*Full Paper***Effect of Selective Serotonin Reuptake Inhibitors via 5-HT_{1A} Receptors on L-DOPA-Induced Rotational Behavior in a Hemiparkinsonian Rat Model**

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Abstract. L-Dihydroxyphenylalanine (L-DOPA) is considered the gold standard for the treatment of Parkinson's disease (PD). However, long-term administration of L-DOPA can induce abnormal side effects. On the other hand, selective serotonin reuptake inhibitors (SSRIs) including fluoxetine have gained tremendous popularity in the treatment of depression in PD. SSRIs are thought to influence motor function in PD via pharmacological modification of interactions between serotonergic and dopaminergic networks, which are complex and not yet fully understood. In this study, intranigral injection of 6-hydroxydopamine (6-OHDA) in rats caused a significant loss of tyrosine hydroxylase immunoreactivity in the striatum and substantia nigra. However, tryptophan hydroxylase immunoreactivity of the striatum and raphe nucleus was unaffected by 6-OHDA. Immunohistochemical analysis reveals that the serotonergic system was unaffected by the injection of 6-OHDA. We demonstrated also that pre-treatment with fluoxetine significantly suppressed L-DOPA-induced rotational behavior. Additionally, fluoxetine suppressed L-DOPA-induced ERK1/2 and histone H3 phosphorylation. These effects of fluoxetine were abolished by pre-treatment with WAY 100135, a 5-HT_{1A} antagonist. These results suggest that fluoxetine may influence motor function in PD via pharmacological modification of interactions between serotonergic and dopaminergic neuronal networks.

Keywords: Parkinson's disease, selective serotonin reuptake inhibitor, fluoxetine, L-dihydroxyphenylalanine, 6-hydroxydopamine

Introduction

The major pathological basis of Parkinson's disease (PD) is the death of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and the degeneration of their nerve terminals in the striatum. This results in a decrease in the dopamine (DA) content in the striatum. L-Dihydroxyphenylalanine (L-DOPA), the precursor of

DA, is considered the gold standard for the treatment of PD. However, long-term administration of this drug can induce abnormal involuntary movements known as "L-DOPA-induced dyskinesias" (LIDs), which are extremely discomforting for the patients. Serotonin (5-hydroxytryptamine, 5-HT) neurons are of particular interest in LIDs, since they can convert L-DOPA to DA, store the newly formed DA in vesicles, and release it in an activity-dependent manner (1 – 6). In dopaminergic terminals of the striatum, DA release is controlled via DA reuptake by the DA transporter and D₂ auto-receptor feedback control. However, in the absence of any auto-regulatory control mechanisms, DA released from 5-HT terminals is likely to show excessive swings that may be particularly prone to induce dyskinesias. Therefore, it is

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likely that L-DOPA-derived DA, released as a “false transmitter” from 5-HT terminals, is the main trigger of the side-effect of dyskinesia (6, 7).

As PD progresses, patients may experience more severe changes in fluctuating non-motor symptoms. Indeed, depression occurs in approximately 10%–45% of patients with PD (8). Selective serotonin reuptake inhibitors (SSRIs) have recently gained tremendous popularity in the treatment of depression because of a lower incidence of serious side-effects (9, 10). Since depression is the most common psychiatric disturbance in PD, SSRIs are now often used for the treatment of depression in PD. However, in a cohort study, van de Vijver and collaborators used a change in anti-parkinsonian drug treatment as a marker for a worsening of symptoms of PD, and they found that patients who were starting a course of treatment with an SSRI had a four-fold higher risk for the worsening of PD compared to those who were starting treatment with a tricyclic antidepressant (TCA) (11). On the other hand, Arbouw and collaborators found no evidence that SSRIs required greater caution than other antidepressants in patients with PD (12). These results suggest that SSRIs may influence motor function in PD via pharmacological modification of interactions between serotonergic and dopaminergic neuronal networks, which are complex and not yet fully understood.

