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# NR2B antagonist modulates the effect of D1R agonist on the reduced and imbalanced bilateral forelimb use in hemiparkinsonian rats

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**Objectives:** The antiparkinsonian effect of N-methyl-D-aspartate (NMDA) receptor subtype 2B (NR2B) antagonists remain controversial. To determine the effects of NR2B antagonist on Parkinson's disease (PD), the effect of a single administration of D1R agonist SKF38393 (SKF) and NR2B antagonist ifenprodil or co-administration of ifenprodil and SKF were investigated using the cylinder test.
**Materials and Methods:** The hemiparkinsonian (hemi-PD) rats were divided into 8 groups. The cylinder test was used to estimate the number of forelimb uses in 15 minutes as a measure of motor activity 30 minutes after administration of SKF (1.0, 2.0, 3.0 mg/kg), 0.1 mg/kg ifenprodil or co-administration of SKF and ifenprodil (2.0 mg/kg SKF + 0.1 mg/kg ifenprodil or 3.0 mg/kg SKF + 0.1 mg/kg ifenprodil). Subsequently, to identify the brain areas influenced by SKF and ifenprodil, neurons with SKF-induced c-Fos expression were analyzed in various brain regions in hemi-PD rats following the administration of SKF, with and without ifenprodil.
**Results:** The administration of SKF increased a frequency of forelimb use in a dose-dependent manner ($F_{(3,36)} = 4.4$, $P = 0.0094$; PD/vehicle (veh) 21.6 ± 6.3, 2.0 mg/kg SKF 103.4 ± 22.1 $P < 0.05$ vs. PD/veh; 3.0 mg/kg SKF 120.2 ± 30.5 $P < 0.01$ vs. PD/veh), mostly via the facilitation of the frequent use of Parkinsonian paw. The combined administration of SKF and ifenprodil completely reversed the SKF-induced abnormality in bilaterally coordinated movement of the forelimb in hemi-PD rats without affecting the facilitatory effect of SKF on motor activity ($F_{(2,46)} = 3.8$, $P = 0.0304$). The co-administered ifenprodil also modulated the SKF-induced c-Fos expression in the striatum ($P < 0.05$) and the subthalamic nucleus (STN) ($P < 0.01$).
**Conclusions:** The ameliorative effect of ifenprodil on the motor deficits in hemi-PD rats resulted from the improvement of the SKF-induced excessive use of Parkinsonian paw, and that the STN and/or the striatum in the lesioned hemisphere are possible targets for the antiparkinsonian effect of the NR2B antagonist.

**Key words:** Parkinson's disease, cylinder test, striatum, subthalamic nucleus, c-Fos, motor deficits

## Introduction

The motor symptoms of Parkinson's disease (PD) have been proved to be caused by dopamine (DA)-depletion in the striatum due to loss of nigral dopaminergic neurons.[1] Therefore, DA replacement therapy with L-DOPA or treatment with D1 receptor (D1R) agonist leads to remarkable recovery from motor deficits in patients with PD during the initial period.[2,3] However, chronic treatment with L-DOPA or D1R agonist leads to the development of uncontrollable movement or motor complications known as L-DOPA-induced dyskinesia (LID) in most patients with PD and animal models of PD.[1-4]

DA-depletion-induced supersensitivity of DA receptors (D1R and D2R) has been suggested as a plausible mechanism for the induction of LID.[5,6] Indeed, a direct relationship between the development of supersensitivity of D1R and the severity of LID has been identified in recent studies.[7] Among the non-

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dopaminergic treatments for PD, N-methyl-D-aspartate (NMDA) receptor antagonists, including amantadine, have improved motor symptoms in PD.[8-12] However, the NMDA receptor antagonists have severe side effects such as mental illness, because they block all types of NMDA receptors, which are widely distributed throughout the brain. Because of the region-specific expression of NMDA receptor subunit NR2, it has been proposed that NR2 subtype NR2B antagonists are viable treatment options for PD to minimize the undesirable side effects.[13] The antiparkinsonian effects of NR2B antagonists have been studied for a couple of decades,[11,14-20] but remains controversial. Previously, we had reported that a single administration of NR2B antagonist, ifenprodil, demonstrated no antiparkinsonian effect in hemiparkinsonian (hemi-PD) rats during a behavioural test in a preliminary study.[21]

It should be noted that the extent of improvement of motor symptoms in PD by NR2B antagonist via NMDA receptor subunit NR2 is not equivalent to that of L-DOPA or D1R agonist via D1R. Therefore, the aim of the present study was to first characterize the effects of D1R agonist SKF38393 (SKF), with and without the NR2B antagonist ifenprodil, on motor deficits in hemi-PD rats, by using the cylinder test. Additionally, to identify the brain areas influenced by SKF and ifenprodil, neurons showing SKF-induced c-Fos expression were analyzed in various brain areas in both DA-depleted hemisphere and intact hemisphere in hemi-PD rats following the administration of SKF, with and without ifenprodil.

