin, 1991; Nabeshima et al., 1989; Noda et al., 1995). Dopamine release in the striatal area including nucleus accumbens increases soon after the acute treatment with PCP (Balla et al., 2001; Greenslade and Mitchell, 2004). The activation of the dopamine-D_2 receptors in the striatum accounts for the positive symptoms of schizophrenia (Abi-Dargham, 2004). In stark contrast to the acute PCP exposure, repeated treatment with PCP reduces the basal extracellular concentration of dopamine and the response of dopamine release to potassium stimulation in the mPFC as shown in our and other researchers' studies (Jentsch et al., 1998a,b; Jentsch and Roth, 1999; Wang et al., 2007b). In the present study, the effects of the individual treatment with risperidone or galantamine at their effective doses and the combined treatment at their non-effective doses were abolished by mPFC-local administration of the D_1 receptor antagonist SCH 23390, indicating the involvement of dopaminergic system in the effects. The present results are consistent with the notion that the dopaminergic

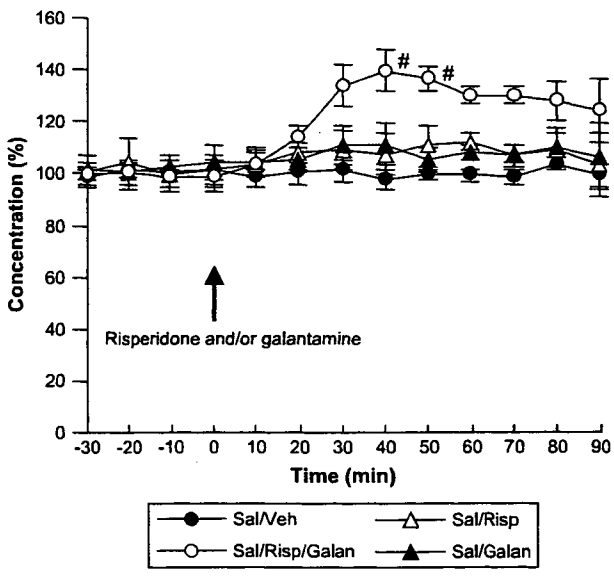

Fig. 8. Synergistic effect of galantamine with risperidone on extracellular concentration of dopamine in mPFC of saline-treated mice. Saline/vehicle-treated mice were used as a control group (Sal/Veh, ●). Galantamine (Galan, ▲, 0.05 mg/kg, p.o.) and risperidone (Risp, △, 0.05 mg/kg, p.o.) were administered individually or together (○, p.o.). Results are expressed as means ± SEM, $n = 4–5$, and analyzed by a two-way ANOVA, followed by the modified Tukey test for multiple comparisons. $^{\#}p < 0.05$, compared between Sal/Veh-treated and Sal/Risp/Galan-treated groups.

dysfunction in the PFC is a characteristic of the neurochemical changes in schizophrenia patients that accounts for the cognitive symptoms (Abi-Dargham, 2004; Albert et al., 2002; Fink-Jensen, 2000; Kolb, 1990).

The cognitive symptoms of schizophrenia are often the most debilitating and difficult to treat in clinical trials. Deficits in attention and information processing mechanisms have been suggested to play a critical role in the pathology of schizophrenia (Noda et al., 2001), and schizophrenia might involve an inability to properly filter incoming sensory information and thus result in sensory inundation and subsequent cognitive fragmentation (McGhie and Chapman, 1961; Noda et al., 2001; Venables, 1960). The water-finding test, which is a latent visual learning paradigm, relates to attention and the ability to sort sensory information (Noda et al., 2001). Repeated treatment with PCP significantly prolonged the finding latency in the water-finding test at the dose of 10 mg/kg, indicating an impairment of latent visuospatial learning and memory that engages attention processes.

Repeated treatment with PCP impaired the latent visuospatial learning and memory in mice as shown in the water-finding test. In the present study, a medicine for Alzheimer's disease, galantamine, ameliorated the cognitive impairment in the PCP-treated mice, and the co-administration of galantamine and risperidone at their non-effective doses showed significant effect on the cognitive impairment in the mice. These results clearly indicated that galantamine may be effective in treating the cognitive symptom of schizophrenia and the combined treatment with galantamine and risperidone may have synergistic effect on the symptom. Since the synergy between galantamine and risperidone in ameliorating the cognitive impairment was blocked by the systemic and mPFC-local administration of the D_1 receptor antagonist SCH 23390, it indicated that D_1 receptor-mediated neurotransmission in the mPFC plays an important role in the synergistic effect of these two drugs on the impairment of latent visuospatial learning and memory induced by repeated PCP treatment.

Ichikawa et al. (2002) have reported that risperidone increases acetylcholine release in the cortex, but not in the striatal areas. This effect of risperidone on the release of acetylcholine may be indispensable for the synergy between galantamine and risperidone in ameliorating the cognitive impairment induced by repeated PCP treatment. Other antipsychotics without an acetylcholine-releasing effect may have none or less synergistic effect with galantamine as recently reported (Lee et al., 2007). Activation of nAChRs promotes the release of DA (Cao et al., 2005; Salminen et al., 2004; Wang et al., 2007a; Wonnacott, 1997; Zhang et al., 2004). In the present study, the effect of the co-administration on dopamine release in the mPFC was antagonized by the nAChR antagonist mecamylamine. These findings indicated the pivotal role of nAChR in regulating dopaminergic neurotransmission in the synergistic effect of galantamine and risperidone. This notion is supported by the result in the present study that donepezil, an AChE inhibitor without the nAChR-potentiating effect, actually did not show synergistic effect with risperidone. At low doses, galantamine potentiates the function of

nAChRs. This acting mode of galantmine at low doses not only makes it possible to avoid the problematic rapid desensitization of receptors generally induced by receptor agonists (Deutsch et al., 2005; Friedman, 2004) but also makes the receptor-mediated cognitive effect be receptor use-dependent, which may be better for learning and memory than general activation of all the nAChRs in the brain caused by agonists. Besides being an allosteric potentiator of nAChR, galantamine is a rapidly reversible and rather modest AChE inhibitor (IC_{50} in the frontal cortex and the hippocampus of mouse and human in the range from 2.8 to 3.9 μM) (Bickel et al., 1991b; Samochocki et al., 2003; Sharp et al., 2004). At the doses used in the present study that are far below those required to reach its IC_{50} value for AChE inhibition (Bickel et al., 1991a; Farlow, 2003; Scott and Goa, 2000; Samochocki et al., 2003), the effect of galantamine on the release of dopamine in the mPFC was mainly resulted from its potentiation of the nAChR, but not from the inhibition of AChE. This notion is supported by the publication that there is only 1–12% brain AChE inhibition 1 h after s.c. injection of 3 mg/kg galantamine (Geerts et al., 2005; Thomsen et al., 1991), and by the fact observed in the study that galantamine at the dose of 3 mg/kg was not more effective than that of 0.3 mg/kg (data not shown).

It has been reported that the repeated treatment with risperidone (0.5 mg, co-administered after 0.5 mg twice daily for 6 days) had no effect on the bioavailability and disposition of galantamine, and the systemic exposure of risperidone active moiety, the most clinically relevant component of risperidone treatment, was not affected by galantamine co-administration. The plasma concentration of risperidone was not changed within 10 h after the co-administration, while the systemic exposure was increased by approximately 10% for risperidone, and mean peak plasma concentration of risperidone active moiety decreased by approximately 10% after co-administration with galantamine (12 mg, co-administered after a serial pretreatment for 6 days) (Huang et al., 2002).

