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Characterization of endogenous serotonin-mediated regulation of dopamine release in the rat prefrontal cortex

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Abstract

Endogenous serotonin (5-hydroxytryptamine, 5-HT)-mediated regulation of dopamine release in the rat prefrontal cortex was pharmacologically characterized using in vivo microdialysis. To increase synaptic 5-HT availability, a selective 5-HT uptake inhibitor fluoxetine was applied via the dialysis probe. Local perfusion of fluoxetine (30 and 100 μM) increased dopamine levels in a concentration-dependent manner. The fluoxetine (100 μM)-induced increases in dopamine release were abolished by pretreatment with the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 (*N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1-biphenyl]-4-carboxamide]) (10 and 100 μM). The facilitation of dopamine release was also prevented by selective inactivation of the mRNA encoding 5-HT_6 receptors using antisense oligonucleotides techniques. These findings suggest that not only 5-HT_{1B} receptors but also 5-HT_6 receptors are associated with the endogenous 5-HT-mediated facilitation of dopamine release. In other words, 5-HT_6 receptors may play, in part, a significant role in the functional interaction between the dopaminergic and serotonergic neuronal system in the rat prefrontal cortex.

Keywords: Dopamine release; 5-HT_{1B} receptor; 5-HT_6 receptor; Cortex, prefrontal; (Rat)

1. Introduction

Anatomical studies have shown that the serotonergic neurons of the dorsal raphe nucleus project to dopaminergic cell bodies in the substantia nigra and ventral tegmental area, and to the dopamine terminal field such as striatum, nucleus accumbens and cortex (Herve et al., 1987). These reciprocal innervations suggest that dopamine neurotransmission is influenced by serotonergic neuronal activity at the somatodendritic or at the terminal levels. Indeed, a large number of electrophysiological or biochemical studies have provided evidence for the functional regulation by serotonin (5-hydroxytryptamine, 5-HT) of the dopamine neuronal system in the rat brain. However, these studies have shown conflicting results depending upon the brain regions or experimental approaches used. For instance, electrophysiological studies have shown that 5-HT exerts an inhibitory input to dopamine cell bodies in the ventral tegmental area (Prisco et al., 1994) and the substantia nigra (Kelland et al., 1990). In the terminal regions, such as the striatum or nucleus accumbens, in vitro studies report that 5-HT causes both inhibitory (Ennis et al., 1981; Westfall and Tittermary, 1982) and facilitatory effects (Blandina et al., 1989). In vivo microdialysis studies consistently indicate that 5-HT stimulates striatal dopamine release, and further suggest that the multiple 5-HT receptors appear to be associated with this facilitation (Benloucif et al., 1993; Santiago et al., 1995; Steward et al., 1996). Although the reasons for these discrepancies are unclear, they may be due to the complex effects exerted by 5-HT acting on different receptor subtypes or different brain regions.

The prefrontal cortex, which receives a dense dopamine innervation originating from the ventral tegmental area, is known to be the site of convergence of dopaminergic and serotonergic projections compared with other cortical re-

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gions. Recently, there has been increasing interest in the contribution of the 5-HT system in the modulation of dopamine neurotransmission in respect to the mechanism of atypical neuroleptics (Meltzer, 1995). Little is known, however, about the physiological regulation of dopamine release by the serotonergic nervous system in this terminal region.

The present study was undertaken to characterize endogenous 5-HT-mediated dopamine release in the rat prefrontal cortex using in vivo microdialysis. For this purpose, a selective 5-HT uptake inhibitor, fluoxetine, was applied locally via the dialysis probe to increase synaptic 5-HT availability. The possible involvement of 5-HT receptors in the modulation of dopamine release was examined by application of 5-HT receptor antagonists. Furthermore, attention was focused on the contribution of the 5-HT_6 receptors because binding studies have revealed that 5-HT_6 receptors exhibit high affinity for a number of antipsychotics and antidepressants (Monsma et al., 1993; Roth et al., 1994). Some of these drugs are suggested to have therapeutic effects as a result of the interaction of 5-HT and dopamine neurotransmission (Meltzer, 1995). Moreover, various histochemical approaches have consistently shown that 5-HT_6 receptor mRNA is abundant in the olfactory tubercle, nucleus accumbens and striatum, and to a lesser extent in the cerebral cortex (Siebert and Larrik, 1992; Gérard et al., 1997; Yoshioka et al., 1998). It is to be noted that these areas are rich in dopaminergic terminals. Although little is known about the physiological role of 5-HT_6 receptors because of the lack of selective drugs, these findings suggest the possible association of 5-HT_6 receptors with a functional interaction between the serotonergic and dopaminergic neuronal systems. The present experiment was performed using the antisense oligonucleotides approach, to selectively inhibit the expression of 5-HT_6 receptors, in combination with in vivo microdialysis.

2. Materials and methods

2.1. Animals

Twelve- to sixteen-week-old male Wistar rats were used. The rats were housed in plastic cages under conditions of constant temperature (21°C) on a 12-h light (7:00 a.m. to 7:00 p.m.)/dark (7:00 p.m. to 7:00 a.m.) cycle with free access to food and water. All handling of animals was performed in accordance with the ''Guidelines for the Care and Use of Laboratory Animals of the Animal Research Committee'' of Hokkaido University School of Medicine.

