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European Journal of Pharmacology 516 (2005) 145 – 150

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# A new approach to finding specific dopamine D4 receptor agonists

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Received 11 April 2005; accepted 14 April 2005
Available online 26 May 2005

**Abstract**

This paper describes a new approach to finding specific dopamine D4 receptor agonists based on pharmacological analysis of the contractile response to ATP in guinea pig vas deferens. A partial cDNA of the dopamine D4 receptor of the vas deferens was identified. In the vas deferens, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis revealed the existence of dopamine D4 receptor mRNA and D4 receptor protein, respectively. ATP ($10^{-7}$ M) induced a transient phasic contraction in the presence of prazosin ($10^{-7}$ M), an $\alpha_1$-adrenoceptor antagonist. This contraction was potentiated by dopamine receptor agonists in a concentration-dependent manner; and was antagonized by 8-Methyl-6-(4-methyl-1-piperazinyl)-11*H*-pyrido[2,3-*b*][1,4]benzodiazepine (JL-18), a dopamine D4 receptor antagonist, but not by raclopride, a dopamine D2 and D3 receptor antagonist. Assay methods utilizing contractile responses to ATP may be available for identifying novel dopamine D4 receptor agonists.


*Keywords:* Dopamine D4 receptor; NPA ((−)-Propylnorapomorphine); JL-18; Guinea pig vas deferens

## 1. Introduction

Dopamine receptors were originally classified into two subtypes, D-1 and D-2 (Kebabian and Calne, 1979). However, molecular cloning has now revealed five dopamine receptor subtypes (Sibley and Monsma, 1992; Sibley et al., 1993; Sokoloff and Schwartz, 1995). The dopamine D1-like receptors include dopamine D1 and D5 receptors, while the dopamine D2-like receptors include dopamine D2, D3 and D4 receptors (Seeman and Van Tol, 1994; Sokoloff and Schwartz, 1995). Recently, it has been shown that the dopamine D4 receptor could be associated with attention deficit hyperactivity disorder (DiMaio et al., 2003;Kirley et al., 2002; Grady et al., 2003).

ATP is released as a co-transmitter with noradrenaline from sympathetic nerve endings in the vas deferens (Sneddon and Burnstock, 1984; Burnstock, 1990), and activates the $P_{2x}$-purinoceptor to induce a phasic contraction (Burnstock and Kennedy, 1985; Furukawa and Morishita, 1997). On the other hand, we found that dopamine D4 receptors are located in the postsynaptic site of guinea pig vas deferens (Morishita and Katsuragi, 1999). Activation of dopamine D4 receptors in the presence of prazosin, an $\alpha_1$-adrenoceptor antagonist, enhances the ATP-induced contraction, which is antagonized by dopamine D4 receptor antagonists (Morishita and Katsuragi, 1999).

*R*(−)-propylnorapomorphine (NPA), N-0434, dopamine, apomorphine, quinpirole, PD 128907 and bromocriptine are dopamine receptor agonists. JL-18, a clozapine analog, is the dopamine D4 receptor antagonist that is more selective than clozapine (Liegéois et al., 1995). Raclopride is an antagonist for dopamine D2 and D3 receptors (Seeman and Van Tol, 1994).

In this paper, we report a novel assay method of searching for dopamine D4 receptor agonists by utilizing the ATP-induced contraction in guinea pig vas deferens.

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doi:10.1016/j.ejphar.2005.04.024

## 2. Materials and methods

### 2.1. Molecular biological analysis

A dopamine D4 receptor cDNA clone was isolated by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was extracted from the guinea pig vas deferens using TRIzol according to the manufacturer's instructions. One microgram of RNA was used as a template for single-stranded cDNA synthesis using MMLV reverse transcriptase with oligo (dt) primers. Two primers were synthesized using published human and mouse dopamine D4 receptor cDNA sequences. The oligonucleotide primers for the dopamine D4 receptor were: 5′-TGTGTGACGCACTCATGGCAATGGAC-GTCA-3′ (human dopamine D4 receptor: 718–747, the 5′ end of the third transmembrane domain, sense primer), and 5′-TCATGC-TACTGCTTTACTGGGCCACTTTCC-3′ (mouse dopamine D4 receptor; 320–349, the 3′ end of the fifth transmembrane domain, antisense primer). PCR was carried out with Taq DNA polymerase using primers under the following conditions: 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C. Amplified cDNA of the guinea pig dopamine D4 receptor gave a fragment 324 bp in length. Guinea pig dopamine D4 receptor cDNA was ligated into the pGEM-T plasmid, further amplified in transformed *E. coli*, and sequenced directly on an automated system using fluorescence-labeled dideoxynucleotides.