SCH 23390 displays an 800 times higher affinity for D_1 receptor than D_2 receptor (Christensen et al., 1984). The fact that SCH 23390 blocks dopamine-stimulated adenylate cyclase at concentrations ($IC_{50} \approx 0.01$ μM) about 2000 times lower than that needed to block spiperone binding ($IC_{50} \approx 24$ μM) and three times lower than that needed to block ketanserin binding ($IC_{50} \approx 0.03$ μM) suggests a more specific antagonism for D_1-receptor than 5-HT_2 receptor (Bischoff et al., 1986; Iorio et al., 1983). SCH 23390 also possesses weak affinities for 5-HT_{1A} ($IC_{50} \approx 2.6$ μM), 5-HT_{1B} ($IC_{50} \approx 0.5$ μM) and α_1-adrenergic receptors ($IC_{50} \approx 4.4$ μM) (Bischoff et al., 1986, 1988). SKF 81297 is an agonist for D_1 receptor with high selectivity. The rank order for dopamine D_1:D_2 receptor selectivity in rhesus striata is: SKF 81297 > SKF 38393 ≫ SKF 82958 > SKF 77434 > *R*(+) 6-BrAPB > *S*(−) 6-BrAPB > dopamine, among which SKF 81297 is the only agonist that displays more than 100 times selectivity for D_1 receptor than D_2 receptor (Weed et al., 1998). Mecamylamine antagonizes all types of nAChRs, but not mAChRs, with greatest affinity for α4β2 receptor, and at higher doses it can also antagonize NMDA receptor (Rabenstein et al., 2006; Xiao and Kellar, 2004). The rank order of nAChR ligands for nAChR-binding affinity in rat forebrain is mecamylamine (K_i > 500,000) ≫ choline ($K_i \approx 43{,}000$) ≫ methyllcaconitine ($K_i \approx 6600$) ≫ carbachol > DHβE > DMPP ≫ acetylcholine ($K_i \approx 45$) > (−)-Nicotine ($K_i \approx 12$) > cytosine ($K_i \approx 1.9$) > A-85380 > (±)-I-epibatidine > I-A-85380 > (±)-epibatidine (Xiao and Kellar, 2004). Scopolamine is a competitive antagonist for all mAChR subtypes with high affinities (Goudie et al., 2004). By citing an unpublished paper, Goudie et al. (2004) reported that scopolamine may also have relatively weak affinity for D_2 receptor. In the present study, the doses that we used are very low, therefore, the effects of the compounds attribute to their principal activity at the receptors, matching the purpose for using them in the present study.

Although galantamine is only a rather modest AChE inhibitor ($IC_{50} \approx 2.8$–3.9 μM) compared with other AChE inhibitors presently used in clinical trials, such as rivastigmine ($IC_{50} \approx 4$ nM) and donepezil ($IC_{50} \approx 15$–24 nM), it increases extracellular concentration of acetylcholine at relatively high doses. In addition, similar as donepezil and rivastigmine, galantamine blocks ACh-activated channels at very high concentration (>10 μM) (Samochocki et al., 2003; Sharp et al., 2004). Based on the above reasons and the fact that galantamine can improve latent visuospatial learning and memory synergistically with risperidone by allosterically potentiating nAChR at relatively low doses, it is preferable to use galantamine at low doses for treating the cognitive symptom in schizophrenia.

The present study indicates that the combined treatment with galantamine and risperidone may have synergistic effect on repeated PCP treatment-induced impairment of latent visuospatial learning and memory by promoting the nAChR activation-dependent increase of dopamine D_1 receptor-mediated neurotransmission, and the combined treatment may be used as a new strategy for treating the cognitive symptom of schizophrenia.

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第8回 感情・行動・認知研究会

脳内エンドカンナビノイドの異常性にもとづくマウスの知的機能障害

山本経之* 縄田陽子* 當原真奈美§

◆はじめに

脳内にはカンナビノイドCB1受容体とその内因性リガンドとしてanandamide（N-arachidonoylethanolamine）ならびに2-AG（2-arachidonoylglycerol）などが同定されている．一方，大麻の活性成分のtetrahydrocannabinol（THC）は認知機能障害を含む統合失調症様症状を誘発すること，また統合失調症患者の脳脊髄液中にはanandamideが増加していることも報告されている．これまで，われわれは内因性カンナビノイドシステムが覚せい剤メタンフェタミン探索行動（"渇望"）の発現[1]ならびにモルヒネ退薬症候の発現に関与すること[2]を報告した．さらにカンナビノイドの作用が，アラキドン酸カスケードの活性化を介してのプロスタグランジンE_2（PGE_2）の産生亢進にもとづくことを，レバー押し行動を用いて明らかにした[3]．これらの知見は精神疾患の病因に内因性カンナビノイドシステム-アラキドン酸カスケードが関与する可能性を示唆している．

本研究では認知機能の障害における内因性カンナビノイドの関与について，カンナビノイド関連薬物ならびにカンナビノイドCB1受容体欠損［CB1（−/−）］マウスを用いて，検討した．さらに，合成麻薬3, 4-methylenedioxy methamphetamine（MDMA）反復投与後の休薬時での認知機能障害における内因性カンナビノイドシステムの関与についてもあわせて検討した．

◆実験方法

本実験ではICR系雄性［CB1（+/+）］マウスならびに，CB1（−/−）マウスを用いた．認知機能の測定にはnovel object recognition課題を使用した．本課題では，①open field装置（直径70 cm）内に同一の金属製物体（高さ5 cmの球状物体）を2つ置き，マウスを10分間放置した（pre-trial）．その3時間後に②片方を形状のまったく異なる物体（円錐状物体）に置き換え，物体から1 cm外につけられた円内にマウスの四肢のいずれかが触れた時間をアプローチ時間として10分間測定した（test-trial）．アプローチ時間は，新奇物体へのアプローチ率（%）$=t_B/(t_A+t_B)\times100$として表記した（t_A：test-trialでの既存物体へのアプローチ時間，t_B：test-trialでの新奇物体へのアプローチ時間）．URB 597（anandamide分解酵素阻害薬）ならびにメロキシカム（COX阻害薬）は各々pre-trial前に投与した．また，MDMAの実験では，単回または7日間の反復投与後，休薬1日目および7日目に本課題実験を施行した．CB1受容体拮抗薬AM 251はURB 597またはMDMAと同時投与した．生化学的検討では，PGE_2量は酵素免疫法，CB1受容体蛋白量はウエスタンブロッティング法にて測定した．

◆結果

URB 597（0.32 mg/kg, i.p.）は，マウスの新奇物

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体へのアプローチ率を有意に低下させ，認知機能障害を惹起した．この障害作用はCB1受容体拮抗薬AM 251（3.2 mg/kg，i.p.）の投与により有意に抑制された．一方，CB1（−/−）マウスではURB 597による認知機能障害は発現しなかった．また，URB 597によるアプローチ率の低下作用は，COX阻害薬メロキシカム（1.0 mg/kg，i.p.）によって有意に抑制された．このURB 597による認知機能障害の発現時では，アラキドン酸カスケードの最終産物であるPGE_2産生量は前頭前皮質および海馬で有意に増加していた．このPGE_2産生増加作用も，メロキシカムにより有意に抑制された．

マウスの新奇物体へのアプローチ率は，MDMA単回投与後の1日目および7日目では変化がなかった（**図1**）．しかし，MDMA（10 mg/kg，i.p.）7日間の反復投与後，休薬1日目および7日目では，マウスの新奇物体へのアプローチ率は有意に低下し，認知機能障害が認められた（**図1**）．この障害も，MDMAとAM 251（3.2 mg/kg，i.p.）との7日間併用投与により抑制された．また，CB1（−/−）マウスでもこのMDMAによる認知機能障害はまったく認められなかった．MDMA休薬時における認知機能の障害発現時では，海馬のCB1受容体の発現量は有意に増加した（休薬7日目）．この増加は併用投与群では抑制される傾向が認められた．一方，前頭前皮質ではその休薬時において海馬同様に増加傾向が認められたが，線条体での変化は認められなかった．

◆考察・結論

脳内における内因性カンナビノイドの増加は認知機能障害を誘発し，この障害はCB1受容体を介してのアラキドン酸カスケードの活性化とこれにもとづくPGE_2産生亢進に基因することが示唆される．一方，MDMA休薬時に起こる認知機能障害もまた内因性カンナビノイド-アラキドン酸カスケードの活性化を介していることが推測される．またこの認知機能障害の発現の責任部位として，CB1受容体が高密度に存在する前頭前皮質および海馬が示唆される．これらのカンナビノイドの知見は，認知機能障害を伴う精神疾患および乱用性薬物による退薬症候の治療薬の開発に向けての新たな糸口を与えるものと期待される．