2.2. Electrical stimulation at the dorsal raphe nucleus

A bipolar electrode was inserted in the dorsal raphe nucleus with a posterial angle of 30° and the following coordinates: posterial, +11.4 mm; lateral, 0.0 mm; ventral, −6.0 mm from bregma and dural surface (in some cases, the position of electrode was as follows: posterial, +7.8 mm; lateral, +2.0 mm; ventral, −5.8 mm from bregma and the dural surface and at an angle of 15°) after the implantation of a dialysis guide cannula into the prefrontal cortex. The electrode was connected via current isolation (ss-302J, Nihon Kohden, Japan) to a stimulator (SEN-3301, Nihon Kohden), the output of which was monitored using an oscilloscope (AD-5141, A and D, Tokyo, Japan). Electrical stimulation was performed at 0.1, 0.3 and 0.6 mA intensity with a constant stimulation frequency at 10 Hz for a period of 20 min. To confirm electrode placement, histological examination by actual recording was performed during the implantation of the electrode.

2.3. In vivo microdialysis procedure

A guide cannula was stereotaxically implanted into the prefrontal cortex (probe tip; rostral–caudal +3.2 mm, lateral +0.7 mm, ventral −1.0 mm, from bregma and dural surface) under ketamine anesthesia (100 mg/kg, i.p.). Two days after surgery, a concentric dialysis probe with a 3-mm tip (0.22 μm O.D. regenerated cellulose 50,000 MW cut-off, Eicom, Kyoto, Japan) was inserted through the guide cannula and perfused continuously (1 μl/min) with Ringer's solution (NaCl 147 mM, KCl 4 mM, $CaCl_2$ 3.4 mM). In this study, we used a high concentration of Ca^{2+} to elevate dialysate dopamine to detectable levels because we could not always identify the dopamine peak with 1–2 mM of Ca^{2+} with the present assay system. Sampling was started 140–180 min after implantation of the probe. Successive 20-μl samples were collected at 20-min intervals in vials containing 10 μl of 0.05 N acetic acid and were injected directly onto the high-performance liquid-chromatography (HPLC) column. Fluoxetine, dissolved in Ringer's solution immediately before use, was perfused locally through the microdialysis probe over a period of 40 min. 5-HT receptor antagonists were applied locally 40 min before fluoxetine administration and co-perfused with fluoxetine. At the end of each experiment, the precise insertion site of the dialysis probe was examined histologically.

Simultaneous determination of extracellular levels of dopamine and 5-HT was done using an HPLC-electrochemical detector (ECD) system as previously described (Matsumoto et al., 1998). Briefly, the HPLC-ECD system consisted of a reverse-phase ODS column (CA-50DS, Eicom, Kyoto, Japan), an ECD-100 (Eicom) attached to a Signal Cleaner (SC77, Sic, Tokyo, Japan). A graphite working electrode (WE-3G, Eicom) was maintained at 450 mV against an Ag/AgCl reference electrode. The mobile phase consisted of 0.1 M sodium dihydrogenphosphate/0.1

M disodium hydrogenphosphate buffer (pH 6.0) with 1.85 mM octanesulfonate and 0.15 mM EDTA-2Na. Depending on column conditions, 18–20% (v/v) methanol was added to this solution. Concentrations of the dopamine metabolite, homovanillic acid (HVA), and the 5-HT metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the dialysate were determined by a different HPLC-ECD system: a reverse-phase ODS column (MA-50DS, Eicom) was attached and the applied potential was maintained at 600 mV against an Ag/AgCl electrode (WE-3G, Eicom). The mobile phase was composed of 0.1 M citric acid/0.1 M sodium acetate buffer (pH 3.5) with 1.06 mM octanesulfonate and 10 μM EDTA-2Na and 20 % (v/v) of methanol.

2.4. Antisense oligonucleotide synthesis and treatment

Oligonucleotide synthesis and treatment were performed following the method reported by Bourson et al. (1995): the antisense oligonucleotide sequence of 5′-GCC TGG CTC TGG AAC CAT-3′ was designed and synthesized based on one through 18 of the rat 5-HT_6 cDNA sequence. As a control, the scrambled oligonucleotide sequence of 5′-CGC TCA GTC ATC GGA GTC-3′ was used. These 18-mer phosphorothiate oligodeoxynucleotide, purified using HPLC, were stored at −70°C. These oligomers were dissolved in artificial cerebrospinal fluid (aCSF) (KCl 2.7 mM, NaCl 140 mM, $CaCl_2$ 1.2 mM, $MgCl_2$ 1mM, NaH_2PO_4 0.3 mM, Na_2HPO_4 1.7 mM) at 6 μg/μl for intracerebroventricular (i.c.v.) injection, which was done by inserting a cannula at the following coordinates: rostral–caudal −0.8 mm, lateral +1.4 mm, ventral −3.3 mm from the bregma and dural surfaces. The injection volume was 2 μl at a flow rate of 1 μl/min twice per day (8:00–9:00 p.m. and 9:00–10:00 a.m.) for 4 days. As the vehicle control, 2 μl of aCSF was administered i.c.v. twice a day for 4 days. After the last i.c.v. injection, the microdialysis experiments were performed as described above.

To ascertain whether antisense oligonucleotide treatment successfully reduced the number of 5-HT_6 receptors, a radioligand binding assay using [^{3}H]lysergic acid diethylamide (LSD) was carried out because of the lack of selective radioligands for 5-HT_6 receptors. Rats treated with antisense oligonucleotides or scrambled oligonucleotides were killed by decapitation immediately after the microdialysis experiment (15 h after the last i.c.v. injection of oligonucleotides). Saturation assays with membranes prepared from whole brain tissue were performed using eight concentrations of [^{3}H]LSD, which consisted of buffer or methiothepine (10^{-5} M) to define nonspecific binding. All assays were performed in the presence of 300 nM spiperone to mask the 5-HT_{2A} and dopamine D_2 receptor binding site. Some rats were decapitated and the dissected cortices were frozen in liquid nitrogen. Within two weeks, tissues were deproteinized with 0.4 N $HClO_4$ and the dopamine and 5-HT contents were determined as described above.