Selective lesioning of rat dopaminergic pathways using the well-known neurotoxic agent 6-hydroxydopamine (6-OHDA) has long been used as an experimental model for studying DA functions in the brain and for evaluating the effects of drugs on DA neurons in the central nervous system (CNS) (13–15). Microinjection of 6-OHDA directly into the brain causes the destruction of nigrostriatal DA-containing neurons. The extent of DA depletion can then be assessed by examining rotational behavior in response to the injection of L-DOPA. The 6-OHDA model has a quantifiable motor deficit (rotation) and has been shown to be useful in the pharmacological screening of agents that affect DA and its receptors (14, 15). On the other hand, the molecular mechanism that underlies drug-induced rotational behavior is also associated with the appearance of L-DOPA-induced dyskinesias (16). DA depletion results in a remarkable increase in the ability of dopaminergic drugs to activate the extracellular signal-regulated kinase (ERK) transduction pathway (17, 18). Indeed, the administration of L-DOPA is accompanied by enhanced levels of phosphorylated ERK in 6-OHDA-lesioned mice (19, 20).

The objective of the present study was to investigate i) whether L-DOPA-induced rotational behavior is influenced by serotonergic neuronal networks, and if so, ii) the molecular mechanisms of signal alterations caused by L-DOPA and serotonergic drugs. In this study, we

used fluoxetine, a major SSRI that is a representative drug that influences serotonergic neuronal networks. Fluoxetine has been clinically used for the treatment of depression in patients with PD. In the present study, we used fluoxetine to demonstrate that the pharmacological attenuation of ERK signaling counteracts L-DOPA-induced rotational behavior.

Materials and Methods

Experimental design

Male Wistar rats (SLC, Shizuoka) weighing approximately 250 g were used in the present study. Rats were acclimated to and maintained at 23°C under a 12-h light/dark cycle (light on 08:00–20:00 h). All animal experiments were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the protocols were approved by the Committee for Animal Research at Kyoto Pharmaceutical University.

At the beginning of the experiment, rats received a unilateral intranigral injection of either 6-OHDA, to lesion the nigrostriatal pathway, or vehicle in the substantia nigra on the left side. Two weeks later, 6-OHDA-injected rats were screened behaviorally in the methamphetamine-induced rotation test. 6-OHDA-injected rats that exhibited more than 300 full body turns / 60 min toward the side of DA deficiency were included in the study. In addition, these selected rats were balanced, according to the results in the L-DOPA-induced rotation test, into the well-matched subgroups. Using 6-OHDA-lesioned rats, we examined the effects of the 5-HT_{1A} agonist 8-OH-DPAT (1 mg/kg; Tocris, Bristol, UK), the 5-HT_{1B} agonist CP94253 (2.5 and 3.5 mg/kg, Tocris), the SSRI fluoxetine (10 mg/kg, Tocris), and the 5-HT_{1A} antagonist WAY 100135 (2 mg/kg, Tocris) on L-DOPA-induced rotational behaviors. We decided the dose of each drug based on previous reports (5, 6, 21). For the agonists experiment, after selection as described earlier, either saline or agonist was injected i.p. 15 min before the injection of L-DOPA plus benserazide. For the SSRIs experiment, either saline or WAY 100135 was injected i.p. 15 min before the injection of saline or fluoxetine, and 15 min later, L-DOPA plus benserazide was injected. After the behavior tests, rats were sacrificed and the brains were quickly removed for immunohistochemical and neurochemical analyses.

6-OHDA lesions

In the nigral 6-OHDA lesion experiment, rats received a unilateral injection of either 6-OHDA or vehicle in the substantia nigra on the left side. For stereotaxic microinjection, rats were anesthetized (sodium pentobarbital, 50

mg/kg, i.p.) and immobilized in a Kopf stereotaxic frame. Subsequently, rats were microinjected with 6-OHDA (Sigma, St. Louis, MO, USA) for a total of 8 μ g in a final volume of 4 μ L of phosphate-buffered saline (PBS) containing 0.02% ascorbic acid at the following coordinates relative to the bregma [4.8-mm anterior-posterior (AP), 1.8-mm left lateral (L), -7.8-mm ventral (V)]. For the vehicle controls, PBS containing 0.02% ascorbic acid was injected into the SNpc. Microinjection was performed with a motor-driven 10- μ L Hamilton syringe using a 26-gauge needle. The infusion rate was 1 μ L/min, and the injection cannula was kept in place for a further 5 min after injection.

