The selective action of D_2 dopamine receptor antisense oligodeoxynucleotide on the expression of the dopamine receptor subtype mRNA in rat striatum

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We have evaluated the effect of the D_2 dopamine receptor antisense oligodeoxynucleotide (D_2 AS ODN) on the gene expression of all five dopamine receptor subtypes including D_1, D_2, D_3, D_4 and D_5 dopamine receptor in the rat striatum. The levels of D_2 dopamine receptor mRNA are significantly decreased at 6, 12, 24 h after the last injection of three time injections of D_2 AS ODN, although D_1, D_3, D_4 and D_5 subtype mRNA levels did not significantly reduced at any time. The present study is the first to demonstrate the selective effect of D_2 AS ODN on D_2 dopamine receptor mRNA among all five dopamine receptor subtypes and the effectiveness of D_2 AS ODN without 6-hydroxydopamine.

Key words: D_2 dopamine receptor, mRNA, antisense oligodeoxynucleotide, real time PCR

INTRODUCTION

A growing body of evidence suggests that dopamine receptors play a critical role in mediating locomotor and behavioral activity. Dysfunction of dopaminergic activity may be responsible for a number of neuropsychiatric disorders including schizophrenia [3, 11], parkinsonism [13] and tardive dyskinesia [1]. Dopamine receptors were initially divided into two general categories, designated as the D_1 dopamine receptor and D_2 dopamine receptor, based on the differences in the pharmacological profile and biochemical mechanisms of signal transduction [7, 14, 16]. Following the application of the techniques of molecular biology, the D_1 and D_2 dopamine receptors were cloned by several groups [2, 25]. Subsequently, additional dopamine receptors with homology to either the D_1 or D_2 dopamine receptor were identified. At present time, two families of vertebrate dopamine receptors, designated as D_1-like and D_2-like, are recognized. The D_1-like family is composed of two distinct receptors (D_1 and D_5 subtypes). The D_2-like family is composed of three distinct receptors (D_2, D_3 and D_4 subtypes) [4, 5, 8, 10, 12, 15]. Dopamine receptor subtypes within the same family are pharmacologically similar, making it difficult to selectively stimulate or block a specific receptor subtype *in vivo*. Thus, the assignment of various physiological or behavioral functions to specific dopamine receptor subtypes using pharmacological tools is difficult. In view of this, many researchers begun to use highly selective genetic approaches to alter the expression of individual dopamine receptor subtypes *in vivo*. Antisense oligodeoxynucleotide (AS ODN), designed to hybridize with specific sequence of the mRNA that encode the targeted protein, has been used to inhibit the synthesis of the transcripts and the proteins, and thereby estimate the function of the protein [6, 17, 18]. Previous studies reported that the intracerebroventricular (i.c.v.) administration of AS ODN against the D_2 dopamine receptor (D_2 AS ODN) inhibited the expression of the D_2 dopamine receptor mRNA but not that of the D_1 dopamine receptor one in the striatum treated unilaterally with 6-hydroxydopamine (6-OHDA), called the unilateral lesioned striatum [19, 24]. However, there are two defects in these results. First, these results showed the effects of D_2 AS ODN on the mRNA expressions of D_1 and D_2 dopamine receptors, but remained to be examined on those of D_3, D_4 and D_5 dopamine receptors. Second, D_2 AS ODN decreased D_2 dopamine receptor mRNA in only the unilateral lesioned striatum, but not the unilateral untreated striatum with 6-OHDA, called the unilateral unlesioned striatum. It is important to recognize that the unilateral lesioned striatum increases the levels of D_2 dopamine receptor mRNA compared with the unilateral unlesioned one [24]. The administration of D_2 AS ODN, in turn, may produce a down-regulation of increase for the levels of D_2 dopamine receptor mRNA in the unilateral lesioned striatum.

In the present study, we have evaluated 1) the effect of D_2 AS ODN on the mRNA expressions of D_1, D_2, D_3, D_4 and D_5 dopamine receptor subtypes and 2) the effectiveness of D_2 AS ODN in the rat striatum without 6-OHDA.