2.5. Drugs

The following drugs were used: GBR 12909; 1-[2-[bis(4-fluorophenyl)methoxy]ethyl]-4-(3-phenylpropyl)piperazine dihydrochloride (RBI Research Biochemicals, MA, USA), ritanserin (RBI), tropisetron (Novartis Pharma., Basel, Switzerland), GR 113808; [1-[2-(methylsulphonylamino)ethyl]-4-piperidinyl]methyl 1-methyl-1*H*-indole-3 carboxylate (RBI), GR 127935; *N*-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2′-methyl-4′-(5-methyl-1,2,4-oxadiazol-3-yl)-[1,1-biphenyl]-4-carboxamide] (Glaxo, Herts, UK). All drugs, which were perfused through the microdialysis probe, were dissolved in deionized water to a concentration of 1 mM or 10 mM and diluted in Ringer's

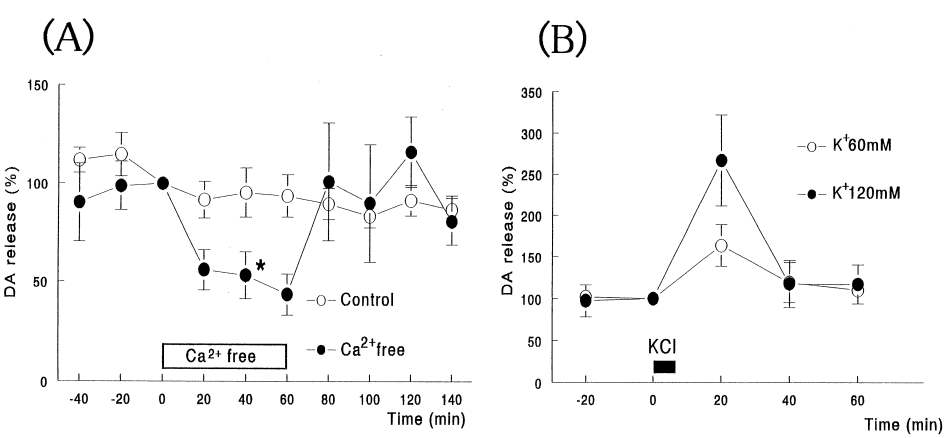

Fig. 1. Characterization of extracellular levels of dopamine (DA) as measured by in vivo microdialysis in the rat prefrontal cortex. Effects of Ca^{2+} removal from the perfusate solution (A) and K^+ (60 and 120 mM) stimulation (B) on dialysate DA levels. $^*P < 0.05$ compared with controls. All results are given as means ± S.E.M. of three to six experiments.

solution to the final concentration. Since ritanserin hardly dissolved in water, subcutaneous (s.c.) administration was performed on the basis of a previous report (Consolo et al., 1996). These drugs, except GR 127935, did not interfere with the present assay system. Only GR 127935 perfused via the dialysis probe produced overlap with the 5-HT peak, but not with the dopamine peak, on the chromatogram.

2.6. Calculations and statistics

Dopamine and 5-HT levels are expressed as percentages of the baseline level determined immediately before addition of fluoxetine to the perfusate. The amount of dopamine or 5-HT was calculated by comparing its peak height in the samples with the peak height of a standard. All results are given as means ± S.E.M. The experimental data were statistically analyzed using the *F*-test to assess the homogeneity of variance. For comparisons of experimental groups with controls, Dunnett's test was used after assessment by repeated measured analysis of variance (rANOVA). For multiple comparisons, the significance of the differences between the experimental groups was evaluated using ANOVA and followed by Tukey's test. Probability values less than 5% were considered significant.