Drug-induced rotation

Drug-induced rotational asymmetry was assessed according to the experimental design as described earlier. Rotational behavior was tested in rotometer bowls (13, 22). Briefly, the total number of full 360° rotations in the ipsilateral and contralateral directions was counted for 60 min after the intraperitoneal administration of methamphetamine (Dainippon Sumitomo Pharma Co., Ltd., Osaka; 2.5 mg/kg) or L-DOPA (Sigma, 10 mg/kg) plus benserazide (Sigma, 6.25 mg/kg).

Tissue preparation and immunohistochemistry

Treated rats were perfused through the aorta with 150 mL of 10 mM PBS, followed by 300 mL of a cold fixative consisting of 4% paraformaldehyde in 100 mM phosphate buffer (PB), under deep anesthesia with pentobarbital (100 mg/kg, i.p.). After perfusion, the brain was quickly removed and postfixed for 2 days with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide at 4°C. The brain slices were cut using a cryostat and collected in 100 mM PBS containing 0.3% Triton X-100 (PBS-T). Primary antibodies included mouse monoclonal antibodies against tyrosine hydroxylase (TH, Sigma) and tryptophan hydroxylase (TPH; Chemicon Int., Temecula, CA, USA). Brain slices were incubated with primary antibody, including TH (diluted 1:10,000) and TPH (1:1,000), for 3 days at 4°C. After several washes, slices were incubated with biotinylated anti-mouse or anti-rabbit IgG antibody (1:2,000), as appropriate, for 2 h at room temperature. The slices were then incubated with avidin peroxidase (ABC Elite Kit, 1:4,000; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. All of the slices were washed several times with PBS-T between each incubation, and labeling was revealed by 3,3'-diaminobenzidine (DAB) with nickel ammonium, which yielded a dark blue color.

Measurement of immunoreactive neurons and areas

TH-positive neurons within the SNpc or TPH-positive neurons within the dorsal raphe nucleus (RN) were estimated using Stereo Investigator software (MBF Bioscience, Williston, VT, USA) based on stereologic principles. Six slices (each 60- μ m-thick), each separated by 300 μ m from the anterior to the posterior, were used for counting in each case. Stereology was performed using a light microscope (BX51; Olympus, Tokyo) coupled to an Optronics Microfire digital camera (CX9000, MBF Bioscience) for the visualization of tissue sections. The total number of immunopositive neurons was estimated from coded slides. For each tissue section analyzed, the section thickness was assessed empirically and 5 μ m-thick guard zones were used at the top and bottom of each section. The SNpc or RN was outlined under low magnification (4 $\times$) and 10 sites in the outlined region were analyzed using a systematic random sampling design generated with the following stereologic parameters: counting frame size, 180 $\times$ 180 μ m; and dissector height, 20 μ m. Neurons were counted under 40 $\times$ magnification. The coefficients of error (CE) were calculated and values < 0.10 were accepted.

The density of TH- or TPH-fibers in the striatum region was determined using a camera (Progres 3008; Carl Zeiss, Jena, Germany) and computerized image-analysis system (WinRoof; Mitani, Fukui) (22). For the analysis of striatal TH- or TPH-immunoreactive intensity, the optical density was measured within a fixed box (0.5 $\times$ 0.5 mm) positioned approximately in the middle of the striatum. Immunoreactive intensity was expressed as a percentage of the intensity recorded from the same area on the contralateral side (14). Subsequently, the average relative intensity in each quadrant was estimated from three striatal slices (1.60, 0.60, and -0.30 mm anterior from the bregma) and a statistical analysis was performed in treated rats.