MATERIAL AND METHODS

Animals and Materials

The present animal experiments were performed in strict accordance with the guidelines of Tokai

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University, and were approved by the Animal Investigation Committee of the University. Male Wistar rats (Clea Japan, Tokyo, Japan) weighing 200 to 250 g were group-housed in laboratory cages and kept in a temperature-controlled room (23 ± 2°C) with a 12 h light / dark cycle (light on: 07:00) with food and water freely available. SUPERSCRIPT First- Strand Synthesis System for RT-PCR containing reverse transcriptase (RTase) (Superscript II, RNase H^-), dithiothreitol (DTT), oligo $(dT)_{12\text{-}18}$ primer, deoxynucleosidetriphosphate (dNTP), RNase H, DNase I and RNase inhibitor were purchased from Invitrogen (San Diego, CA, USA). SV Total RNA Isolation System and PCR Master Mix from Promega (Madison, WI, USA). DyNAmo SYBER green qPCR Kit were from Finnzymes (Espoo, Finland), DNA 1000 Lab Chips Kit (Agilent Technologies; Palo Alto, CA, USA) were from Takara Bio Co. (Tokyo, Japan). PCR primers were synthesized by Sigma Genosys Japan (Tokyo, Japan). All other chemicals and reagents were purchased from Wako Chemical Co. (Tokyo, Japan) unless otherwise noted.

Oligodeoxynucleotide (ODN)

Based on the cDNA sequence for the D_2 (Bunzow *et al.*, 1988), a 20-mer ODN was designed and synthesized. The sequence of D_2 AS was 5'-GTG GAT CCA TTG GGG CAG TG-3' as reported previously [24]. And the sequence of random ODN was 5'-GCT GGT AGC GTG ACG GTG TC-3'. Metabolically stable phosphorothioate analogues of ODN were used in the present study. Gel-filtrated and HPLC-purified ODNs were dissolved in sterilized water and stored at –20°C.

Administration of ODN into striatum

Male Wistar rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.) and mounted on a stereotaxic frame (Narishige, Tokyo). Stainless-steel guide cannulae (outer diameter, 0.55 mm) were implanted. Four days after operation, an injection cannula (outer diameter, 0.33 mm; 16 mm in length) was inserted into left striatum through the guide cannula at the following stereotaxic coordinates: AP, +0.5 mm; V, +7.0 mm; L; −3.0 mm [9]. Ten micrograms of AS, sense or random ODN in 1 µl of saline, or 1 µl of saline alone were microinjected through an injection cannula at a flow rate of 0.5 µl/min. Injection cannula was released from guide cannula 2 min after each injection. Rats were administrated AS ODN, sense ODN, random ODN, or saline three times at 48 h interval.

Dissection of striatum

Before the dissection of the striatum, 1 µl of 0.1% bromophenol blue was injected into the striatum through injection cannula at flow rate of 0.5 µl/min. Twenty minutes after the dye injection, rats were decapitated, and the brain was removed and placed on an ice-cooled glass petri dish. By observing the dye staining, the striatum block (1 × 1 × 3 mm) was dissected out.

RNA extraction and reverse transcription

The total RNA was extracted by a method similar to the one described previously [22]. The total RNA in the striatum was extracted by using SV Total RNA Isolation System. Total RNA (0.5 µg) was incubated with 200 units of reverse transcriptase in a buffer containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 2.5 mM $MgCl_2$, 10 mM ditiothreitol, 0.5 mM of each dNTP, and 0.5 µg oligo $(dT)_{12-18}$ primer in the final volume of 20 µl. The mixture was incubated at 42°C for 50 min, and the reaction was stopped by heating at 70°C for 15 min. The RNA was removed by adding 2 units of ribonuclease H to facilitate the synthesis of double stranded cDNA.

Table 1

Gene		primer sequence
GAPDH	upper	GTG GAC CTC ATG GCC TAC AT
	lower	TGT GAG GGA GAT GCT CAG TG
D1	upper	CTC CTG ATG GAA CCC TAC CA
	lower	CAG GAT GAG CAG TGA CAG GA
D2	upper	TGG ATC CAC TGA ACC TGT CC
	lower	TTG TAG TGG GGC CTG TCT G
D3	upper	TTA GCC CAC ATT GCT GTC TG
	lower	GGA GTT GAG GTG GGT GCT TA
D4	upper	ATG GCC CCT GAC TGC AAA TC
	lower	AGT CCG GTG CCA GTA CCT AA
D5	upper	AGC ATG CTC AGA GTT GCC GG
	lower	ACA AGG GAA GCC AGT CTT TGG

Quantitative Real-Time Polymerase Chain Reaction (PCR)

D_2 receptor gene expression was determined by real-time PCR using the glyceraldehydes 3-phosphated dehydrogenase (GAPDH) [20, 21] (GenBank accession number NM_017008) gene as an internal control and primers specific for D_1 mRNA (accession number NM_012546), D_2 mRNA (accession number NM_012547), D_3 mRNA (accession number NM_017140), D_4 mRNA (accession number NM_012944) and D_5 mRNA (accession number NM_012768) (Table 1).