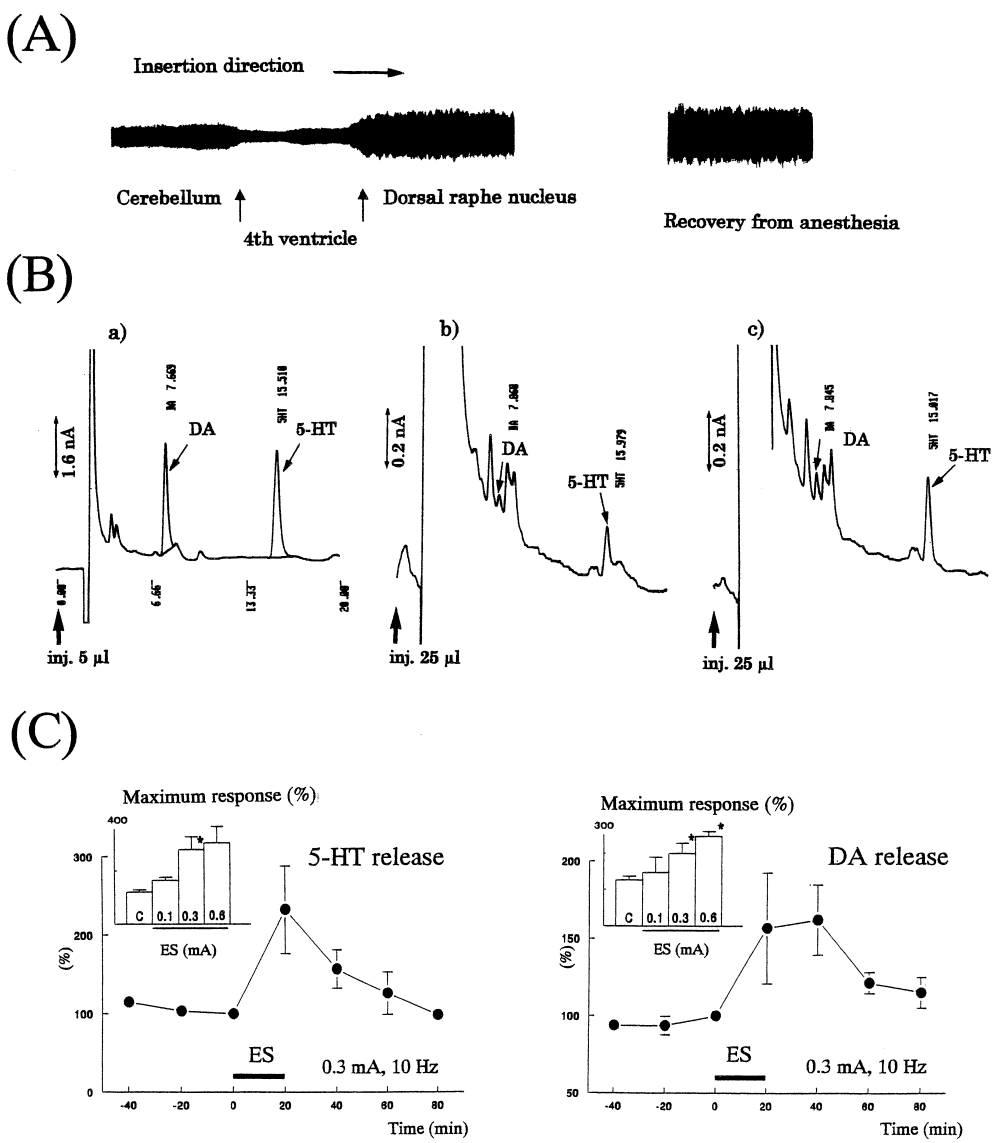

Fig. 2. Effects of electrical stimulation of the dorsal raphe nucleus on serotonin (5-HT) and dopamine (DA) release in the rat prefrontal cortex. Actual recording through a stimulating electrode in the dorsal raphe nucleus (A) and chromatograms of dialysate samples (B). Chromatograms indicate 50 pg of DA and 5-HT standard peak (a), dialysate sample before (b) and after (c) electrical stimulation at an intensity of 0.3 mA with a frequency of 10 Hz for 20 min. (C) shows the time course of 5-HT and DA release elicited by electrical stimulation (ES) of the dorsal raphe nucleus. ES was performed at 0.3 mA intensity with a constant frequency at 10 Hz for 20 min. Inset: maximum response of ES with stimulatory intensity of 0.1, 0.3 and 0.6 mA. $^{*}P < 0.05$ compared with controls. All results are given as means ± S.E.M. of three to six experiments.

3. Results

3.1. Characterization of dialysate dopamine levels in the rat prefrontal cortex

The neuronal contribution to dialysate dopamine levels was examined. Removal of Ca^{2+} from the Ringer's solution, by replacement of EDTA-2Na^{+}, caused a decrease in dialysate dopamine levels, an effect which was reversed by replacement of the Ca^{2+}-free perfusion fluid with normal Ringer's solution (Fig. 1A). High concentrations of KCl (60 and 120 mM) added to the perfusate for 5 min elicited increases in the dopamine output in a concentration-dependent manner (Fig. 1B). The extracellular dopamine and 5-HT levels in the rat prefrontal cortex were 10.4 ± 1.6 and 31.9 ± 5.8 fmol/sample (means ± S.E.M., $n = 23$), respectively.

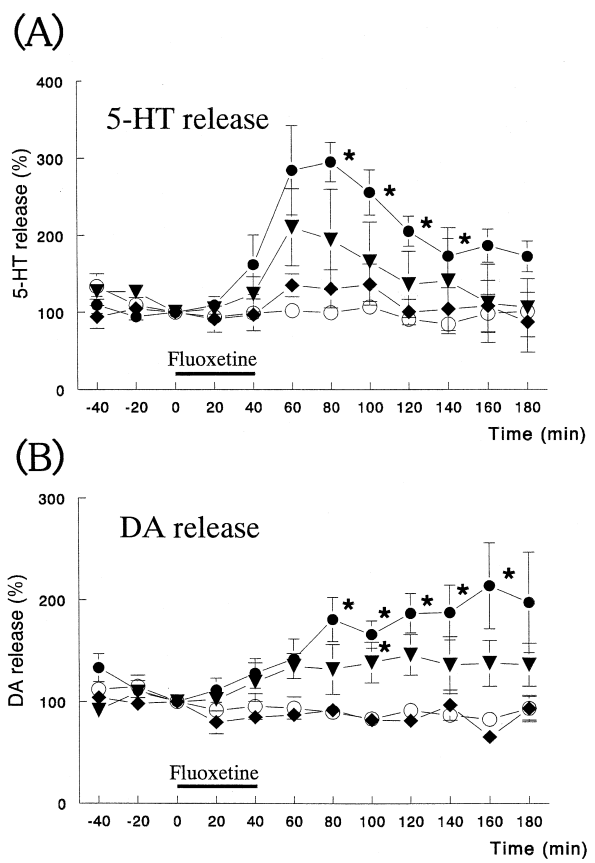

Fig. 3. Time course of serotonin (5-HT) (A) and dopamine (DA) release (B) from the rat prefrontal cortex after local application of fluoxetine, 10 μM (♦, $n = 4$), 30 μM (▼, $n = 4$) and 100 μM (●, $n = 8$). Fluoxetine was perfused through the microdialysis probe for 40 min. $^{*}P < 0.05$ compared with controls (○, $n = 7$). All results are given as means ± S.E.M. The basal DA levels determined immediately before addition of fluoxetine (10, 30 and 100 μM) to the perfusate were 8.9 ± 2.2 ($n = 4$), 16.6 ± 6.6 ($n = 4$) and 8.3 ± 1.6 fmol/sample ($n = 8$), respectively, which did not differ from those of controls (11.2 ± 2.8 fmol/sample, $n = 6$).

