*Short Communication***Pramipexole Upregulates Dopamine Receptor D₂ and D₃ Expression in Rat Striatum**

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Received April 16, 2012; Accepted August 17, 2012

Abstract. Randomized clinical trials have shown that pramipexole has an antidepressant effect in patients with Parkinson's disease. We investigated the comparative efficacy of pramipexole toward dopamine receptor D₂ and D₃ expression in rat brain. Groups of rats were treated subacutely with pramipexole (1 mg/kg), imipramine (10 mg/kg), or bromocriptine (5 mg/kg), with appropriate controls. Using real-time RT-PCR and immunoblotting, dopamine receptor D₂ and D₃ expression was up-regulated in the striatum following pramipexole treatment, while imipramine and bromocriptine had no significant effects. These findings support that pramipexole exerts additional therapeutic benefits such as decreasing depression by increasing dopamine receptor D₃ expression in the striatum.

Keywords: dopamine receptor, Parkinson's disease, pramipexole

Pramipexole is a dopamine agonist used for the treatment of Parkinson's disease. Among the members of the dopamine D₂-like-receptor family (1), pramipexole showed higher selectivity for the dopamine receptor D₃ (DRD3) than for dopamine receptor D₂ (DRD2) and D₄ (2). Despite this affinity profile, it has been assumed that the antiparkinsonian activity of pramipexole is mainly due to DRD2 stimulation, while the role of DRD3 remains unclear (3). The aim of the current study was to investigate the effects of pramipexole on DRD2 and DRD3 expression profiles to reveal potential and additional functions for this dopamine agonist in the field of neuropharmacology.

Male Wistar rats (8-week-old) were purchased from CLEA, Inc. (Tokyo). The animals were housed two per cage under controlled environmental conditions (temperature of 24°C ± 2°C, humidity of 50% ± 5%, and 7 AM – 7 PM / 7 PM – 7 AM light/dark cycle). The animals had free access to food and water. Pramipexole monohydrate was generously provided from Boehringer

Ingelheim GmbH (Ingelheim, Germany). Imipramine hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka) and bromocriptine mesylate (Sigma-Aldrich Corp., St. Louis, MO, USA) were purchased commercially. Pramipexole and imipramine were dissolved in 0.9% saline and injected subcutaneously. Bromocriptine was ultrasonically diffused in 0.25% methyl cellulose 400 solution (Wako Pure Chemical Industries, Ltd.).

In Experiment 1, nine rats (298 – 336 g) received pramipexole at 1 mg/kg or imipramine at 10 mg/kg in a volume of 1 ml/kg subcutaneously twice daily at 9 AM and 4 PM for 14 days. Nine control rats (288 – 312 g) received 1 ml/kg 0.9% saline alone using the same schedule.

In Experiment 2, ten rats (262 – 284 g) received bromocriptine at 5 mg/kg in a volume of 1 ml intraperitoneally twice daily at 9 AM and 4 PM for 14 days. Ten control rats (266 – 282 g) received 1 ml/kg 0.25% methyl cellulose 400 using the same schedule.

The brains were dissected out on the day after the final injection, and the hypothalamus and striatum were isolated. Isolated brain tissues were immediately treated with the RNeasyTM RNA stabilization reagent (Qiagen GmbH, Hilden, Germany) and incubated overnight at 4°C. The solution was then removed and tissues were stored at –80°C until assayed.

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Published online in J-STAGE on September 15, 2012 (in advance)

doi: 10.1254/jphs.12096SC

Frozen tissues were ultrasonically disrupted and total RNA was extracted using RNeasy[®] Lipid Tissue Mini Kit combination with optional on-column DNase digestion (Qiagen, Inc., Valencia, CA, USA). Extracted RNA was reverse-transcribed with PowerScript[™] Reverse Transcriptase (Clontech Laboratories, Inc., Mountain View, CA, USA) in Experiment 1 and Sprint[™] RT Complete-Random Hexamer kit (Clontech Laboratories, Inc.) in Experiment 2 according to the manufacturer's instructions.

Quantification of DRD2 and DRD3 transcripts was performed with Power SYBR[®] Green PCR Master Mix and the 7500 Real-Time PCR System (both supplied by Applied Biosystems, Carlsbad, CA, USA) by using the standard curve method. Primer sets for dopamine DRD2 and DRD3 amplification were purchased from Perfect Real Time PCR support system (Takara Bio, Inc., Shiga). Internal control gene to normalize quantities of cDNA was determined using Rat Housekeeping Gene Primer Set (Takara Bio, Inc.). To determine the absolute copy

number of the target transcripts, cloned plasmid DNA for dopamine DRD2 and DRD3, Gapdh, Ppia, and Atp5f1 were used to generate standard curves. Each sample was run in duplicate.