To examine the distribution of guinea pig dopamine D4 receptor mRNA, RT-PCR was carried out using total RNA prepared from vas deferens, brain and urinary bladder. PCR products were separated by electrophoresis in a 2.0% agarose gel and transferred to a nylon membrane. PCR products were labeled by random primer labeling and then used as hybridization probes.

TRIzol (Gibco BRL, Rockville, MD), MMLV reverse transcriptase with oligo (DT) primers (Perkin-ELmer Cetus, Norwalk, CT) and pGEM-T plasmid (Protégé, Madison, WI) were used.

### 2.2. Western blot analysis for dopamine receptors

Tissues were homogenized in phosphate-buffered saline (PBS), 1 mM benzamidine and 1 mM phenylmethylsulfonyl fluoride. Part of these homogenized tissues was centrifuged at 3000×*g* for 15 min, and the resultant supernatant centrifuged at 10,000×*g* for 30 min. Pellet fractions were resuspended in PBS.

Homogenates and membranes were mixed with sodium dodecyl sulfate-polyacrylamide polyacrylamide gel electrophoresis (SDS-PAGE). They were then subjected to SDS-PAGE on 8.5% acrylamide gels, and blotted to transfer membranes (Immobilon™-P). Proteins were immunostained with rabbit anti-mouse dopamine D4 receptor polyclonal antibody (Chemicon international) and visualized using the Amersham enhanced chemiluminesence (ECL) detection system (Amersham Biosciences).

Rabbit anti-mouse dopamine D4 receptor affinity purified polyclonal antibody (Chemicon, Temecula, CA) and membranes from CHO cells transfected with the human recombinant D4.4 dopamine receptor (Research Biochemicals International) were obtained commercially.

### 2.3. Pharmacological analysis

Male guinea pigs (280–550 g) were killed by stunning and exsanguination, and vasa deferentia isolated. The preparations were dissected from the surrounding connective tissue and suspended in a 20-ml muscle chamber containing Krebs–bicarbonate solution (pH 7.35 to 7.40) maintained at 37 °C, with a gas mixture of 5% $CO_2$ in $O_2$ continuously bubbled through the fluid. Longitudinal contractions of vasa deferentia were recorded isometrically with a force displacement transducer (Nihon Kohden, SB-1T) linked to a polygraph. The resting tension was adjusted to 0.80 g and the preparation was allowed to equilibrate for 90 min to obtain a steady tension before the start of the experiment (Morishita and Katsuragi, 1998). During this period, the bathing solution was changed three times. The composition of the Krebs–bicarbonate solution used was (mM): NaCl 117.7, KCl 4.7, $CaCl_2$ 2.5, $KH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 24.4 and dextrose 10.0.

Solutions of dopamine receptor agonist were made daily. Working solutions of the desired concentration for experimental use were freshly prepared by diluting the stock solution with Krebs–bicarbonate solution before the experiments. Drugs were added to the organ bath in volumes of 0.25 ml or less. All concentrations in the text refer to final concentrations of drugs in the muscle chamber, and are expressed in terms of molarity. Prazosin ($10^{-7}$ M) was always added to the bathing solution 15 min before ATP to block the $\alpha_1$-adrenoceptor agonistic activity of dopamine receptor agonists. To observe the effects of NPA (propylnorapomorphine) on the ATP-induced contraction, NPA was added to the organ bath 5 min prior to ATP. Further, to observe antagonism to potentiate the effect of NPA, dopamine receptor antagonists were added 5 min prior to NPA.

NPA, N-0434, dopamine, apomorphine, PD 128907 and raclopride were dissolved in a 0.01 M HCl solution containing $NaHSO_3$ 0.1 mM to prevent oxidation. Prazosin and clozapine were dissolved in a 0.01 M HCl solution. Bromocriptine was dissolved in 30% ethyl alcohol, while other drugs were dissolved in distilled water.