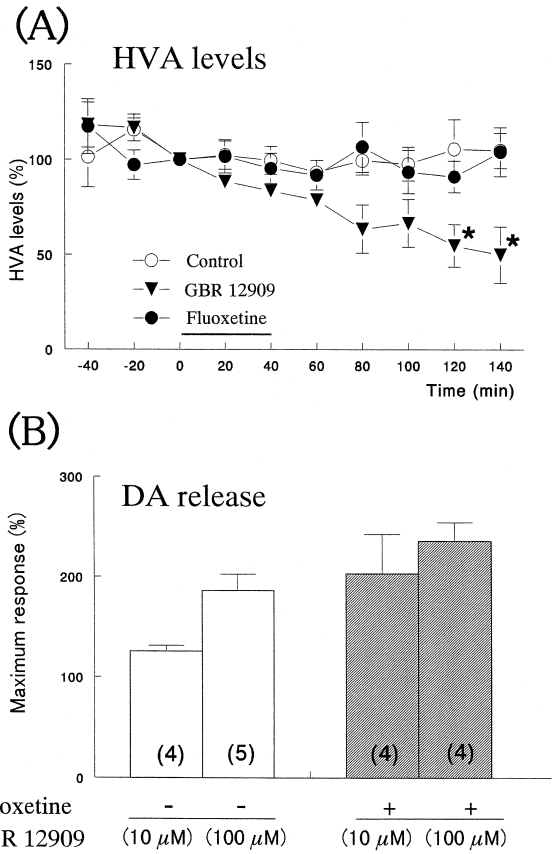

Fig. 4. (A) Time course response of extracellular levels of homovanillic acid (HVA) in the rat prefrontal cortex after local application of fluoxetine (100 μM) (●, $n = 7$) or the dopamine (DA) uptake inhibitor, GBR 12909 (100 μM)(▼, $n = 5$). $^{*}P < 0.05$ compared with controls (○, $n = 5$). All results are given as means ± S.E.M. The basal levels of HVA in fluoxetine - and GBR 12909-treated rats were 0.68 ± 0.17 ($n = 7$) and 0.56 ± 0.03 pmol/sample ($n = 5$), which did not differ from those of controls (0.64 ± 0.17 pmol/sample, $n = 5$). (B) Effects of coperfusion of fluoxetine (100 μM) and GBR 12909 (10 and 100 μM) on DA release in the rat prefrontal cortex. GBR 12909 was added to the perfusate 20 min before fluoxetine administration and co-perfused with fluoxetine over a period of 40 min. All results are given as means ± S.E.M. The number (n) of experiments is shown in parentheses.

3.2. Effects of electrical stimulation of the dorsal raphe nucleus on dopamine release in the rat prefrontal cortex

Bipolar electrode placement was confirmed by recording firing activity during implantation of the electrode and after recovery from anesthesia (Fig. 2A). Fig. 2B shows chromatograms of the dialysate before and after electrical stimulation of the dorsal raphe nucleus. Both 5-HT and dopamine levels increased after electrical stimulation (intensity; 0.3 mA; frequency; 10 Hz) for 20 min: 5-HT concentrations peaked during the electrical stimulation and returned to the basal level, whereas dopamine levels peaked after stimulation. Electrical stimulation with a constant frequency at 10 Hz caused an increase in 5-HT and dopamine release in an intensity-dependent manner (Fig. 2C).

3.3. Effects of local application of fluoxetine on dopamine release in the rat prefrontal cortex

The effects of endogenous 5-HT on dopamine release were estimated by administration of the 5-HT uptake inhibitor fluoxetine. Local administration of fluoxetine (10, 30 and 100 μM) produced concentration-dependent increases in extracellular 5-HT levels with a maximum effect of $164.2 \pm 17.8\%$ ($n = 4$), $218.5 \pm 57.7\%$ ($n = 4$) and $319.4 \pm 48.5\%$ ($n = 8$), respectively. Thirty and 100 μM of fluoxetine significantly increased dopamine release with a maximum effect of $173.7 \pm 17.5\%$ ($n = 4$) and $231.7 \pm 18.9\%$ ($n = 8$), respectively, while 10 μM had no effect. As shown in Fig. 3, dialysate 5-HT levels were markedly enhanced after fluoxetine administration and then gradually returned to the basal levels, whereas dopamine levels increased significantly after the peak response of 5-HT was observed.

To clarify whether fluoxetine elicited dopamine release due to the nonselective blockade of dopamine uptake, two sets of experiments were performed with application of a selective dopamine uptake inhibitor GBR 12909 (Andersen, 1989). First, the extracellular levels of metabolites were determined as a reflection of intraneuronal metabolism of neurotransmitter release. Local administration of GBR 12909 (100 μM) significantly reduced the concentrations of the dopamine metabolite HVA, whereas fluoxetine (100 μM) did not affect these levels (Fig. 4A). The levels of the 5-HT metabolite 5-HIAA decreased to approximately 75% after fluoxetine administration, but did not change after perfusion of GBR 12909 (data not shown). Second, the effects of GBR 12909 on dopamine release were examined in the presence of fluoxetine. GBR 12909 was added to the perfusate 20 min before fluoxetine and co-perfused with fluoxetine. As shown in Fig. 4B, dopamine release elicited by GBR 12909 (10 and 100 μM) was additionally increased by co-perfusion of fluoxetine (100 μM).