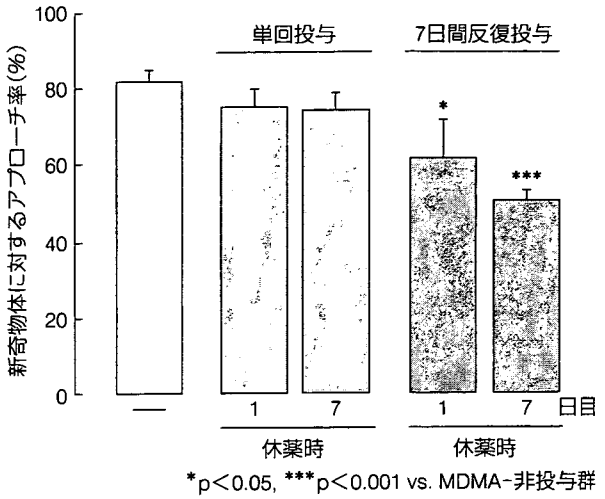

図1. ICR系マウスを用いてのMDMA（10 mg/kg）単回・反復投与後の休薬時での新奇物体に対するアプローチ時間（%）の変容

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Implication of protein kinase C in the orexin-induced elevation of extracellular dopamine levels and its rewarding effect

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Keywords: addiction, Ca^{2+}, orexin, PKC, reward

Abstract

In the present study, we investigated the role of orexinergic systems in the activation of midbrain dopamine neurons. In an *in vitro* study, exposure to either orexin A or orexin B under superfusion conditions produced a transient increase in the intracellular Ca^{2+} concentration through the phospholipase C (PLC)/protein kinase C (PKC) pathway via $G_{q11}\alpha$ or $G\beta\gamma$ subunits in midbrain cultured neurons, which were shown to be tyrosine hydroxylase (TH)-positive cells, but not in purified midbrain astrocytes. Here we show that *in vivo* injection with a selective PKC inhibitor chelerythrine chloride or 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido [1,2-a]indol-3-yl}-3-1-methyl-1H-indol-3-ylmaleimide HCl (Ro-32–0432) into the ventral tegmental area (VTA) significantly suppressed the place preference and increased levels of dopamine in the nucleus accumbens (NAcc) induced by intra-VTA injection of orexins. These results strongly support the idea that activation of the orexin-containing neuron in the VTA leads to the direct activation of mesolimbic dopamine neurons through the activation of the PLC/PKC pathway via $G_{q11}\alpha$ or $G\beta\gamma$-subunit activation, which could be associated with the development of its rewarding effect.

Introduction

Drugs of abuse can alter synaptic plasticity in the mesolimbic dopaminergic system, a region that is implicated in a variety of addictive behaviours. In particular, long-lasting changes at excitatory synapses in the nucleus accumbens (NAcc) and the ventral tegmental area (VTA) result from *in vivo* administration of drugs of abuse (Vanderschuren & Kalivas, 2000; Narita *et al.*, 2001a; Thomas *et al.*, 2001; Ungless *et al.*, 2001; Borgland *et al.*, 2004). Mesolimbic dopamine pathways are critical for a similar repertoire of motivated behaviours. In addition, excitatory synaptic transmission in VTA dopamine neurons is an important locus of neural plasticity induced by psychostimulant administration.

Orexins are neuropeptides synthesized in the neurons of the lateral hypothalamus (LH) that can elicit arousal, feeding, and appetitive behaviours (de Lecea, *et al.*, 1998; Sakurai *et al.*, 1998). There are two known orexins, orexin A and orexin B, whose actions are mediated by two G-protein-coupled receptors, termed orexin receptor type 1 (OX1R) and orexin receptor type 2 (OX2R). OX1R shows higher affinity for orexin A, while OX2R shows equal affinity for the two ligands (Sakurai *et al.*, 1998). The VTA and NAcc receive massive input from the LH area, including projections from neurons containing orexin A and orexin B (Fadel & Deutch, 2002).

Protein kinase C (PKC), which is activated by 1,2-diacylglycerol in the presence of Ca^{2+} and phospholipids, acts as a key enzyme for signal transduction in various physiological processes (Nishizuka, 1988, 1992, 1995). The protein phosphorylation catalysed by PKC may exert profound modulation of various processes, such as the release of neurotransmitters, cell proloferation and differentiation, potentiation and desensitization of several kinds of receptor systems (Nishizuka, 1988, 1992, 1995). Activators of PKC enhance both basal and depolarization-mediated dopamine release (Zurgil & Zisapel, 1985; Shu & Selmanoff, 1988; Giambalvo, 1989; Davi & Patrick, 1990; Kantor & Gnegy, 1998; Cowell *et al.*, 2000). Furthermore, an electrophysiological study has recently shown that orexins may directly activate VTA dopamine neurons (Korotkova *et al.*, 2003). In addition, we previously reported that *in vivo* treatment with orexin induced hyperlocomotion and dopamine release in the NAcc of rodents (Narita *et al.*, 2006). In the present study, we show that orexins activate the mesolimbic dopamine neurons through the activation of PKC via G-proteins, leading to the development of reward in rodents.

Materials and methods

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Every effort was made to minimize the number of animals used in the following experiments.

Animals

In the present study, we used male Sprague–Dawley rats and ICR mice (Tokyo Laboratory Animals Science Co, Ltd, Tokyo, Japan). Animals

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were housed in a room maintained at 23 ± 1 °C with a 12-h light : 12-h dark cycle (light on at 08:00 h to 20:00 h). Food and water were available *ad libitum*.

Surgery and microinjection

After 3 days of habituation to the main animal colony, all rats were anaesthetized with sodium pentobarbital (50 mg/kg, i.p.). The anaesthetized animals were placed in a stereotaxic apparatus. The skull was exposed and a small hole was made using a dental drill. A guide cannula (AG-9, Eicom Co, Kyoto, Japan) was implanted into the unilateral side of VTA (from bregma; posterior −5.3 mm, lateral −0.9 mm, ventral −7.7 mm) and/or unilateral side of NAcc (from bregma; anterior +4.0 mm, lateral −0.8 mm, ventral −6.8 mm, angle 16°) according to the atlas of Paxinos & Watson (1998). The guide cannula was fixed to the skull with cranioplastic cement. Three to five days after surgery, the animals were injected with an orexin A or orexin B (Peptide institute, Inc. Osaka, Japan) into the VTA. In the microinjection method, we used the injection cannula (AMI-9.5, Eicom Co) extended beyond the guide cannula by 0.5 mm. A stainless steel injection cannula was inserted into that guide cannula for each animal. The injection cannula was connected through polyethylene tubing to a 10-μL Hamilton syringe that was preloaded with orexin A, orexin B (0.1 or 1 nmol/0.3 μL) or saline. The orexin or saline was delivered by motorized syringe pump in a volume of 0.3 μL over 60 s.

Place conditioning

Place conditioning was conducted as described previously (Suzuki *et al.*, 1990). The apparatus was a shuttle box (30 cm wide × 60 cm long × 30 cm high) that was made of acrylic resin board and divided into two equal-sized compartments. One compartment was white with a textured floor and the other was black with a smooth floor to create equally preferable compartments. The place conditioning schedule consisted of three phases (preconditioning test, conditioning and postconditioning test). The preconditioning test was performed as follows; the partition separating the two compartments was raised to 7 cm above the floor, a neutral platform was inserted along the seam separating the compartments, and mice that had not been treated with either drugs or saline were then placed on the platform. The time spent in each compartment during a 900-s session was then recorded automatically with an infrared beam sensor (KN-80, Natsume Seisakusyo Co, Tokyo, Japan). Conditioning sessions (3 days for orexin, 3 days for saline) were conducted once daily for 6 days. Immediately after orexin intra-VTA microinjection, these animals were placed in the compartment opposite that in which they had spent the most time in the preconditioning test for 1 h. On alternative days, these animals received saline and were placed in the other compartment for 1 h. On the day after the final conditioning session, a postconditioning test that was identical to the preconditioning test was performed. The sensitive GTP-binding protein petussis toxin (PTX, Calbiochem-Novabiochem Corp, San Diego, CA, USA), a selective PKC inhibitor, chelerythrine chloride (Sigma–Aldrich Co. St. Louis, MO, USA) or 2-{8-[(dimethylamino)methyl]-6,7,8,9-tetrahydropyrido[1,2-a]indol-3-yl}-3-1-methyl-1H-indol-3-ylmaleimide HCl (Ro-32–0432, Calbiochem-Novabiochem Corp) was injected into the VTA 10 min before the start of conditioning with orexin. These inhibitors were delivered by motorized syringe pump in a volume of 0.3 μL over 60 s.