Adenosine 5′-triphosphate disodium salt (ATP) (Boehringer Mannheim), dopamine hydrochloride (Nakarai Chem.), *R*(−)-apomorphine hydrochloride, (+)-bromocriptine methanesulfonate, (±)-2-(*n*-phenyl-*n*-propyl)amino-5-hydroxytetralin hydrochloride (N-0434), *R*(−)-propylnorapomorphine hydrochloride (NPA), (*S*)-(+)-(4*aR*,10*b*-Tetrahydro-4-propyl-2*H*,5*H*-[1]benzopyrano-[4,3-*b*]-1,4-oxazin-9-ol hydrochloride (PD 128907 hydrochloride), (−)-quinpirole hydrochloride, 8-methyl-6-(4-methyl-1-piperazinyl)-11*H*-pyrido[2,3-*b*][1,4]benzodiazepine (JL-18),prazosin hydrochloride, *S*(−)-raclopride L-tartrate, (Research Biochemicals International) were all obtained commercially.

### 2.4. Data and statistical analysis

The maximum contraction induced by ATP ($10^{-7}$ M), at 90 min, was regarded as the control (100%). The results are expressed as mean values ± S.E.M.

Statistical significance of differences between values were determined by analysis of variance (ANOVA) followed by Dunnett's test for multiple comparisons with a single control or by Student's paired *t*-test for paired values. *P* values of 0.05 or less were considered to be significant.

## 3. Results

### 3.1. Cloning of dopamine D4 receptor in guinea pig vas deferens

As seen in Fig. 1, the predicted amino acid sequence of the guinea pig dopamine D4 receptor displayed 91.7% homology with the human one.

```
      TRANSMEMBRANE DOMAIN 3   INTRACELLULAR
a   CDALMAMDVMLCTASIFNLCAISVDRFVAVAVPLRYNRQGGSRR
b   CDALMAMDVMLCTASIFNLCAISVDRFVAVTVPLSYGRQG--RR

      TRANSMEMBRANE DOMAIN 4   EXTRACELLULAR
a   QLLLIGATWLLSAAVAAPVLCGLNDVRGRDPAVCRLED
b   QLLLIGATWLLSAAVAAPVLFGLNDVRGRDPTVCRLED

      TRANSMEMBRANE DOMAIN 5   INTRACELLULAR
a   RDYVVYSSVCSFFLPCPLMLLLYWATFRG------
b   RDYVVYSSVCSFFLPCPVMLLLYWATF---------
```

Fig. 1. Deduced amino acid sequence comparison between the human (a) and guinea pig (b) dopamine D4 receptors. Transmembrane domains are shown in bold. Boxes represent amino acid differences.

### 3.2. Distribution of dopamine D4 receptor mRNA

The distribution of dopamine D4 receptor mRNA in vas deferens, brain and urinary bladder was examined by Southern blot analysis. As seen in Fig. 2, high levels of receptor mRNA were detected in vas deferens and brain, but only a weak signal was observed in urinary bladder.

### 3.3. Western blot analysis for dopamine D4 receptors

In Western blot analysis Fig. 3, a major band with an apparent molecular mass of 55 kDa was predicted to correspond to dopamine D4.4 receptor protein. The 38-kDa band is likely a degradation product, as has been reported by Lanau et al. (1997).

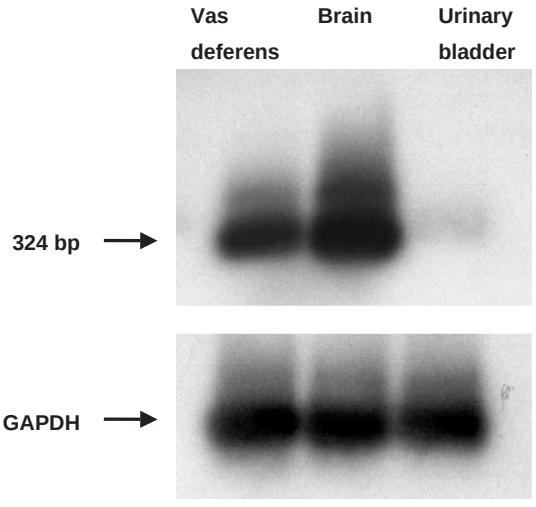


Fig. 2. Tissue distribution of guinea pig dopamine D4 receptor mRNA. Southern blot analysis was performed on PCR products amplified using primers for dopamine D4 receptor from different guinea pig tissues. GAPDH: glyceraldehyde-3-phosphate dehydrogenase; bp: base pair.