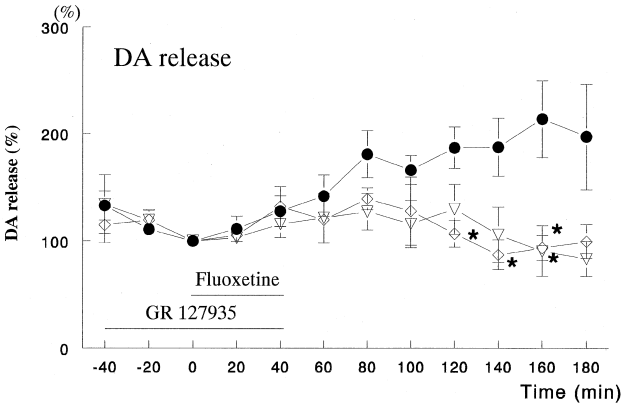

Fig. 6. Time course of fluoxetine (100 μM)-induced increases in dopamine (DA) release from the rat prefrontal cortex after administration of the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 (◇, 10 μM, $n = 6$ and ▽, 100 μM, $n = 6$). GR 127935 was added to the perfusate 40 min before fluoxetine administration and co-perfused with fluoxetine over a period of 40 min. To compare the time course of the response of fluoxetine in the presence or absence of GR 127935, fluoxetine (100 μM)-induced enhancement of DA release in Fig. 3 was used as a control. $^*P < 0.05$ compared with fluoxetine-administered groups in the absence of GR 127935 (●, $n = 8$). All results are given as means ± S.E.M. The basal DA levels determined immediately before addition of fluoxetine in the GR 127935 (10 and 100 μM)-treated rats were 13.5 ± 4.0 and 14.1 ± 5.0 fmol/sample, respectively.

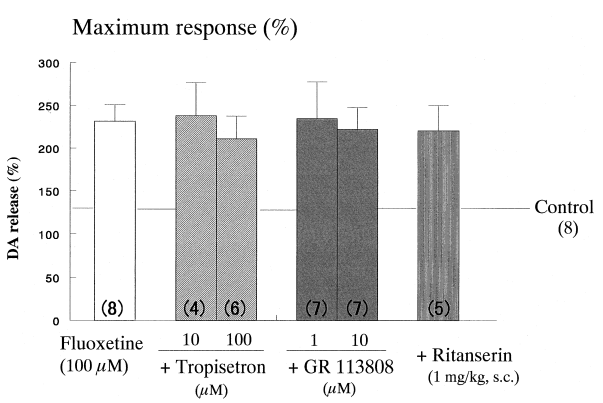

Fig. 5. Effects of 5-HT receptor antagonists on fluoxetine (100 μM)-induced increases in dopamine (DA) release from the rat prefrontal cortex. Tropisetron, a 5-HT_3/5-HT_4 receptor antagonist, or GR 113808, a 5-HT_4 receptor antagonist, was added to the perfusate 40 min before fluoxetine administration and co-perfused with fluoxetine over a period of 40 min. The 5-$HT_{2A/2C}$ receptor antagonist ritanserin was injected 20 min before perfusion of fluoxetine. All results are given as means ± S.E.M. The number (n) of experiments is shown in parentheses. The basal DA levels in the presence of 5-HT receptor antagonists were as follows: tropisetron, 10 μM, 15.0 ± 4.6 fmol/sample ($n = 4$) and 100 μM, 17.2 ± 8.1 fmol/sample ($n = 6$). GR 113808, 1 μM, 14.2 ± 7.8 fmol/sample ($n = 7$) and 10 μM, 11.4 ± 1.6 fmol/sample ($n = 7$) and ritanserin, 9.6 ± 1.2 fmol/sample ($n = 5$).

3.4. Antagonism of fluoxetine-induced increases in dopamine release by application of 5-HT receptor antagonists

The possible involvement of 5-HT receptors in the fluoxetine (100 μM)-induced facilitation of dopamine release was elucidated by application of 5-HT receptor antagonists. Antagonist concentrations were chosen based on previous in vivo microdialysis studies (Consolo et al., 1996; Iyer and Bradberry, 1996; Steward et al., 1996). Pretreatment with either the 5-HT_3/5-HT_4 receptor antagonist, tropisetron (Richardson et al., 1985) (10 and 100 μM) or the 5-HT_4 receptor antagonist, GR 113808 (Grossman et al., 1993) (1 and 10 μM), did not influence the facilitation of dopamine release produced by fluoxetine. None of these 5-HT receptor antagonists by themselves altered the spontaneous dopamine levels; maximum dopamine release was $121.7 \pm 8.8\%$ and $134.0 \pm 29.4\%$ in the tropisetron (100 μM, $n = 3$)- and the GR 113808 (10 μM, $n = 3$)-treated groups, respectively. Systemic administration of a potent antagonist at the 5-HT_{2A} and 5-HT_{2C} receptor, ritanserin (1 mg/kg, s.c.), also did not significantly affect the fluoxetine-induced increases in dopamine

release (Fig. 5). The elevation of 5-HT levels induced by fluoxetine was not affected by pretreatment with these 5-HT receptor antagonists.

Further characterization of the 5-HT receptors involved in the facilitation of dopamine release was achieved by administering the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 (Clitherow et al., 1994). Pretreatment with GR 127935 (10 and 100 μM) prevented the fluoxetine-induced increases in dopamine release. As shown in Fig. 6, the significant inhibition by GR 127935 (10 μM) was observed 120 min after commencement of the perfusion of fluoxetine. A high concentration of GR 127935 (100 μM) abolished the facilitation of dopamine release, but not in a concentration-dependent manner.