Immunoblotting

The striatum in treated rats was rapidly removed and homogenized with 7 volumes of 50 mM Tris-HCl buffer (pH 7.4) containing 0.32 M sucrose, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM orthovanadic acid, 1 mM phenyl methyl sulfonyl fluoride (PMSF), 1 μ M okadaic acid, and 1 μ g/mL leupeptin. After centrifugation at 800 $\times$ g for 15 min, the pellets were used as the nuclein fraction. The supernatants were centrifuged at 15,000 $\times$ g for 20 min. The supernatants were then collected as the cytosol fraction and the resulting precipitates were used to prepare particulate fractions. The protein concentration was measured using a Bradford assay. Aliquots of cytosolic or nuclein fraction containing 10 μ g of protein

were subjected to sodium dodecyl sulfate–polyarylamide gel electrophoresis (SDS–PAGE) and then immunoblotting using mouse monoclonal antibodies against phosphohistone H3 (Ser10) (1:2,000; Millipore, Billerica, MA, USA), 67-kDa glutamic acid decarboxylase (GAD67, 1:5,000; Millipore), TH (1:5,000, Sigma), and β -actin (1:2,000, Sigma), or rabbit polyclonal antibodies against phospho-p44/42 MAPK (phospho-ERK1/2, 1:2,000; Cell Signaling Technology, Danvers, MA, USA), p44/42 MAPK (ERK1/2, 1:1,000; Cell Signaling Technology) and histone H3 (Ser10) (1:500, Cell Signaling Technology). For a semi-quantitative analysis, the bands of these proteins on radiographic films were scanned with a CCD color scanner (DuoScan; AGFA, Leverkusen, Germany) and then analyzed. Densitometric analysis was performed using the public-domain program NIH Image 1.56.

Statistical evaluation

Drug-induced rotational asymmetry, the number of immunopositive neurons, and the optimal density in the striatum are given as the mean $\pm$ standard error of the mean (S.E.M.). The significance of differences was determined by an analysis of variance (ANOVA). Further statistical analysis for *post hoc* comparisons was performed using the Bonferroni/Dunn test (StatView; Abacus Concepts, Berkeley, CA, USA).

Results

Effect of 6-OHDA on DA and 5-HT neurons

First, to confirm the degree of dopaminergic and/or serotonergic neurodegeneration in 6-OHDA-injected rats, we performed an immunohistochemical analysis (Figs. 1 and 2). As shown in representative photomicrographs (Fig. 1), TH immunoreactivity was markedly decreased in both the ipsilateral striatum (Fig. 1B) and substantia nigra (Fig. 1D) of rats that received intranigral injections with 6-OHDA compared to those injected with vehicle (Fig. 1: A and C). A semi-quantitative analysis showed that the intensity of TH immunoreactivity in the striatum was significantly decreased in 6-OHDA-injected rats (6-OHDA: $42\% \pm 13\%$, vehicle: $100\% \pm 3.5\%$, $P < 0.001$ vs. vehicle; Fig. 1E). Similarly, a stereological analysis of nigral TH-positive neurons showed that the microinjection of 6-OHDA caused a significant loss of TH-positive neurons (6-OHDA: 592 ± 278 cells, vehicle: 8766 ± 529 cells, $P < 0.001$ vs. vehicle; Fig. 1F).

As shown in representative photomicrographs (Fig. 2), TPH immunoreactivity was not changed in both the ipsilateral striatum (Fig. 2B) and dorsal raphe nucleus (Fig. 2D) of rats that were injected with 6-OHDA compared to those that were injected with vehicle (Fig. 2: A and C). A semi-quantitative analysis showed that the intensity of

Fig. 1. Effect of 6-OHDA on dopaminergic neurons. Treated rats were sacrificed and the brains were quickly removed for immunohistochemical analysis. Brain slices of the striatum (A, B) and mid-brain (C, D) were immunostained by anti-TH antibody. Scale bar: 2 mm in A, 1 mm in C. E: For the semi-quantitative analysis of TH-immunoreactive intensity, we analyzed coronal sections of the striatum. F: Stereological analysis of TH-positive neurons in the substantia nigra. Each value is the mean $\pm$ S.E.M. ($n = 7$ in each group). Significance: *** $P < 0.001$ vs. vehicle.