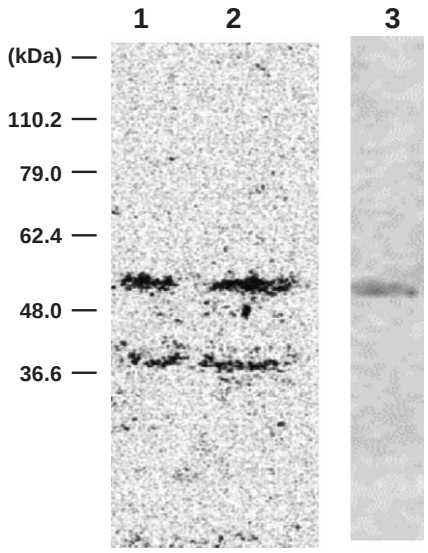


Fig. 3. Western blotting analysis of dopamine D4 receptors immunoreactivity in vas deferens of guinea pigs. Homogenates (lane 1: 50 μg/lane; lane 2: 100 μg/lane) were prepared from vas deferens. The human recombinant D4.4 dopamine receptors (lane 3: 50 μg/lane) were obtained commercially as control. Molecular-mass markers are shown on the left.

### 3.4. Effects of dopamine receptor agonists on ATP-induced contraction in the presence of prazosin

As seen in Fig. 4, ATP ($10^{-7}$ M) induced a transient phasic contraction in the presence of prazosin ($10^{-7}$ M), the mean increase in tension being 0.640±0.025 g from 80 preparations. The ATP-induced contraction in the presence of prazosin ($10^{-7}$ M) was potentiated by NPA, a dopamine D4 receptor agonist (Fig. 4). Similar results were obtained with other dopamine receptor agonists. As seen in Fig. 5, the concentration–response curves were obtained by noncumulative methods. As high concentrations of NPA and apomorphine ($5\times10^{-4}$ M or more) induced rhythmic contractions, the order of the maximum response at a concentration of $10^{-4}$ M appeared as follows: NPA ($n=5$)=N-0434 ($n=8$) ≫ dopamine ($n=8$)=apomorphine ($n=5$) ($P<0.001$)>quinpirole ($n=4$) ($P<0.001$). However, PD 128907 ($n=5$) and bromocriptine ($n=5$) exhibited little activity toward dopamine D4 receptors.

### 3.5. Effects of JL-18 and raclopride on the potentiation of the ATP-induced contraction by NPA

Muscle tone was not affected by JL-18 ($10^{-6}$ M) and raclopride ($10^{-6}$ M) (data not shown). Further, the contractile response to ATP ($10^{-7}$ M) was not altered by these drugs (data not shown). As seen in Fig. 6, the potentiation of the ATP-induced contraction by N-0434 ($1.5\times10^{-6}$ M), NPA ($2.5\times10^{-6}$ M) and dopamine ($2.5\times10^{-5}$ M) in the presence of prazosin ($10^{-7}$ M) was antagonized by JL-18 ($10^{-7}$ M or $5\times10^{-7}$ to $10^{-6}$ M). However, raclopride ($10^{-6}$ M) had no influence on potentiation.