In vivo *microdialysis study and quantification of dopamine*

Three to 5 days after the surgery, microdialysis probes (AI-8–2; 2 mm membrane length, Eicom Co) were slowly inserted into the NAcc through guide cannulas under anaesthesia with diethyl ether and rats were settled in the experimental cages (width 30 cm × depth 30 cm × height 30 cm). The probes were perfused continuously at a flow rate of 2 μL/min with artificial cerebrospinal fluid (aCSF) containing 0.9 mM $MgCl_2$, 147.0 mM NaCl, 4.0 mM KCl and 1.2 mM $CaCl_2$. The outflow fractions were taken every 20 min. Following the collection of three baseline fractions in the rat NAcc, orexin A, orexin B or saline was treated into the rat VTA using a 10-μL Hamilton syringe and motorized syringe pump. For these experiments, dialysis samples were collected for the next 180 min after orexin A or orexin B treatment. The PTX, chelerythrine chloride or Ro-32–0432 was injected into the VTA 10 min before the start of challenge treatment with orexins. These inhibitors were delivered by motorized syringe pump in a volume of 0.3 μL over 60 s. Dialysis fractions were then analysed using high-performance liquid chromatograph (HPLC; Eicom Co) with electrochemical detection (ECD; Eicom Co) system. Dopamine was separated by a column with mobile phase containing sodium acetate (4.05 g/L), citric acid monohydrate (7.35 g/L), sodium 1-octane sulphonate (170 mg/L), EDTA (2Na; 10 mg/L), and 17% methanol. The mobile phase was delivered at a flow rate of 210 μL/min. Identification of dopamine was determined according to the retention times of dopamine standard, and amounts of dopamine were quantified by calculating with peak areas.

Tissue processing and Ca^{2+} imaging

Midbrain neuron/glia cocultures were grown as follows; the midbrain including the VTA was obtained from newborn ICR mice, minced, and treated with papain (9 unit/mL; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. Animals were deeply anesthetized with diethyl ether and killed. After enzyme treatment at 37 °C for 15 min, cells were seeded at a density of 2×10^6 cells/cm^2. The cells were maintained for 7 days in Dulbecco's modified Eagle's medium supplemented with 10% precolostrum newborn calf serum, 10 U/mL penicillin and 10 μg/mL streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. On day 8, the cells were treated with drugs. Cells were loaded with 10 μM fluo-3AM (Wako Pure Chemical Ind, Ltd, Osaka, Japan) for 60 min at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance, 2000, Bio-Rad Laboratories, Inc, Hercules, CA, USA). Fluo-3 was excited with the 488 nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm. To compensate for the uneven distribution of fluo-3, self-ratios were calculated ($R_s = F/F_0$).

Delivery of $G_{q11}\alpha$ or Gβ-subunit antibody into neurons/glia

Antibody for $G_{q11}\alpha$- or Gβ-subunit (Santa Cruz Biotechnology, Inc) was incorporated using a protein-transfection kit (ChariotTM, Active Motif Japan, Tokyo, Japan). We used the manufacturer's protocol for the incorporation of antibody. Briefly, cocultured neurons/glia were incubated with various concentrations of $G_{q11}\alpha$- or Gβ-subunit antibody (1 : 500 to 1 : 2000) and an appropriate concentration of Chariot™ for 1 h at 37 °C in serum-free culture medium. Cells were then incubated with serum-containing medium for an additional 2 h and used for Ca^{2+} imaging experiments.

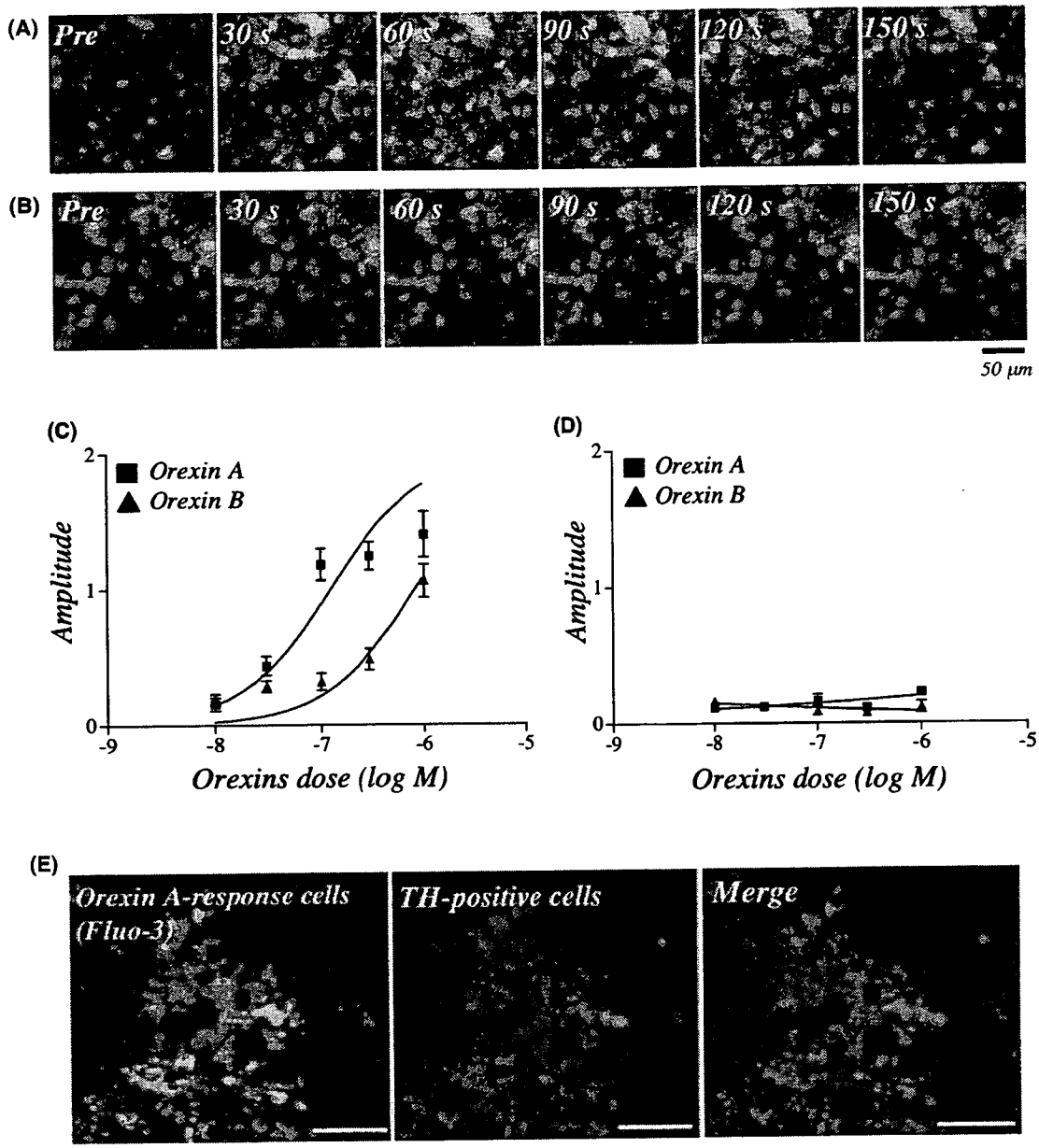

FIG. 1. Mouse midbrain neurons show calcium influx in response to orexins. A and B show sequential images of the orexin A-evoked increase in the intracellular Ca^{2+} concentration in the neuron/glia coculture cells or purified midbrain astrocyte cells as measured by fluo-3 based on the confocal Ca^{2+} imaging method. Scale bar, 50 μm. (C–D) Dose–response curve for orexin A- or orexin B-induced intracellular Ca^{2+} concentration increase in mouse midbrain primary neuron/glia cocultures (C) or mouse midbrain purified astrocyte cells (D). (E) Double-labelling experiments showed that orexin A response cells (fluo-3AM response cells) were highly overlapped with a selective dopamine neuron marker, tyrosine hydroxylase (TH), in midbrain culture cells (≥ over 80% overlap). Scale bar, 50 μm.

Drugs

The drugs used in the present study were orexin A or orexin B (Peptide institute, Inc), Ro-32–0432, PTX, 1-[6-[[(17β)-3-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1H-pyrrole-2,5-dione (U-73122), 2′-amino-3′-methoxyflavone (PD98059, Calbiochem-Novabiochem Corp), chelerythrine chloride, nifedipine and thapsigargin (Sigma–Aldrich Co).