3.5. Effects of antisense oligonucleotide treatment on fluoxetine-induced increases in dopamine release

The possible involvement of 5-HT_6 receptors in fluoxetine (100 μM)-induced dopamine release was elucidated by treatment with antisense oligonucleotides, complementary to the coding region of 5-HT_6 receptor mRNA. Fluoxetine caused increases in 5-HT release in both antisense oligonucleotide-treated rats and scrambled oligonucleotide-treated controls, with a maximum response of 339.4 ± 56.0% ($n = 7$) and 364.0 ± 70.9% ($n = 6$), respectively. Dopamine release was also increased after fluoxetine administration, with a maximum response of 206.9 ± 27.5% in scrambled oligonucleotide-treated rats ($n = 6$), whereas

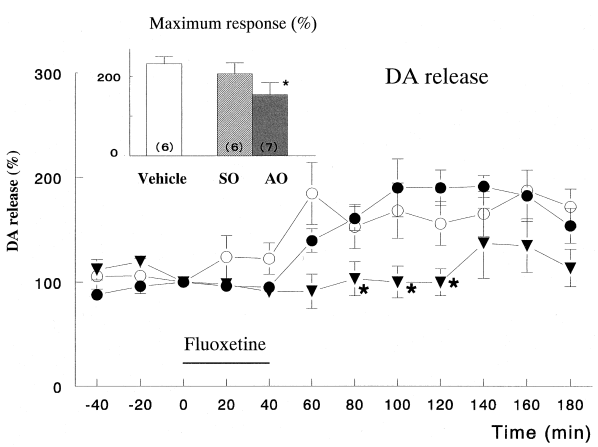

Fig. 7. Time course response of fluoxetine (100 μM)-induced increases in dopamine (DA) release from the rat prefrontal cortex in antisense oligonucleotide (AO) (▼, $n = 7$) or scrambled oligonucleotide (SO) (○, $n = 6$)-treated rats. AO (12 μg)- or SO (12 μg)-treated rats were microinjected i.c.v. twice a day for 4 days. Injection volume was 2 μl at a flow rate of 1 μl/min. $^*P < 0.05$ compared with vehicle- treated controls (●, $n = 6$). All results are given as means ± S.E.M. Basal levels of DA in AO treated rats (15.2 ± 7.8 fmol/sample, $n = 7$) did not significantly differ from those in SO-treated rats (9.7 ± 3.3 fmol/sample, $n = 6$) and vehicle-treated controls (11.2 ± 2.8 fmol/sample, $n = 6$). Inset: maximum response of DA release after fluoxetine (100 μM) administration in AO- and SO-treated rats. The number (n) of experiments is shown in parentheses.

Table 1
B_{max} and K_d values of membrane prepared from the whole brain in antisense oligonucleotide (AO)- and scrambled oligonucleotide (SO)-treated rats

[^{3}H]LSD	(n)	B_{max} (fmol/mg of protein)	K_d (nM)
Control	(5)	82.5 ± 4.8	8.5 ± 1.0
AO treatment	(5)	57.4 ± 9.5[a]	6.2 ± 0.5
SO treatment	(5)	88.3 ± 24.3	7.9 ± 1.3

[a] $P < 0.05$ vs. non-treated control rats. Data presented are means ± S.E.M.

significant inhibition was observed during 80–120 min after commencement of the perfusion of fluoxetine in antisense oligonucleotide treated rats. The maximum effect of dopamine release in antisense oligonucleotide-treated rats was 154.8 ± 29.7% ($n = 7$), which was significantly lower than that in vehicle-treated rats (233.2 ± 18.9%, $n = 6$) (Fig. 7).

A binding study using [^{3}H]LSD was performed to ascertain whether antisense oligonucleotide treatment reduced the number of 5-HT_6 receptors (Bourson et al., 1995). The K_d (nM) value of membranes prepared from whole brains from antisense oligonucleotide-treated rats did not significantly differ from that of membranes prepared from brains from either scrambled oligonucleotide-treated or intact controls, whereas B_{max} decreased compared with the control value (Table 1). With respect to the cortical dopamine content, there were no significant differences between antisense oligonucleotide-treated rats (312.9 ± 9.6 ng/g wet weight, $n = 3$), scrambled oligonucleotide-treated rats (298.6 ± 35.7 ng/g wet weight, $n = 4$) and non-treated rats (257.4 ± 50.6 ng/g wet weight, $n = 4$).

4. Discussion

In this study, dialysate dopamine levels were Ca^{2+}-dependent and were increased by perfusion of high concentrations of K^+, indicating that the extracellular dopamine levels in the rat prefrontal cortex were of neuronal origin. Electrical stimulation of the dorsal raphe nucleus produced increases not only in 5-HT but also in dopamine release in an intensity-dependent manner. These findings support the hypothesis that 5-HT has an excitatory effect, rather than an inhibitory effect, on dopamine release. Serotonergic neurons from the dorsal raphe nucleus project to dopamine cell bodies such as the ventral tegmental area and terminate in the terminal field, prefrontal cortex. Therefore, the facilitatory regulation by the 5-HT neuronal system might take place at the level of cell bodies and/or at the terminal regions. The present finding that local application of fluoxetine caused concentration-dependent increases in dopamine release suggests that the elevated 5-HT levels in the

extracellular space stimulated dopamine neurotransmission. Furthermore, there was a difference in the time course of the maximum response between the 5-HT and dopamine levels after fluoxetine administration: dopamine release increased gradually and significantly after the peak effect of 5-HT had appeared. These findings strengthen the possibility that endogenous 5-HT elicited by fluoxetine enhanced dopamine release in the nerve terminal field.