## 4. Discussion

We previously demonstrated that dopamine D4 receptors exist in the smooth muscle of guinea pig vas deferens in a

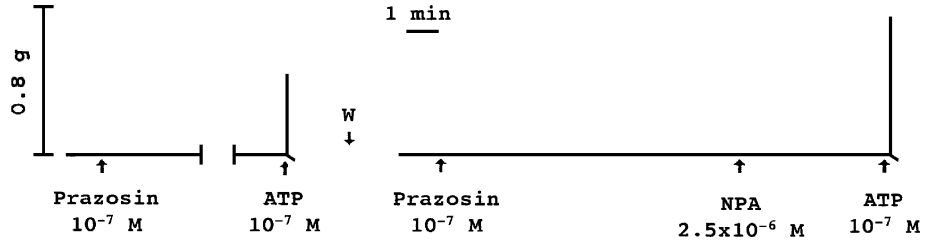


Fig. 4. Typical recording showing potentiation of the ATP-induced contraction by NPA in the presence of prazosin. Prazosin ($10^{-7}$ M) was added to the bathing solution 15 min before ATP ($10^{-7}$ M). NPA ($2.5 \times 10^{-6}$ M) was added 5 min before ATP. The arrows indicate drug administration and wash out (W). The calibration marks indicate 1 min (horizontal) and 0.8 g (vertical), respectively.

pharmacological analysis (Morishita and Katsuragi, 1999). However, the existence of dopamine D4 receptor has not been examined by other methods such as molecular biological analysis.

In this study, a partial cDNA of dopamine D4 receptor was identified, and compared with the amino acid sequence in humans. The predicted amino acid sequence of the guinea pig dopamine D4 receptor clone had high homology with the human one reported by Van Tol et al. (1991). Further, the distribution of dopamine D4 receptor mRNA in tissue was examined by Southern blot analysis. Relatively high levels of mRNA were observed in vas deferens and brain. Further, Western blot analysis showed the existence of dopamine D4.4 receptor protein in vas deferens. Therefore, the results obtained here show that dopamine D4 receptors exist in the smooth muscle of guinea pig vas deferens.

Potentiation of ATP-induced contraction by dopamine receptor agonists was described in the introduction. It has been reported that synergistic cross-talk interaction between G-protein-coupled receptors specific for different neurotransmitters results in augmentation of smooth muscle contraction (Selbie and Hill, 1998). Therefore, the potentiation of ATP-induced contraction by dopamine D4 receptor agonists may be induced by a similar mechanism. Potentiation by NPA, N-0434 and dopamine of the ATP-induced contraction in the presence of prazosin was antagonized by JL-18, a dopamine D4 receptor antagonist,

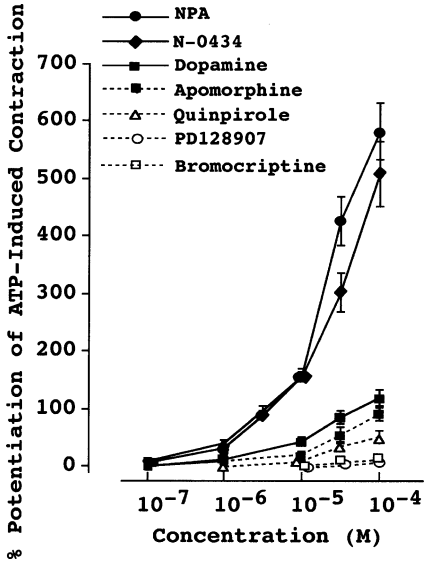


Fig. 5. Concentration-dependent potentiating effects of dopamine receptor agonists on ATP-induced contraction. Results are expressed as percent potentiation of the contraction induced by ATP ($10^{-7}$ M) in the presence of prazosin ($10^{-7}$ M). Concentration-dependent potentiating curves for dopamine receptor agonists were obtained by noncumulative methods. After the contractile response to ATP ($10^{-7}$ M) was observed in the absence and presence of dopamine receptor agonists, preparations were washed out with Krebs–bicarbobate solution. Contractile responses to ATP ($10^{-7}$ M) were observed at intervals of 25 min. Dopamine receptor agonists were added as a single concentration to the bath solution 5 min before ATP ($10^{-7}$ M) in the presence of prazosin ($10^{-7}$ M). Each curve represents the mean values of 4 to 8 experiments.