DRD2 mRNA expression was up-regulated in the striatum following pramipexole administration compared to controls, but there was no change in the hypothalamus (Fig. 1A). There was no significant change in DRD2 mRNA expression in either striatum or hypothalamus following imipramine administration. Expression of DRD3 mRNA was also up-regulated in the striatum following pramipexole administration, but was not significantly changed in the hypothalamus compared to controls (Fig. 1B). Imipramine treatment did not affect DRD3 mRNA expression in the striatum or hypothalamus.

Considering the effects of pramipexole on mRNA expression of both DRD2 and DRD3, we decided to investigate another dopamine agonist, namely bromocriptine, on DRD2 and DRD3 mRNA expression in the hypothalamus and striatum using quantitative real-time RT

Fig. 1. Effects of pramipexole and imipramine on A) dopamine D₂ receptor (DRD2) and B) dopamine D₃ receptor (DRD3) mRNA expression in rat striatum and hypothalamus. Animals received pramipexole at 1 mg/kg or imipramine at 10 mg/kg, subcutaneously, twice daily for 14 days. The control group received vehicle alone using the same schedule. The relative mRNA expression levels of DRD2 and DRD3 were calculated by dividing the average value of dopamine receptor transcripts by that of the housekeeping genes: peptidylprolyl isomerase A (cyclophilin A) (Ppia) or ATP synthase H⁺-transporting mitochondrial F0 complex subunit B1 (Atp5f1). Expression levels are indicated as relative values that assumed expression levels in the hypothalamus of control animals to be 100. The data of quantitative real-time RT PCR were analyzed with one-way analysis of variance (ANOVA) followed by the Tukey test. Each bar represents the mean $\pm$ S.E.M. using the average of two analyses per animal ($n = 8 - 9$ animals). Asterisks: $P < 0.05$ vs. control group.

PCR. However, when compared to controls, no statistically significant change was found in DRD2 and DRD3 mRNA expression in either the hypothalamus or striatum following bromocriptine treatment (Fig. 2A, B). Given the results of the three antiparkinsonian drugs on mRNA expression encoding DRD2 and DRD3, we decided to confirm the DRD2 and DRD3 protein expression profiles in the striatum of pramipexole-treated rats using immunoblotting analysis. Two groups of rats comprising five rats (272 – 340 g) were treated with pramipexole or saline at the same dose and schedule as described above. All rats were decapitated on the day after final injection and striata were isolated.

The striata of rat brains were ultrasonically homogenized with SDS solution in 10 volumes of Laemmli's sample solution containing 3% SDS. Thereafter, samples were immediately heated at 100°C for 5 min. The lysates were electrophoresed using a 15% polyacrylamide gel and transferred to methanol-treated polyvinylidene fluoride (PVDF) membranes. Then, the membranes were

incubated with primary antibodies to Ppia (rabbit polyclonal antibody; Sigma, St. Louis, MO, USA), DRD2 (rabbit polyclonal antibody; Millipore, Temecula, CA, USA), and DRD3 (rabbit polyclonal antibody; Abcam, Cambridge, UK) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG antibody (Promega, Madison, WI, USA). Immunoreaction enhancer solution (Toyobo, Osaka) was used for diluting primary and secondary antibodies. The immunoreaction was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Immunoreactive bands were analyzed by densitometry using Image J 1.43u (Wayne Rasband, National Institute of Health, Bethesda, MD, USA). The densitometry data were standardized using Ppia as an internal standard.

Notably, pramipexole-treatment increased DRD3 protein expression (Fig. 3B). Indeed, there was no statistically significant difference in DRD2 protein expression between pramipexole and control groups, but pramipexole treatment showed a tendency for up-regulation of

Fig. 2. Effects of bromocriptine on A) dopamine D₂ receptor (DRD2) and B) dopamine D₃ receptor (DRD3) mRNA expression in rat striatum and hypothalamus. Animals received bromocriptine at 5 mg/kg, intraperitoneally, twice daily for 14 days. The control group received vehicle alone using the same schedule. The relative mRNA expression levels of DRD2 and DRD3 were calculated by dividing the average value of dopamine receptor transcripts by that of the housekeeping genes: peptidylprolyl isomerase A (cyclophilin A) (Ppia) or glyceraldehyde-3-phosphate dehydrogenase (Gapdh). Expression levels are indicated as relative values that assumed expression levels in the hypothalamus of control animals to be 100. The data of quantitative real-time RT PCR were analyzed with Student's *t*-test. Each bar represents the mean ± S.E.M. using the average of two analyses per animal (*n* = 9 – 10 animals).