Although fluoxetine has selective high affinity for the 5-HT uptake carrier (Jacobs and Fornal, 1991), a high concentration of this drug might produce increases in dopamine levels by nonspecific blockade of the dopamine transporters. In this experiment, fluoxetine reduced the 5-HIAA levels as a reflection of the intraneuronal metabolism of 5-HT. A selective dopamine uptake inhibitor, GBR 12909, also caused decreases in the concentrations of HVA, whereas fluoxetine did not induce any changes in these levels. We also observed that the GBR 12909-induced increases in dopamine release were augmented by co-perfusion with fluoxetine. If fluoxetine enhanced dopamine release by acting on the dopamine transporter, then fluoxetine would not affect dopamine levels when the dopamine uptake site was already blocked. Therefore, the possibility that fluoxetine acted directly on the transporter located on dopamine terminals which subsequently increased dopamine levels was excluded. In other words, these findings indicate that the dopamine release elicited by fluoxetine was due to the effects of endogenous 5-HT elevated by the selective blockade of the 5-HT uptake site.

In the present experiment, pretreatment with either the 5-HT_3/5-HT_4 receptor antagonist, tropisetron, or the selective 5-HT_4 receptor antagonist, GR 113808, did not influence the fluoxetine-induced increases in dopamine release. Blockade of 5-HT_2 receptors by ritanserin also did not affect the facilitation of dopamine release. A previous study (Chen et al., 1992) showed the involvement of 5-HT_3 receptors in the facilitation of dopamine release, i.e., a selective 5-HT_3 receptor agonist, 1-phenylbiguanide, increased dopamine release in the rat medial prefrontal cortex. We also found that local application of 1-phenylbiguanide (10 and 100 μM) produced significant increases in dopamine release with a maximum effect of 238.9% ($n = 3$) and 308.0% ($n = 4$), respectively. However, the 1-phenylbiguanide (100 μM)-induced increase in dopamine release was not reduced by co-perfusion of the sodium channel blocker tetrodotoxin (10 μM) (maximum effect; 282.3%, $n = 4$). Thus, the 1-phenylbiguanide-induced facilitation of dopamine release might not be due to a neuronal mechanism but due to carrier-mediated processes. This observation is in agreement with the findings of in vivo (Santiago et al., 1995) and in vitro studies (Schmidt and Black, 1989) of the rat striatum. Taken together, the present findings suggest that 5-HT_3 receptors are not involved in endogenous 5-HT-mediated dopamine release, at least in the rat prefrontal cortex.

Consistent with the report by Iyer and Bradberry (1996), the 5-$HT_{1B/1D}$ receptor antagonist GR 127935 abolished the facilitation of dopamine release. They showed that dopamine release elicited by exogenously applied 5-HT was completely prevented by pretreatment with GR 127935. In our study, however, the inhibitory effect of GR 127935 was partial despite its high concentration. Although the reason for this discrepancy is unclear, one possible explanation is that fluoxetine and GR 127935 have an additional effect as a consequence of a combination of inhibitory effects for both the uptake site and the terminal 5-HT_{1B} autoreceptors (Engel et al., 1986). Co-perfusion of fluoxetine and GR 127935 might cause greater increases in synaptic 5-HT levels than those caused by fluoxetine administration alone, which consequently overcome the antagonistic action of GR 127935 alone. Indeed, several studies using guinea pigs have revealed that the local perfusion of GR 127935 causes an increase in cortical 5-HT release (Skingle et al., 1995, Roberts et al., 1997).

However, pretreatment with antisense oligonucleotide to prevent the expression of 5-HT_6 receptors also inhibited the fluoxetine-induced increase in dopamine release. Treatment with antisense oligonucleotide caused a reduction of [^{3}H]LSD binding sites whereas the affinity of these sites was not changed, suggesting that this treatment successfully decreased the number of 5-HT_6 receptors (Monsma et al., 1993). These findings indicate the possible involvement of 5-HT_6 receptors in the endogenous 5-HT-mediated regulation of dopamine release. Pretreatment with antisense oligonucleotide did not influence the spontaneous dopamine level. This indicates that no tonic regulation of dopamine release via 5-HT_6 receptors existed in this region. The inhibition of dopamine release mediated via 5-HT_6 receptors appeared to be partial: a significant inhibitory effect was observed at 80–120 min after perfusion of fluoxetine in antisense oligonucleotide-treated rats. This might have been due to the partial inactivation of 5-HT_6 receptors because the number of [^{3}H]LSD binding sites in antisense oligonucleotide-treated rats was reduced to approximately 30% of that of intact rats. Hypothetically, the endogenous 5-HT-mediated dopamine release might be subject to dual control by 5-HT_{1B} and 5-HT_6 receptors in a different manner.

In this study, we could not identify the location of 5-HT_{1B} or 5-HT_6 receptors underlying the 5-HT-mediated regulation of dopamine release. 5-HT_{1B} receptors are known to be inhibitory heteroreceptors (Hoyer et al., 1994). Therefore, the facilitation of dopamine release mediated via 5-HT_{1B} receptors might be caused indirectly via interneurons such as γ-aminobutyric acid (GABA)ergic (Johnson et al., 1992) or glycinergic neurons (Umemiya and Berger, 1995). Although it has been suggested that 5-HT_6 receptors are synthesized in the targets of dopaminergic projections (Gérard et al., 1996), further studies will be required.

In summary, the present study demonstrated that there is facilitatory regulation of dopamine release by endogenous 5-HT in the rat prefrontal cortex. This facilitation was prevented by pretreatment with a 5-$HT_{1B/1D}$ receptor antagonist or by selective inactivation of the mRNA encoding the 5-HT_6 receptor. These findings suggest that not only 5-HT_{1B} receptors but also 5-HT_6 receptors might be involved in the endogenous 5-HT-mediated regulation of dopamine release. Although little is known of the physiological role of 5-HT_6 receptors, the present findings indicate the possible involvement of 5-HT_6 receptors in the cortical dopamine neuronal system. In other words, 5-HT_6 receptors may play a significant role in the functional relationship between the dopaminergic and serotonergic neuronal systems in the rat prefrontal cortex.

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