Fig. 2. Effect of 6-OHDA on serotonergic neurons. Treated rats were sacrificed and the brains were quickly removed for immuno-histochemical analysis. Brain slices of striatum (A, B) and midbrain (C, D) were immunostained by anti-TPH antibody. Scale bar: 2 mm in A, 1 mm in C. E: For the semi-quantitative analysis of TPH-immunoreactive intensity, we analyzed coronal sections of the striatum. F: Stereological analysis of TPH-positive neurons in the dorsal raphe nucleus. Each value is the mean $\pm$ S.E.M. ($n = 7$ in each group).

TPH immunoreactivity in the striatum did not differ between 6-OHDA-injected and vehicle-injected rats (6-OHDA: $101\% \pm 14\%$, vehicle: $100\% \pm 14\%$; Fig. 2E). Similarly, a stereological analysis of TPH-positive neurons showed that there were no differences between 6-OHDA-injected and vehicle-injected rats (6-OHDA: 23193 ± 2043 cells, vehicle: 26950 ± 3430 cells; Fig. 2F).

Effect of fluoxetine on L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats

The results of the immunohistochemical analysis (Figs. 1 and 2) suggest that the serotonergic system was unaffected by the injection of 6-OHDA. If so, drugs associated with serotonergic neural activation (tone) should also be able to control L-DOPA-induced rotational behavior. As shown in Fig. 3A, the injection of L-DOPA plus benserazide induced contralateral rotation in 6-OHDA-lesioned hemiparkinsonian rats (289 ± 42 in 6-OHDA-injected rats treated with L-DOPA plus benser-

azide). Pre-treatment with 8-OH-DPAT, a 5-HT_{1A} agonist, inhibited L-DOPA-induced rotational behavior (total rotations: 84 ± 26 and 289 ± 42 in 6-OHDA-injected rats pre-treated with 8-OH-DPAT and saline, respectively, $P < 0.001$ vs. saline), while pre-treatment with CP94253 (2.5 mg/kg), a 5-HT_{1B} agonist, did not (total rotations: 245 ± 90 in 6-OHDA-injected rats pre-treated with CP94253 at 2.5 mg/kg) (Fig. 3A). We also examined whether pre-treatment with the high dose of CP94253 (3.5 mg/kg) inhibited L-DOPA-induced rotational behavior. However, pre-treatment with CP94253 (3.5 mg/kg) did not inhibit L-DOPA-induced rotational behavior (total rotations: 330 ± 135 in 6-OHDA-injected rats pre-treated with CP94253 at 3.5 mg/kg) (data not shown). On the other hand, pre-treatment with fluoxetine significantly suppressed L-DOPA-induced rotational behavior in 6-OHDA-injected rats (total rotations: 46 ± 26 and 266 ± 61 in 6-OHDA-injected rats pre-treated with fluoxetine and saline, respectively; $P < 0.001$ vs. saline) (Fig. 3B). Moreover, the combination of pre-treatment

Fig. 3. Effect of serotonergic drugs on L-DOPA-induced rotational behavior in rats with intranigral 6-OHDA lesion. Using 6-OHDA-lesioned rats, we examined the effects of 8-OH-DPAT, CP94253, fluoxetine, and WAY 100135 on L-DOPA-induced rotational behavior. A: Either 8-OH-DPAT or CP94253 was injected i.p., and 15 min later, L-DOPA plus benserazide was injected to evaluate rotational behavior. B: Either saline or WAY 100135 was injected i.p. 15 min before the injection of either saline or fluoxetine, and 15 min later, L-DOPA plus benserazide was injected to evaluate rotational behavior. Each value is the mean $\pm$ S.E.M. ($n = 7$ in each group). Significance: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. 6-OHDA alone. ††† $P < 0.001$ vs. L-DOPA-induced rotational behavior in 6-OHDA injected rats. ### $P < 0.001$ vs. L-DOPA-induced rotational behavior in 6-OHDA-injected rats that were treated with fluoxetine.

with fluoxetine and WAY 100135, a selective 5-HT_{1A} antagonist, did not inhibit L-DOPA-induced rotational behavior (total rotations: 46 ± 26 and 303 ± 85 in 6-OHDA-injected rats pre-treated with fluoxetine and pre-treated with the combination of fluoxetine and WAY 100135, respectively, $P < 0.001$ vs. pre-treated with fluoxetine) (Fig. 3B).