The cDNA was amplified by real-time PCR using DyNAmo SYBER green qPCR Kit (Finnzymes, Espoo, Finland) on the DNA Engine Opticon 2 System (MJ Research, Inc. MA, USA) running 35 cycles of the following protocol: 10 min predenaturation at 95 °C, 15 sec denaturation at 95 °C, 20 sec at 58 °C for D_1, D_4 and D_5, 60 °C for D_2 and D_3, or 62 °C for GAPDH, followed by a 20 sec extension at 72 °C. The PCR products were separated by an Agilent 2100 Bioanalyzer (Agilent Technologies; Palo Alto, CA, USA) which utilizes chip-based nucleic separation technology. DNA 1000 Lab Chip (Agilent Technologies) was loaded with the samples as recommended by the manufacture. Furthermore, the identification of the amplified PCR products of the dopamine receptors and GAPDH cDNAs were determined by the dye terminator cycle sequencing.

Statistical analysis

The results are given as mean with standard error of the mean (S.E.M.) of the data. A statistical analysis

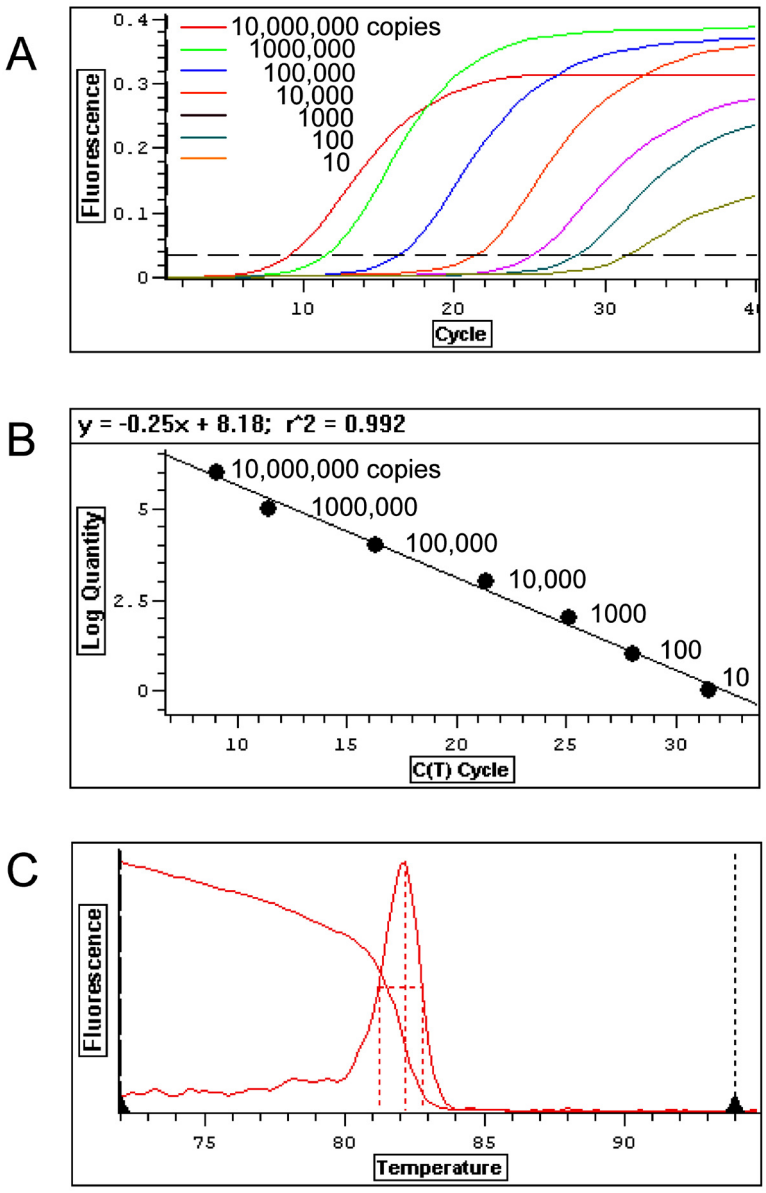

Fig. 1 Amplification (**A**), standard curve (**B**) and melting curve of D_2 mRNA by real-time quantitative PCR. **A:** PCR curves of serially diluted D_2 cDNA from 10^6 to 10^1 are presented. **B:** Standard curve generated from the Ct values of the amplification plots. **C:** Melting curve analysis of PCR product amplified from cDNA confirms specificity of the reaction as a single peak.

was conducted using computer software (Statview; SAS Institute, Cary, NC, USA) for comparison across the experimental conditions. A statisitical evaluation was carried out using Mann-Whitney U test. A P-value $<$ 0.05 was considered as reaching statistical significance.