Histology

Determination of the location of the infusion cannula placements and drug diffusion was assessed at the completion of the experiments. Rats were deeply anaesthetized with sodium pentobarbital (50 mg/kg, i.p.) at the end of the experiment, given microinjections of ink for anatomical localization of cannula sites (0.3 μL). The brain was then removed by decapitation and cut into coronal sections. Cannula placements were mapped onto a stereotaxic atlas (Paxinos & Watson, 1998) and confirmed to be in the VTA.

Statistical data analysis

The data are presented as the mean ± SEM. The statistical significance of differences between the groups was assessed by one-way or two-way analysis of variance (ANOVA) followed by the Bonferroni/Dunnett test or Student's *t*-test.

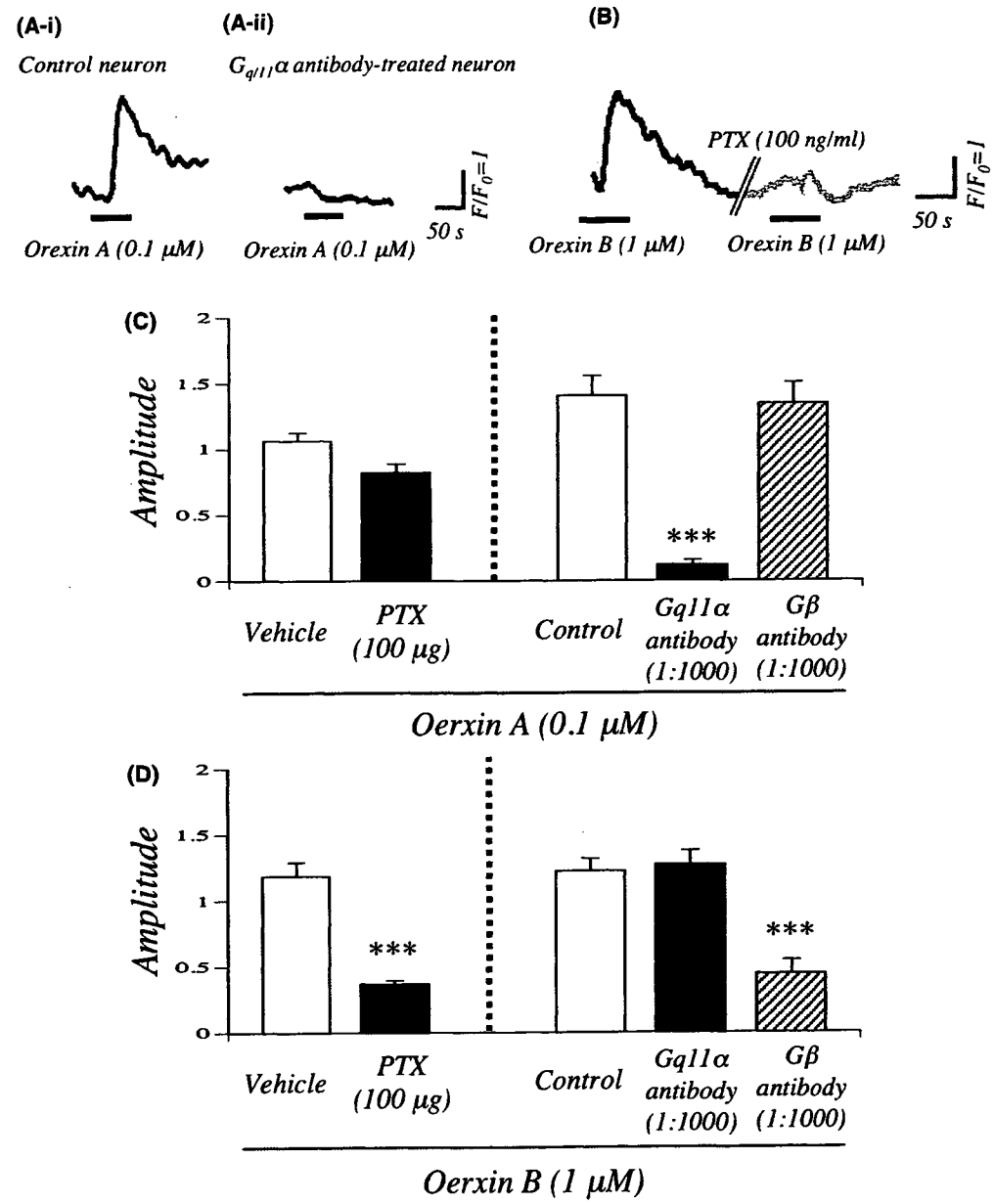

FIG. 2. Orexin A-induced Ca^{2+} response in mouse midbrain neurons was $G_{q11}\alpha$-dependent, whereas Gβ was implicated in the effect of orexin B. Traces in A-i-ii and B show the orexin A- or B-evoked increase in intracellular Ca^{2+} concentration and the blocking effect of $G_{q11}\alpha$ antibody or PTX on this response. C and D show intracellular Ca^{2+} concentration in response to orexin A and orexin B in control, PTX-treated neurons and $G_{q11}\alpha$ or Gβ antibody-transfected neurons. $G_{q11}\alpha$ and Gβ antibodies were incorporated into neurons using a protein-transfection reagent according to the manufacturer's protocol. The intracellular Ca^{2+} concentration response to orexin B was suppressed by PTX, whereas the orexin A-induced intracellular Ca^{2+} concentration response was not affected by PTX. The response of intracellular Ca^{2+} concentration to orexin A was almost abolished by $G_{q11}\alpha$-subunit antibody, whereas the orexin B-evoked intracellular Ca^{2+} concentration increase was significantly suppressed by Gβ-subunit antibody, but not by $G_{q11}\alpha$-subunit antibody. Significant differences with respect to the control were evaluated by Student's *t*-test. Data represent the mean ± SEM of 54–118 cells from two separated observations. ***$P < 0.001$ vs. control.

Results

Orexin-induced Ca^{2+} response in tyrosine hydroxylase-positive neurons in the mouse midbrain primary culture but not midbrain purified astrocyte

Exposure to either orexin A (0.01–1 μM) or orexin B (0.01–1 μM) under superfusion conditions produced a transient increase in the intracellular Ca^{2+} concentration on a dose-dependent manner in midbrain dopamine neurons (Fig. 1), which were subsequently shown to be TH-positive cells (over 80% overlap). However, neither an orexin A- nor an orexin B-evoked intracellular Ca^{2+} concentration increase was observed in purified midbrain astrocytes.

Ca^{2+} response of orexins was induced by activation of PLC/PKC pathway via $G_{q11}\alpha$- or $G\beta$-protein activation in the primary culture neuron

Pretreatment with PTX (100 ng/mL), which is sensitive to GTP-binding proteins Gi and Go, significantly inhibited orexin B-evoked intracellular Ca^{2+} concentration increase in neurons (Fig. 2C and D; $P < 0.001$ vs. vehicle-treated cells). However, PTX had no effect on the orexin A-evoked Ca^{2+} influx. The intracellular Ca^{2+} concentration response to orexin A was almost abolished by $G_{q11}\alpha$-subunit antibody ($P < 0.001$ vs. control cells), whereas orexin B-evoked intracellular Ca^{2+} concentration increase was suppressed by $G\beta$-subunit antibody ($P < 0.001$ vs. control cells), but not $G_{q11}\alpha$-subunit antibody. This orexin A- or orexin B-evoked $[Ca^{2+}]_i$ increase was blocked by a PLC inhibitor, U-73122 (5 μM; $P < 0.001$ vs. vehicle-treated cells, Fig. 3A and B) and a specific PKC inhibitor, chelerythrine chloride (1 μM; $P < 0.001$ vs. vehicle-treated cells) and Ro-32–0432 (1 μM; $P \leq 0.001$ vs. vehicle-treated cells), but not specific extracellular signal-regulated kinase (ERK) kinase (MEK) inhibitors, PD98059 (50 μM) and U-0126 (data not shown). In addition, either orexin A- or orexin B-induced intracellular Ca^{2+} concentration increase was suppressed by nifedipine, a voltage dependent calcium channel blocker (50 μM; $P < 0.001$ vs. vehicle-treated cells) and thapsigargin, a sarcoplasmic-endoplasmic reticulum (ER) Ca^{2+}-ATPase inhibitor (100 nM; $P < 0.001$ vs. vehicle-treated cells).