Effect of fluoxetine on L-DOPA-induced ERK phosphorylation in rats with intranigral 6-OHDA lesion

To investigate the molecular mechanisms that underlie signal alterations caused by L-DOPA and fluoxetine, we examined the effect of fluoxetine on L-DOPA-induced ERK1/2 phosphorylation in the striatum of 6-OHDA-injected rats. ERK1/2 phosphorylation was significantly increased by the administration of L-DOPA in 6-OHDA-lesioned rats, although the total levels of ERK1/2 were unaffected (p-ERK1 / ERK1: $276\% \pm 15\%$ and $100\% \pm 13\%$ in L-DOPA and saline, respectively, $P < 0.001$ vs. saline; p-ERK2 / ERK2: $254\% \pm 5.1\%$ and $100\% \pm 11\%$ in L-DOPA and saline, respectively, $P < 0.001$ vs. saline; Fig. 4). Pre-treatment with fluoxetine significantly suppressed L-DOPA-induced ERK1/2 phosphorylation (p-ERK1 / ERK1: $141\% \pm 24\%$ in pre-treatment with fluoxetine, $P < 0.001$ vs. L-DOPA; p-ERK2 / ERK2: $108\% \pm$

35% in pre-treatment with fluoxetine, $P < 0.001$ vs. L-DOPA). This effect of fluoxetine was abolished by pre-treatment with WAY 100135 (p-ERK1 / ERK1: $260\% \pm 17\%$ in pre-treatment with the combination of fluoxetine and WAY 100135, $P < 0.01$ vs. pre-treatment with fluoxetine; p-ERK2 / ERK2: $218\% \pm 25\%$ in pre-treatment with the combination of fluoxetine and WAY 100135, $P < 0.01$ vs. pre-treatment with fluoxetine).

Effect of fluoxetine on L-DOPA-induced histone H3 phosphorylation in rats with intranigral 6-OHDA lesion

The activation of ERK results in sequential histone H3 phosphorylation (23). Therefore, we examined whether pre-treatment with fluoxetine inhibited histone H3 phosphorylation. Histone H3 phosphorylation was significantly increased by the administration of L-DOPA plus benserazide in 6-OHDA-lesioned rats, although the total level of histone H3 was unaffected (p-histone H3 / histone H3: $220\% \pm 14\%$ and $100\% \pm 18\%$ in L-DOPA and saline, respectively, $P < 0.001$ vs. saline; Fig. 5). Pre-treatment with fluoxetine significantly suppressed L-DOPA-induced histone H3 phosphorylation (p-histone H3 / histone H3: $76\% \pm 17\%$ in pre-treatment with fluoxetine, $P < 0.001$ vs. L-DOPA). This effect of fluoxetine

Fig. 4. Effect of fluoxetine on L-DOPA-induced ERK phosphorylation in rats with intranigral 6-OHDA lesion. Either saline or WAY 100135 was injected i.p. 15 min before the injection of either saline or fluoxetine, and 15 min later, L-DOPA plus benserazide was injected. A: Representative phosphorylated (left) and total (right) ERK1/2. Quantification of phospho-ERK1 (p-ERK1, B) and phospho-ERK2 (p-ERK2, C) by western blotting. Each value is the mean $\pm$ S.E.M. ($n = 5$ in each group). Significance: *** $P < 0.001$ vs. 6-OHDA alone. ††† $P < 0.001$ vs. L-DOPA-induced ERK phosphorylation in 6-OHDA-injected rats. ## $P < 0.01$ vs. L-DOPA-induced ERK phosphorylation in 6-OHDA-injected rats treated with fluoxetine.