## Materials and Methods

*Animals*

All experiments were performed on Male Wistar Hannover rats (CLEA Japan, Tokyo) weighing 250－300 g. Rats were housed in clear plastic cages in groups of 2－3 under a 12 h light/dark cycle with constant temperature (25 ± 1℃) and humidity (55 ± 5%), and allowed free access to food and water throughout the experiments. All experiments were approved by Kitasato University Institutional Animal Care and Use Committee. Animals were treated in accordance with the rules of the National Research Council for the Care and Use of Laboratory Animals (Authorization number: Ei Ken 11-19, 12-12, 13-12). All efforts were made to minimize the number of animals and their suffering.

*Preparation of hemi-PD rats*

To prepare hemi-PD rats, stereotaxic 6-hydroxydopamine (6-OHDA, Sigma-Aldrich, St. Louis, MO, USA) injections were conducted under pentobarbital sodium anesthesia (40 mg/kg, *i.p.*; Abbott Lab, North Chicago, IL, USA). Rats received 8 μg of 6-OHDA (2 μg/μl,) dissolved in 0.1% L-ascorbic acid/saline into the medial forebrain bundle in the right hemisphere with the following coordinates; AP, 2.1 mm caudal to the bregma; L, 2.0 mm right of the midline; V, 8.0 mm ventral to the dural surface, in accordance with Akita et al.[22] In total, 4 μl of 6-OHDA solvent was slowly injected by a glass micropipette (tip size: 30 μm, 20 μl, Drummond, Broomall, PA, USA) connected to an air pressure system. Before pulling the micropipette from rats' brain, the micropipette was retained for 5 minutes at least. Rats received desipramine HCl (15 mg/kg, *i.p.*; Sigma-Aldrich) intraperitoneally for the preservation of noradrenergic neurons 30 minutes prior to the 6-HODA injection.

*Rotation test*

To evaluate the severity of motor deficits of hemi-PD rats caused by dopamine depletion, apomorphine-induced rotation test was performed after 3 weeks interval from 6-OHDA injection. Rats were placed in a square open box (50 cm each side) and habituated for 15 minutes in the environment. After the habituation, apomorohine (1.0 mg/kg, *i.p.*; Sigma-Aldrich) dissolved in 0.1% L-ascorbic acid/saline was injected intraperitoneally. Thirty minutes after drug administration, rat behavior was recorded and scored for a 15-minute test period. During the test period, the number of turns, a rotation of more than 180° (1 half full-body turn), was counted. The number of turns is a measure of the severity of apomorphine-induced rotation behavior. Rats demonstrating more than 100 turns per 15 minutes were defined as a complete hemi-PD rat and used for further experiments.[22,23]

*Cylinder test*

The cylinder test, first described by Schallert and Lindner,[24] was performed to evaluate the ameliorative effect of SKF38393 (SKF; Sigma-Aldrich) and/or ifenprodil (Sigma-Aldrich) against abnormality of forelimb use of hemi-PD rats. The rats were placed and allowed to move freely in a transparent cylinder (20 cm diameter, 30 cm height) on a transparent stage. Rats' behavior was recorded from beneath to the transparent stage for 15 minutes using a charge-coupled device (CCD) camera. The test was performed in late afternoon without habituation in order to make animal to explore inside of the cylinder. During the test period, rats demonstrate rearing behavior and single, consecutive, and

simultaneous wall touches by forepaws. An observer blinded to animal identities observed the recorded behavior by CCD camera and scored wall touches. As described by Shi et al.,[25] wall touches with the forepaws were classified into three behaviors: (1) ambidextrous forelimb-use-rat touches the wall with both forelimbs alternately or simultaneously; (2) ipsilateral forelimb-use-rat touches the wall with its ipsilateral forelimb (intact paw) independently; and (3) contralateral forelimb-use-rat touches the wall with its contralateral forelimb (Parkinsonian paw) independently. The total number of wall touches (TWT) per 15 minutes was estimated by summing ipsilateral, contralateral, and ambidextrous touches. The rate of forelimb use (RFU) was expressed by three parameters: the ratio of ipsilateral forelimb use to TWT; the ratio of contralateral forelimb use to TWT; and the percent use of ambidextrous forelimbs to TWT.