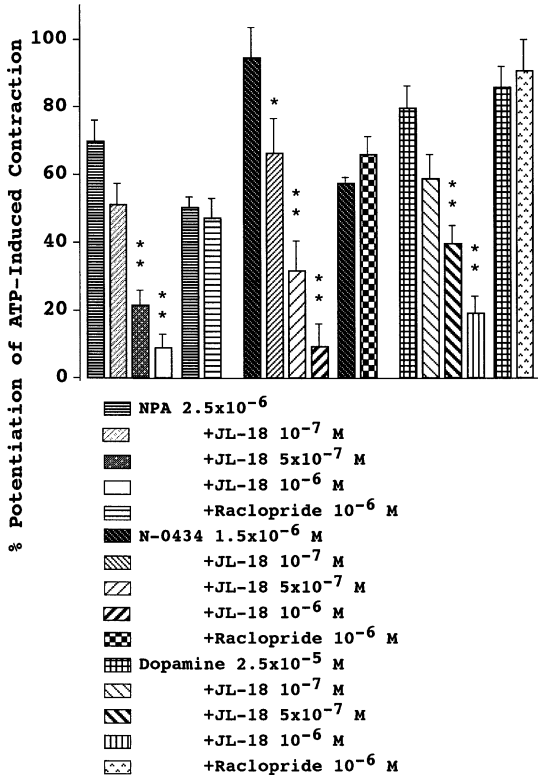


Fig. 6. Effects of JL-18 and raclopride on the potentiation of the ATP-induced contraction by NPA. JL-18 and raclopride were added 10 min before ATP. NPA ($2.5 \times 10^{-6}$ M) was added 5 min before ATP ($10^{-7}$ M). Results are expressed as percent potentiation of the contraction induced by ATP ($10^{-7}$ M). Each column represents the mean percent potentiation from 5 to 6 experiments. Vertical bars indicate standard errors. Significant difference from NPA (*$P<0.05$, **$P<0.001$).

but not by raclopride, an antagonist for dopamine D2 and D3 receptors. This shows that dopamine D4 receptors exist on the smooth muscle of guinea pig vas deferens, and that activation of dopamine D4 receptors potentiates the ATP-induced contraction.

In this study, among the seven dopamine receptor agonists used, NPA and N-0434 showed the most potent agonist activities. NPA is a selective dopamine D4 receptor agonist (Seeman and Van Tol, 1993), whereas N-0434 is a dopamine D2-like receptor agonist, but its selectivity to dopamine receptor subtypes has not been determined (Andersen and Jansen, 1990). However, it has been reported that N-0434 exhibits dopamine D4 receptor agonist activity in neurohypophysial slices of rat (Wilke et al., 1998). The potentiation of ATP-induced contraction by dopamine was much weaker than that of NPA and N-0434. Apomorphine behaved as a partial agonist for dopamine receptors (Gazi et al., 2000). Quinpirole shows higher affinity to dopamine D2 receptor than to dopamine D3 and D4 receptors (Seeman and Van Tol, 1994). The weak potentiation of ATP-induced contraction by apomorphine and quinpirole can be explained from the above facts. Bromocriptine is a dopamine D2 and D3 receptor agonist (Seeman and Van Tol, 1994), and PD 128907 is a dopamine D3 receptor agonist (Pugsley et al., 1995). Thus, the ability of stimulation to dopamine D4 receptors seems to be related to potentiation of the ATP-induced contraction. Therefore, an assay method utilizing contractile responses to ATP on the basis of the unique characteristics of dopamine D4 receptors may be suitable for identifying novel dopamine D4 receptor agonists in guinea pig vas deferens.

As to the attention deficit hyperactivity disorder, spontaneous locomotor activity of mice was reduced dose-dependently by apomorphine and NPA (Costall et al., 1981). Further, stimulation of dopamine D4 receptor can enhance novelty seeking in C57BL/6J mice (Powell et al., 2003). Dopamine D4 receptor-knock-out mice exhibit reductions in behavioral responses to novelty, reflecting a decrease in novelty-related exploration (Dulawa et al., 1999). Recently, it has been reported that selective dopamine D4 receptor agonist facilitates penile erection in rats (Brioni et al., 2004). Thus, selective dopamine D4 receptor agonists may be useful for treatments of attention deficit hyperactivity disorder and male erectile dysfunction.

In conclusion, pharmacological methods that utilize the unique characteristic of dopamine D4 receptors of guinea pig vas deferens may greatly contribute to the development of novel dopamine D4 receptor agonists.

## Acknowledgement

We thank Dr. Takahiro Iwamoto for technical advice of molecular biological analysis.

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