Fig. 5. Effect of fluoxetine on L-DOPA-induced histone H3 phosphorylation in rats with intranigral 6-OHDA lesion. Either saline or WAY 100135 was injected i.p. 15 min before the injection of either saline or fluoxetine, and 15 min later, L-DOPA plus benserazide was injected. A: Representative phosphorylated and total histone H3. B: Quantification of phospho-histone H3 (p-histone H3) by western blotting. Each value is the mean $\pm$ S.E.M. ($n = 5$ in each group). Significance: ** $P < 0.01$, *** $P < 0.001$ vs. 6-OHDA alone. ††† $P < 0.001$ vs. L-DOPA-induced histone H3 phosphorylation in 6-OHDA-injected rats. ### $P < 0.001$ vs. L-DOPA-induced histone H3 phosphorylation in 6-OHDA-injected rats treated with fluoxetine.

was abolished by pre-treatment with WAY 100135 (p-histone H3 / histone H3: $202\% \pm 17\%$ in pre-treatment with the combination of fluoxetine and WAY 100135, $P < 0.001$ vs. pre-treatment with fluoxetine).

Discussion

In the early stage of PD, it is generally assumed that L-DOPA acts by being taken up into spared DA neurons and terminals. As dopaminergic degeneration progresses, fewer and fewer DA terminals can contribute to the conversion of peripherally administered L-DOPA. Under these conditions, other neuronal and non-neuronal cell types have been suggested to play a role in the production of DA (24–26). In the normal brain, the majority of 5-HT innervation of the basal ganglia comes from the dorsal and, to lesser extent, medial raphe nuclei (27, 28). The dopaminergic and 5-HT systems are functionally connected and 5-HT, via its numerous receptors, modulates dopaminergic transmission and DA levels in the striatum (29). Additionally, 5-HT neurons represent an interesting element because they express aromatic amino acid decarboxylase and vesicular monoamine transporter 2 (3). Indeed, 5-HT neurons can store and release DA after the peripheral administration of L-DOPA, both in vivo and in vitro (1, 2). Previous reports showed that dyskinesia induced by chronic L-DOPA treatment in 6-OHDA-lesioned rats was critically dependent on the integrity and function of the serotonergic system (5, 6). Previous studies also showed that the 5-HT₁- as well as 5-HT₂-receptor super-family are involved in a variety of behaviors and disease including PD (30). Among of them, 5-HT₁-receptor agonists have been studied in PD for their potential effects. On the contrary, 5-HT_{2A}- and also 5-HT_{2C}-receptor antagonists, but not their respective agonists, have been frequently studied in PD for their potential effects on both motor and non-motor complications of treatment. In this study, we examined effect of fluoxetine on L-DOPA-induced rotational behavior. Therefore, we speculated that 5-HT increased by fluoxetine might modulate L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats via the stimulation of 5-HT receptors. Fluoxetine suppressed L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats, and this effect of fluoxetine was almost completely inhibited by pre-treatment with a 5-HT_{1A} antagonist. Indeed, we showed that L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats was significantly decreased when these rats were pretreated with a 5-HT_{1A}-receptor agonist, but not 5-HT_{1B}-receptor agonist. Therefore, these results suggest that fluoxetine modulates the pharmacological actions of L-DOPA such as rotational behavior via 5-HT_{1A} receptors.

SSRIs, including fluoxetine, exert their pharmacological effects by increasing the extracellular 5-HT content around serotonergic neurons through blockade of the 5-HT transporter. In the acute phase of SSRIs administration, the blockade of 5-HT reuptake by SSRIs induces an increase in extracellular 5-HT content, preferentially around the cell bodies of serotonergic neurons, which results in the activation of 5-HT_{1A} receptors located at these sites, leading to a decrease in the firing rate of serotonergic neurons (21, 31). In the present study, pre-treatment with fluoxetine significantly suppressed L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats. In addition, this effect of fluoxetine was abolished by pre-treatment with WAY 100135. Activation of 5-HT_{1A} receptors reduces serotonergic nerve discharge, which consequently leads to a reduction in 5-HT release from the corresponding afferent nerve terminals, including those originating from the dorsal raphe nucleus to the striatum (32, 33). Indeed, L-DOPA-derived extracellular DA contents were attenuated by treatment with fluoxetine (31). The effect of fluoxetine observed in the present study may be due to its indirect 5-HT_{1A} agonistic property, and L-DOPA-induced rotational behavior was inhibited through the reduced efflux of L-DOPA-derived extracellular DA.