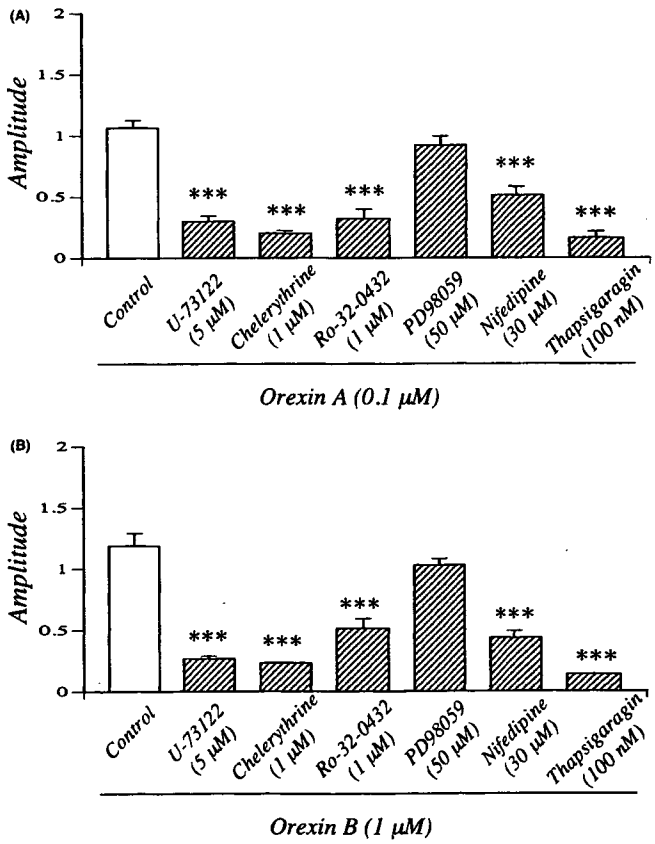

FIG. 3. Characterization of orexin-evoked Ca^{2+} signalling in mouse midbrain neurons. (A and B) Effect of U-73122, chelerythrine chloride, Ro-32–0432, PD98059, nifedipine or thapsigargin on the orexin A- or orexin B-induced increase in intracellular Ca^{2+} concentration in primary cultured midbrain neurons. Data represent the mean ± SEM of 54–118 cells from two separated observations. ***$P < 0.001$ vs. vehicle-treated cells.

Orexin-induced PKC-dependent place preference and dopamine release in rats

To clarify further orexin-activated dopamine neurons, rats were treated with intra-VTA microinjection of orexin A or orexin B. Figure 4A shows the placement of microinjection cannulas within the rat VTA. Guide cannulas were localized above the VTA. Only data from rats in which guide cannulas had been accurately inserted in the VTA were used for subsequent statistical analysis. The diffusion of the microinjection with 0.3 μL volume was strictly observed inside the VTA area. An intra-VTA microinjection with saline produced no preference for either place; the mean conditioning score was 16.6 ± 16.8 s (Fig. 4B and C). An intra-VTA microinjection of orexin A (0.1 and 1 nmol/rat) produced a dose-dependent place preference (Fig. 4B, 0.1 nmol/rat 93.6 ± 12.6 s, $P < 0.01$ vs. saline-conditioned group; 1 nmol/rat 126.4 ± 13.6 s, $P < 0.001$ vs. saline-conditioned group). Similarly, a significant place preference was also seen with an intra-VTA microinjection of orexin B at 0.1 and 1 nmol/rat (Fig. 4C, the mean conditioning scores were

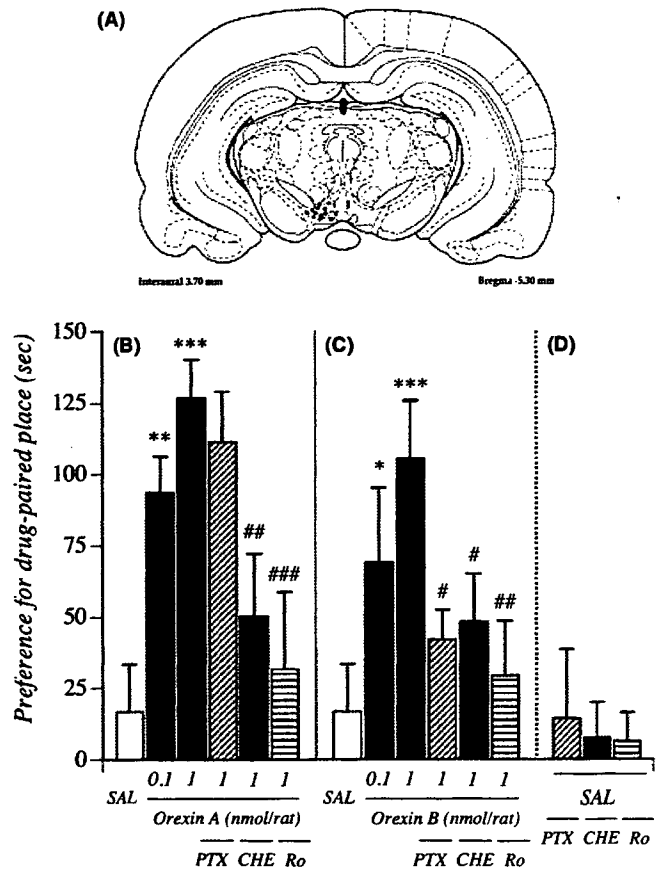

FIG. 4. PKC-dependent rewarding effect induced by orexins in rats. (A) Localization of microinjection sites in the rat ventral tegmental area (VTA). Closed black circles show the region in the rat brain in which a cannula was inserted. (B–D) Effect of the microinjection of PTX, chelerythrine chloride (CHE) or Ro-32–0432 (Ro) into the VTA on the orexin-induced place preference. Rats were pretreated with PTX or chelerythrine chloride 10 min before the start of conditioning with orexin. Ordinate – preference for drug-paired place, as defined by the postconditioning test score minus preconditioning test score in the drug-treated side. The data represent the mean ± SEM of 4–8 rats. *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs. saline-conditioned rats. #$P < 0.05$, ##$P < 0.01$ and ###$P < 0.001$ vs. orexin A- or orexin B-conditioned rats.

68.9 ± 26.3 and 105.6 ± 20.1 s, $P < 0.05$, $P < 0.001$ vs. saline-conditioned rats, respectively). Under these conditions, intra-VTA pretreatment with PTX (0.5 μg/rat) suppressed the place preference induced by orexin B, but not orexin A (orexin A 111.3 ± 17.6 s, orexin B 41.8 ± 10.5 s, $P < 0.05$ vs. orexin B 1 nmol-conditioned rats). In addition, pretreatment with the intra-VTA administration of chelerythrine chloride (50 ng/rat) or Ro-32–0432 (1 nmol/rat) markedly suppressed the place preference induced by orexins (chelerythrine chloride + orexin A; 50.2 ± 22.0 s, $P \leq 0.01$ vs. orexin A 1 nmol-conditioned rats, chelerythrine chloride + orexin B; 48.0 ± 16.8 s, $P \leq 0.05$ vs. orexin B 1 nmol-conditioned rats, Ro-32–0432 + orexin A; 31.5 ± 27.2 s, $P \leq 0.001$ vs. orexin A 1 nmol-conditioned rats, Ro-32–0432 + orexin B; 29.2 ± 19.1 s, $P \leq 0.05$ vs. orexin B 1 nmol-conditioned rats). Neither PTX, chelerythrine chloride nor Ro-32–0432 alone induced either significant place preference or place aversion in rats; the mean conditioning scores were 14.1 ± 24.2 s for PTX, 7.4 ± 12.4 s for chelerythrine chloride and 6.1 ± 10.2 s for Ro-32–0432 (Fig. 4D).

The effect of intra-VTA administration of orexin A or orexin B on the dialysate dopamine level in the NAcc is shown in Figs 5 and 6. In the microdialysis study, there was no difference in the basal dialysate levels of dopamine, DOPAC and HVA in the NAcc between saline-pretreated and chelerythrne chloride-pretreated rats (Table 1). The dopamine level was markedly increased by the injection of orexin A or orexin B compared to saline treatment ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ vs. saline + saline-treatment rats). The injection of orexins into the VTA also produced a significant increase in a major dopamine metabolite DOPAC ($P \leq 0.05$ and $P \leq 0.01$ vs. saline-pretreated saline-treatment rats). Similarly, orexins increased the level of another dopamine metabolite, HVA ($P \leq 0.05$ and $P \leq 0.01$ vs. saline + saline-treatment rats). Under these conditions, the release of dopamine and its metabolites induced by orexins was significantly suppressed by intra-VTA pretreatment with chelerythrine chloride or Ro-32–0432 ($P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$ vs. saline + orexin A- or orexin B-treatment rats).