RESULTS

Results of real-time PCR

The PCR of the rat brain cDNAs with primers for five distinct dopamine receptors gave a single product of the expected size determined by the Agilent 2100 Bioanalyzer, respectively. Tenfold serial dilutions of the cDNA were used to construct a standard curve from 10^7 copies to 10^1 copies. Figure 1A presents typical amplification plots for standards of D_2 receptor. A standard curve was then generated from the Ct values (threshold cycle) (Fig. 1B). Linear regression analysis, plotting the cycle number versus the log quantity of the PCR products, gave a straight-line plot and a correlation coefficient of 0.992, which indicated that our system was able to accurately quantify the cDNAs ranging from 10^7 to 10^1 copies for the D_2 dopamine receptor. A melting curve analysis also revealed that the D_2 dopamine receptor-specific primer pair amplified a single predominant product with a melting temperature of 82.3 °C (Fig. 1C).

Selective decrease in D_2 mRNA by D_2 AS ODN

As shown in Fig. 2A, microinjection of D_2 AS ODN caused a gradual decrease in the expression of D_2 subtype mRNA relative to that of GAPDH mRNA in the striatum of rats treated with saline was expressed as 100%: 6 h (64%), 12 h (47%), 24 h (14%), and 48 h (69%). However, D_1, D_3, D_4 and D_5 dopamine receptor subtype mRNA levels did not significantly reduce at any time. As shown in Fig. 2B and 2C, microinjection of D_2 sense ODN or random ODN did not significantly change the gene expression of five dopamine receptor subtypes (D_1, D_2, D_3, D_4 and D_5) at any time.