All of the dopamine receptor subtypes (D₁ – D₅) are present in the striatum, although D₁ and D₂ receptors are the most abundant. G_s-coupled D₁ receptors are mainly expressed on striatonigral ('direct') medium spiny neurons, while G_i-coupled D₂ receptors are enriched in striatopallidal ('indirect') neurons (34). It is commonly accepted that the balance of signal control through these dopamine receptors is essential for the striatal control of movement and reward. Previously, it was clearly shown that the D₁ receptor was critical for the development of L-DOPA-induced dyskinesias in mice and in the underlying molecular changes in the denervated striatum, while the D₂ receptor had, at most, only a slight role (35). A contralateral rotational response, a commonly used measure of behavioral sensitization to L-DOPA, is triggered by the supersensitivity of DA receptors in the DA-depleted striatum. This molecular mechanism is also associated with the appearance of L-DOPA-induced dyskinesias (16). Previous studies in 6-OHDA-lesioned rats support the notion that D₁ receptors play a major role in the development of contralateral rotation and dyskinesias under treatment with L-DOPA (36–40). Reports in humans and other primates have confirmed that D₁ receptors play an important role in the development of dyskinesias (41–43). DA depletion also remarkably enhances the ability of dopaminergic drugs to activate the ERK transduction pathway (17, 18). In the present study, we showed that ERK1/2 phosphorylation was increased by

the administration of L-DOPA in 6-OHDA-lesioned rats and that L-DOPA-induced phosphorylation of ERK1/2 was abolished by pre-treatment with fluoxetine. These results suggest that dysregulation of the ERK pathway via D₁ receptors is closely associated with L-DOPA-induced behavioral impairment in 6-OHDA-lesioned rats. Previous studies have shown that the L-DOPA-induced activation of ERK mediates the sequential phosphorylation of histone H3 in 6-OHDA-lesioned mice (19, 20). In the present study, we also demonstrated that in 6-OHDA-lesioned rats, the L-DOPA-induced activation of ERK mediated sequential histone H3 phosphorylation and L-DOPA-induced phosphorylation was inhibited by pre-treatment with fluoxetine. Therefore, these results suggest that the inhibitory effect of fluoxetine may be caused by a decrease in the efflux of L-DOPA-derived extracellular DA via 5-HT_{1A} receptors and attenuation of the activation of signaling of D₁ receptors.

Postranslational histone modification, including phosphorylation, appears to modulate both the accessibility of chromatin for RNA transcription and how well chromatin acts as a template for transcription (44). In particular, phosphorylation of Ser10 of histone H3 is sufficient to change heterochromatin to the more open euchromatin (45). Phosphorylation alters the electrostatic interactions between the N-terminal domain of histone H3 and DNA due to the additional, negatively charged, phosphate group and thus promotes accessibility to transcription factors (46). It may also result in the recruitment of other enzymes involved in further chromatin remodeling such as histone acetyltransferases (47). In the present study, phosphorylation of Ser10 of histone H3 was increased by the administration of L-DOPA. This phosphorylation was inhibited by pretreatment with fluoxetine. This chromatin remodeling has been proposed to play a role in the development of LID (20, 48). Therefore, fluoxetine may influence the side-effects of L-DOPA treatment for PD via modulating 5-HT_{1A} receptor.

In the present study, we demonstrated that pre-treatment with fluoxetine significantly suppressed L-DOPA-induced ERK1/2 and histone H3 phosphorylation, as well as L-DOPA-induced rotational behavior in 6-OHDA-lesioned rats. These effects of fluoxetine were abolished by pre-treatment with WAY 100135, a 5-HT_{1A} antagonist. Since depression is the most common psychiatric disturbance in PD, SSRIs are now often used for the treatment of depression in PD. However, the effect of SSRI on motor function in PD patients via pharmacological modification of interactions between serotonergic and dopaminergic neuronal networks is still contradictory (9–12). Although the 6-OHDA lesion does not exactly reflect PD in time course and pathophysiology, our results suggest that fluoxetine could help to prevent

or reverse the side-effects of L-DOPA treatment for PD via modulating 5-HT_{1A} receptors.

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