Discussion

Using primary neuronal cell cultures obtained from newborn mice, the activation of orexin receptors induced by either orexin A or orexin B produced a robust increase in the intracellular Ca^{2+} concentration through activation of PKC via G-proteins in the mouse dopamine neuron. Furthermore, intra-VTA administration of orexin yielded a marked increase in the rewarding effect and release of dopamine in the

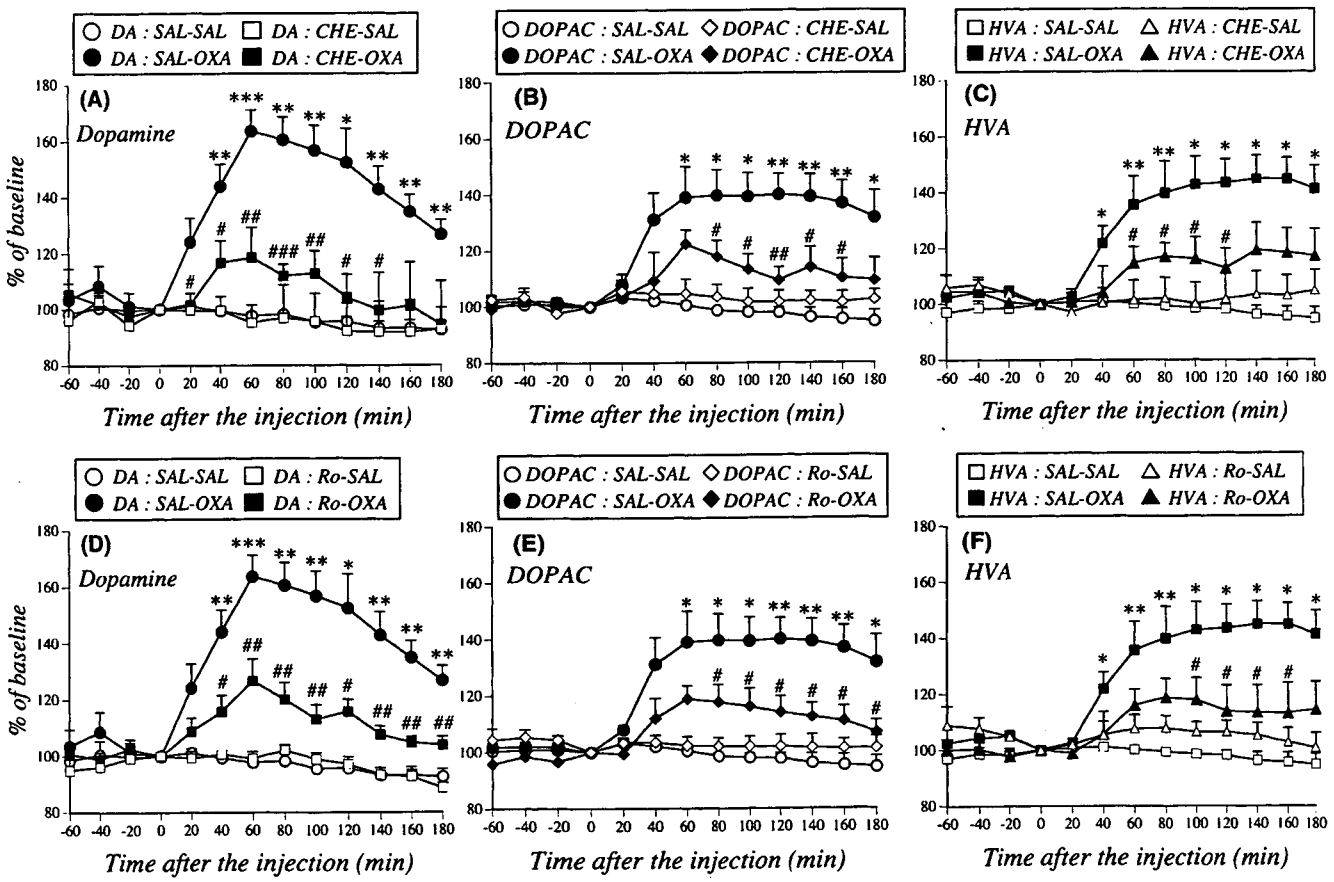

FIG. 5. Implication of PKC in the VTA on the release of dopamine in the NAcc induced by intra-VTA treatment with orexin A. (A–F) Effect of the microinjection of chelerythrine chloride (CHE) or Ro-32–0432 (Ro) into the VTA on the orexin A (OXA)-induced increase in dialysate dopamine and its metabolites level in the NAcc. Following the collection of baseline fractions, rats were injected with saline (SAL) or orexin A (OXA, 1 nmol) into the VTA at time 0 to evoke the release of dopamine. The chelerythrine chloride was injected into the VTA 10 min before the start of challenge treatment with orexin A. Data are expressed as percentages of the corresponding baseline levels with SEM for three to six rats. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. saline + saline-treatment rats. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ vs. saline + orexin A-treatment rats. The statistical significance of differences was assessed with Student's *t*-test.