Fig. 3. Effects of pramipexole on A) dopamine D₂ receptor (DRD2) and B) dopamine D₃ receptor (DRD3) protein expression in rat striatum. Animals received pramipexole at 1 mg/kg, subcutaneously, twice daily for 14 days. The control group received vehicle alone using the same schedule. Graphs show the densitometric analysis of the immunoblot data presented as the mean $\pm$ S.E.M. (n = 5 per group). Expression levels are indicated as relative values that assumed expression levels of control animals to be 1. Statistical analysis was performed using the Mann-Whitney U-test. Asterisk: $P < 0.05$ vs. control group. Pictures show the representative immunoblot data (n = 1) of DRD2 and DRD3 protein. Ppia was used as the loading internal standard.

DRD2 protein expression (Fig. 3A).

In the present study, we found that pramipexole up-regulated DRD2 and DRD3 mRNA expression in the striatum (Fig. 1), and the increased expression of DRD3 expression was confirmed (Fig. 3B). However, we did not find any statistically significant up-regulation of DRD2 protein levels despite an increased tendency following pramipexole treatment. It is suggested that pramipexole increases both striatal DRD2 and DRD3 mRNA expression, although two weeks may not be sufficient to complete the post-transcriptional phase of protein synthesis for the DRD2. A previous study has shown an effect of pramipexole on dopamine receptor binding and/or behavior in vivo, and pramipexole administration increased DRD3 binding by the agonist in the islands of Calleja and nucleus accumbens in rats. However, the concentration of DRD3 mRNA in the islands of Calleja was not changed by pramipexole administration, and it was suggested that pramipexole administration increased the responsiveness of DRD3 to the agonist and also raised sensitivity to endogenous dopamine (4). In our study, the expression of DRD2 and DRD3 was also unchanged in the hypothalamus following pramipexole administration, although the expression of both genes was up-regulated in the striatum (Fig. 1). As a preliminary study, we administered pramipexole with 1 mg/kg once daily for 5 days, but no effect of pramipexole on DRD2 and DRD3 mRNA was observed in the rat stri-

tum, nor in the hypothalamus. We therefore applied the drug treatment regimen of twice daily for 14 days as described. These results suggest that long-term treatment is necessary to see an effect of pramipexole on dopamine-receptor mRNA expression.

On the other hand, both imipramine and bromocriptine had no significant effect on DRD2 and DRD3 mRNA expression in either the hypothalamus or the striatum. Indeed, previous studies have reported that imipramine up-regulates DRD2 expression in the striatum (5, 6), but no significant changes were observed in our study (Fig. 1). A possible explanation for this discrepancy might be the experimental method. We used quantitative real-time RT PCR instead of an *in situ* hybridization method. Bromocriptine had a tendency to down-regulate DRD2 mRNA expression in the striatum (Fig. 2). In accordance with this, it has been reported that the acute administration of bromocriptine reduced striatal DRD2 mRNA expression by 21% compared to controls in mice (7).

With respect to our findings on pramipexole, it is unclear what physiological effect would result from increased DRD2 and DRD3 mRNA and protein expression. The increased expression might contribute to enhanced binding activity of dopamine receptors, as described above. Previous studies have suggested that, as an anti-parkinsonian drug, the pharmacological action of pramipexole is mainly related to the stimulation of DRD2 (8, 9), while the role of the DRD3 has not been clarified.

The loss of DRD3 could be a more important factor for the loss of responsiveness to dopaminergic drugs rather than DRD2 changes; stimulation of DRD3 is considered a potential therapeutic target in PD (3).

To date, this appears to be the first report showing up-regulation of DRD2 and DRD3 in the striatum in vivo following the administration of the pramipexole. Our findings, taken together, indicate that pramipexole has an additional therapeutic value such working against depression (10) in its activity on motor symptoms by increasing DRD3 expression in addition to increasing DRD2 expression.

Acknowledgments

This work was supported by Grants-in-Aid from the Research Committee of CNS Degenerative Diseases, the Ministry of Health, Labour and Welfare of Japan, and GJTS. Funding sources had no involvement in the conduct of the current research or preparation of its publication.

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