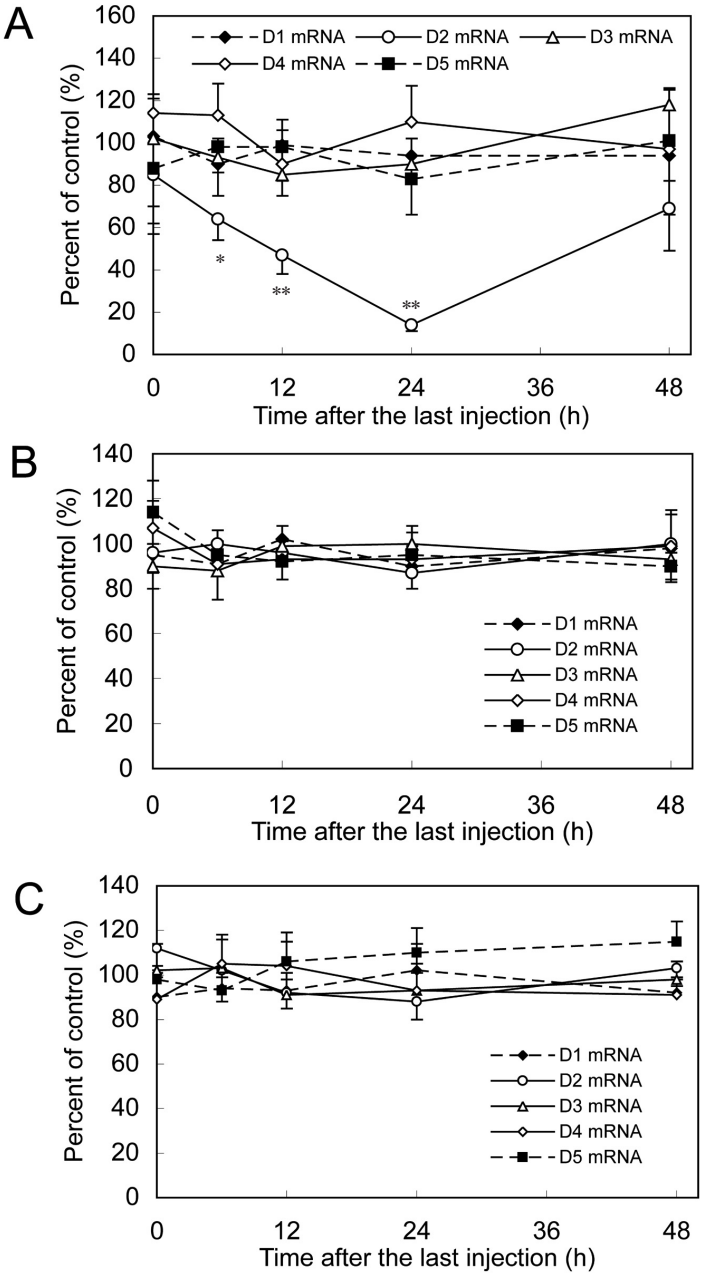

Fig. 2 Time courses of changes in the D_1, D_2, D_3, D_4 and D_5 mRNA levels in striatum of rat brain after the last injection of three injections of D_2 AS ODN (**A**), D_2 sense ODN (**B**), or random ODN (**C**). D_2 AS ODN, D_2 sense ODN, or random ODN (10 μg in 1 μl of saline) were microinjected into striatum three times at 48 h interval. Rats were decapitated 0, 6, 12, 24 and 48 h after the last injection of three injections, and total RNA from striatum was prepared. The levels of gene expression for D_1, D_2, D_3, D_4 and D_5 subtypes were determined by real-time PCR using GAPDH gene as an internal control and primers specific for D_1, D_2, D_3, D_4 and D_5 mRNA. The levels of gene expression for the D_1, D_2, D_3, D_4 and D_5 subtypes in rats injected with the D_2 AS ODN (A), D_2 sense ODN (B), or random ODN (C) were expressed as a percentage of that of saline. Results are means with S.E.M. of data obtained from five rats. $^*P < 0.05$ and $^{**}P < 0.01$ by Mann-Whitney U test when compared with the values of a group that had received saline.

DISCUSSION

The present study demonstrates 1) the administration of D_2 AS ODN into the striatum transiently and markedly decreases the D_2 dopamine receptor mRNA and 2) D_2 AS ODN does not reduce D_3, D_4 dopamine receptor mRNA, nor the D_1-like (D_1 and D_5 subtypes) dopamine receptor mRNA in the striatum at any time (Fig. 2A). As previous studies examined the effect of D_2 AS ODN on the mRNA expressions of only D_1 and D_2 dopamine receptors [19, 24], the present study is the first to demonstrate the specific effect of D_2 AS ODN among the mRNA expressions of all five dopamine receptor subtypes including D_1, D_2, D_3, D_4 and D_5 dopamine receptor. In addition to denial of the effect of D_2 AS ODN on the mRNA expressions of D_1, D_3, D_4 and D_5 dopamine receptor, there is an important benefit in our study. As the treatment of the striatum with 6-OHDA increases the levels of the D_2 dopamine receptor mRNA [24], the previous study showed that D_2 AS ODN inhibited the increase for the levels of D_2 dopamine receptor mRNA in the unilateral lesioned striatum. In contrast, the present study shows that D_2 AS ODN significantly decreases the D_2 dopamine receptor mRNA in the untreated striatum from the non-elevated levels.

Figure 2A shows that the direct administration of D_2 AS ODN into the striatum significantly and markedly decreases the D_2 dopamine receptor mRNA. In contrast, previous studies demonstrated that the i.c.v. administration of D_2 AS ODN did not decrease the levels of D_2 dopamine receptor mRNA in the unilateral unlesioned striatum [19, 24]. This discrepancy of results in the striatum between by the direct and i.c.v. administration may depend on the differences in 1) the concentration of the AS ODN at the sites of action and/or 2) the time after the last administration of AS ODN to analyze the level of the D_2 dopamine receptor mRNA. First, the importance of the concentration of

AS ODN at the site of action is clear from the results that the magnitude of inhibitory effect of AS ODN on the expression of target gene is a dose dependent manner [22, 24]. The concentration of AS ODN in the striatum after the direct administration is suggested to be higher than that after the i.c.v. administration, although total doses administrated in the present study (1.5 nmol × 3 times injections = 4.5 nmol) are lower than those in the previous study (2.5 nmol × 15 times injections = 37.5 nmol). Second, the importance of the time to analyze the level of mRNA expression after the last administration of AS ODN is demonstrated by a fact that the onset (6 h) of action is slow, in other words, the AS ODN does not easily penetrate into the cell (Fig. 2A). The previous studies merely showed the D_2 dopamine mRNA levels 2 h after the last injection of D_2 AS ODN [19, 24]. Considering the rout of administration of AS ODN, the onset or offset time of the inhibitory effect of the i.c.v. administration may be slower than the direct administration into the striatum. Further studies such as in situ hybridization are needed to determine how widespread the effects of the direct administration of the AS ODN compared with the i.c.v. administration.

The present study indicate that the D_2 AS ODN is one of the useful agents to elucidate the role of the D_2 dopamine receptor in the striatum as realizing a difference among all five dopamine receptor subtypes and is effective without 6-OHDA increasing D_2 mRNA expression.

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