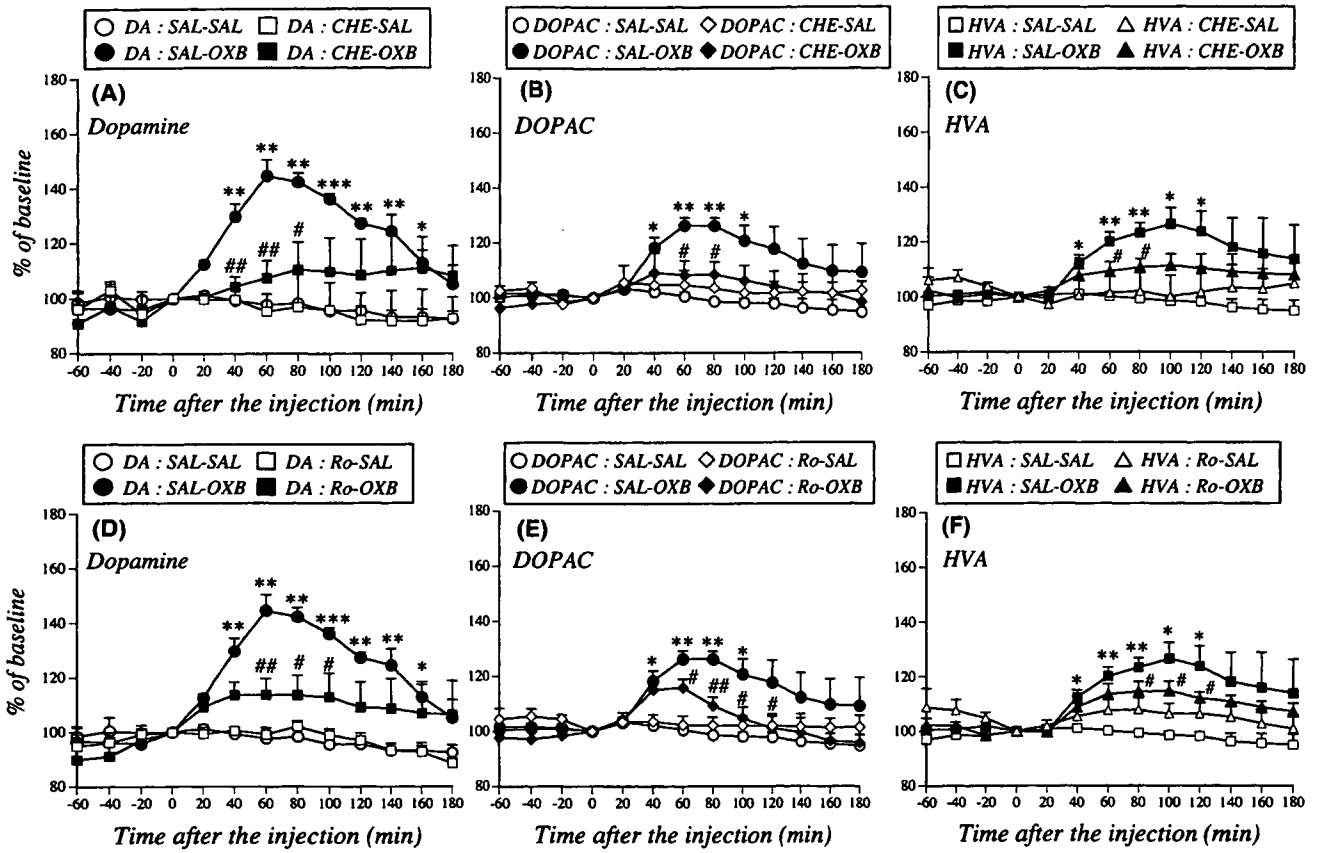

FIG. 6. Implication of PKC in the VTA on the release of dopamine in the NAcc induced by intra-VTA treatment with orexin B. (A–F) Effect of the microinjection of chelerythrine chloride (CHE) or Ro-32–0432 (Ro) into the VTA on the orexin B-induced increase in dialysate dopamine and its metabolites level in the NAcc. Following the collection of baseline fractions, rats were injected with saline (SAL) or orexin B (OXB, 1 nmol) into the VTA at time 0 to evoke the release of dopamine. The chelerythrine chloride was injected into the VTA 10 min before the start of challenge treatment with orexin B. Data are expressed as percentages of the corresponding baseline levels with SEM for three to six rats. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. saline + saline-treatment rats. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ vs. saline + orexin B-treatment rats. The statistical significance of differences was assessed with Student's *t*-test.

TABLE 1. Basal dialysate levels of dopamine and its metabolites in the nucleus accumbens in saline-, cherelythrine chloride- and Ro-32–0432-pretreated rats

	Basal dialysate levels (nM)		
Treatment group	Dopamine	DOPAC	HVA
Saline-pretreated	1.18 ± 0.17	215.7 ± 24.7	105.8 ± 6.2
Cherelythrine-pretreated	1.16 ± 0.12	236.4 ± 21.1	101.9 ± 6.3
Ro-32–0432-pretreated	1.21 ± 0.12	266.6 ± 14.1	116.0 ± 16.0

The data represent the mean ± SEM of 10–14 rats. The statistical significance of differences between groups was assessed with one-way analysis of variance (ANOVA) followed by the Boneferroni/Dunnett test.

NAcc. Although it has been already reported that *in vitro* treatment with the selective PKC inhibitor prevents the orexin-induced increase in intracellular Ca^{2+} concentration (Uramura *et al.*, 2001; Muroya *et al.*, 2004), in the present study we found for the first time, that *in vivo* treatment with the selective PKC inhibitor, chelerythrine chloride, significantly suppressed the development of the rewarding effect and reduced the increased dopamine release in the NAcc induced by orexins in rodents.

Activation of the orexin receptor results in PLC-catalysed phosphoinositide (PI) hydrolysis, which leads to the release of Ca^{2+} from intracellular sources and stimulation of PKC (Uramura *et al.*, 2001; Muroya *et al.*, 2004). As previously reported, orexin increases the intracellular Ca^{2+} concentration in rat VTA dopamine neurons via phosphatidylcholine-specific PLC and PKC (Uramura *et al.*, 2001). PKC is a key regulatory enzyme that modulates both presynaptic and postsynaptic neuronal function, the synthesis and release of neurotransmitters, and the regulation of receptors (Narita *et al.*, 2001b). Taken together, these findings support the idea that the activation of PKC in the VTA dopamine neuron following treatment with orexins leads to the increase in dopamine release in the NAcc, which is responsible for the development of the orexin induced-rewarding effect.

The VTA contributes to many forms of drug reinstatement (Shalev *et al.*, 2002). The systemic administration of psychostimulants and opioids has been shown to yield an increase in the firing rate of dopaminergic neurons in the VTA and to produce a dopamine receptor antagonist-sensitive rewarding effect (Matthews & German, 1984; Bals-Kubik *et al.*, 1993; Vanderschuren & Kalivas, 2000; Narita *et al.*, 2001a). We previously reported that the development of morphine-induced rewarding effect was blocked by intra-VTA injection of an orexin receptor antagonist and was abolished in prepro-orexin

knockout mice (Narita *et al.*, 2006). Furthermore, a double-label immunohistochemical study demonstrated that morphine- or cocaine-conditioned rats showed the enhancement of Fos activation in lateral hypothalamus orexin neurons (Harris *et al.*, 2005). Taken together, activation of VTA orexin neurons, which directly originates from the LH, may be implicated in the development of the rewarding effect induced by drugs of abuse.

Neurons respond to various electrical and chemical stimuli, including neurotransmitters, neuromodulators and hormones, with an increase in the intracellular Ca^{2+} concentration. These Ca^{2+} responses result from the co-ordinated activity of several molecular cascades responsible for Ca^{2+} movement into or out to the cytoplasm by way of either the extracellular space or intracellular stores. In addition, increased intracellular Ca^{2+} concentration plays a key role in neural plasticity relevant to addiction. Furthermore, PKC activity is stimulated by a rise in the intracellular Ca^{2+} concentration. Activation of PKC in the neuron is also involved in neural plasticity as well as increase in intracellular Ca^{2+} concentration in neurons. Accumulating evidence suggests that repeated administration of psychostimulants induces long-lasting synaptic and neuronal plasticity (Nestler, 2001). Long-lasting synaptic and/or neural adaptations in dopaminergic neurotransmission provide a neuronal framework for altered behavioural processing which underlies the development of psychological dependence on drugs of abuse. Orexin potentiates *N*-methyl-D-aspartate (NMDA) receptor excitatory postsynaptic currents (EPSCs) in VTA dopamine neurons via activation of orexin receptors and stimulation of PLC/PKC signal transduction pathways (Borgland *et al.*, 2006). In the present study, we found that either orexin A or orexin B increased intracellular Ca^{2+} concentration via stimulated Ca^{2+} influx through voltage-gated Ca^{2+} channels and sarcoplasmic-endoplasmic reticulum in the VTA dopamine-containing neurons. This effect was mediated by activation of phosphatidylcholine-specific PLC and PKC via $G_{q11}\alpha$- or $G\beta\gamma$ (probably Gi/o protein)-subunit activation. Collectively, these findings suggest a potential role of orexin signalling in neural and/or synaptic plasticity related to dopaminergic network.

In the present Ca^{2+} imaging study, we found that the potency of orexin A for either orexin-induced intracellular Ca^{2+} response was greater than that of orexin B. Furthermore, the orexin A-induced Ca^{2+} response was $G_{q11}\alpha$-dependent, whereas $G\beta$ (probably $G\beta\gamma$ complex) was implicated in the effect of orexin B. As previously reported, OX1R can be activated by endogenous orexin A and orexin B. In contrast, OX2R is predominantly responsible for the effect of orexin B. It has been proposed that OX1R and OX2R play quite distinct roles within the CNS, in that they depolarize distinct populations of monoaminergic neurons in the CNS, with orexin 1 receptor playing a greater role in noradrenergic neurons and OX2R playing a greater role in serotonergic neurons (Soffin *et al.*, 2004). Recently, an electrophysiological study indicated that both orexin A and orexin B directly depolarize and activate VTA dopamine neurons (Korotkova *et al.*, 2003). In the present study, when orexin A and orexin B were administered to the VTA, they both produced place preference and dopamine release in the NAcc. Taken together, these findings support the idea that although orexin A and orexin B may differentially regulate their related receptors with quite distinct intracellular mechanisms, both endogenous orexin A and orexin B are likely to be involved in the activation of dopamine neurons.

In conclusion, activation of orexin-containing neurons in the VTA leads to the direct activation of mesolimbic dopamine neurons through the activation of the PLC/PKC pathway via $G_{q11}\alpha$ or $G\beta\gamma$ (probably Gi/o protein)-subunit activation, which could be associated with the development of its rewarding effect.

Acknowledgements

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Abbreviations

LH, lateral hypothalamus; MEK, extracellular signal-regulated kinase kinase; NAcc, nucleus accumbens; OX1R, orexin receptor type 1; OX2R, orexin receptor type 2; PKC, protein kinase C; PLC, phospholipase C; PTX, petussis toxin; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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