*Drug administration and treatment groups*

Thirty minutes prior to the cylinder test, administration of SKF or ifenprodil and combined administration of SKF with ifenprodil were performed *i.p.* to hemi-PD rats. The hemi-PD rats were divided into 8 treatment groups: SKF, hemi-PD rats administered with various doses of SKF (1.0 mg/kg, 2.0 mg/kg, or 3.0 mg/kg); PD/0.1 mg/kg ifenprodil, hemi-PD rats administrated with 0.1 mg/kg ifenprodil; PD/2.0 mg/kg SKF + ifenprodil, hemi-PD rats co-administered with 2.0 mg/kg SKF and 0.1 mg/kg ifenprodil; PD/3.0 mg/kg SKF + ifenprodil, hemi-PD rats co-administered with 3.0 mg/kg SKF and 0.1 mg/kg ifenprodil; and PD/veh, hemi-PD rats administered with the solvent of SKF and ifenprodil as the vehicle (veh). The data on the PD/0.1-mg/kg ifenprodil-treated group was also presented in a previous study by co-author (Igarashi).[21] The solvent of SKF and ifenprodil was 0.1% L-ascorbic acid/saline.

*c-Fos immunohistochemistry*

Seventy minutes after the cylinder test, rats were subject to transcardial perfusion with 200 mL of heparin saline followed by 200 ml of 4% paraformaldehyde buffered with a 0.1 M phosphate buffer (pH 7.4) under deep anesthesia with ethyl carbamate (1.4 g/kg, *i.p.*; WAKO, Osaka). Brains were collected, and post-fixed for 1－2 hours in the same solution, and transferred in 30% sucrose solution for 12 hours. Coronal 40 $\mu$m sections were sampled every 80 $\mu$m throughout the brain area including the striatum, the motor cortex, and the subthalamic nucleus (STN) using a freezing-stage microtome. Sections were kept in a cryoprotectant solution (20 mM phosphate buffer containing 30% ethylene glycol and 30% glycerin) at -20℃. c-Fos immunohistochemical staining was performed using the avidin-biotin peroxidase reaction. Anti-c-Fos rabbit polyclonal antibody (sc-52; Santa Cruz Biotech, Santa Cruz, CA, USA) was used for the staining. To prevent the reaction of endogenous peroxidase, coronal sections were preincubated in 0.3% hydrogen peroxide solution diluted in 50 mM phosphate buffered saline (PBS), and then sections were transferred to a blocking solution (3% goat serum and 0.25% triton X-100) and followed by incubation at 4℃ in a 1:2,000 dilution of anti-c-Fos antibody in 50 mM PBS and 0.25% triton X-100 overnight. After incubation in PBS, sections were transferred in a 1:400 dilution of biotinylated anti-rabbit-IgG antibody in 50 mM PBS and 0.25% triton X-100 and followed by the 1:100 ABC reagent (ABC Kit, Vector Labs, Burlingame, CA, USA ) diluted in 0.5% triton X-100 in 50 mM PBS. The reactions were visualized by incubation with 0.05% 3,3'-diaminobenzidine (Sigma-Aldrich) and 0.015% hydrogen peroxide solution for 5 minutes, and then were mounted on gelatin coated slides.

*Quantitative analysis of c-Fos expression*

Coronal sections showing c-Fos expression were sampled from the motor cortex (AP, 3.7 mm rostral to the bregma), the striatum (AP, 0.0 mm caudal to the bregma), and the STN (AP, 3.6 mm caudal to the bregma). Photomicrographs of the coronal section were taken by optical microscope and saved as digital data. The digitized photomicrographs were subject to quantitative analysis of c-Fos expression in the 3 regions in the cortico-basal ganglia-thalamocortical loop for motor regulation using automated imaging software (cellSens; Olympus, Tokyo) by following procedures: the dorsal area of the striatum, the M1 and M2 cortex and the STN was enclosed in the square as shown in Figure 5. And then dark c-Fos positive nuclei, which exceed the threshold level of a density, were automatically counted by the software. Finally, the software automatically estimated the density (cells/mm$^2$) of c-Fos-positive neurons in each enclosed brain area.

*Statistical analysis*

All values indicated in the figures were expressed as mean ± SEM. Cylinder test scores were compared using one-way or two-way ANOVA followed by Bonferroni/Dunnett's multiple comparisons test for more than 2 groups or the Student's *t*-test for 2 groups, if significant. The TWT scores and the c-Fos quantitative scores were compared using the Student's *t*-test. Statistical significance was set at $P < 0.05$ for all statistical analyses.

## Results

*Characteristics of abnormalities in the forelimb use in the hemi-PD rats by the cylinder test*

To define the motor deficits in hemi-PD rats using the cylinder test, the abnormality in the forelimb use in the hemi-PD rats (PD/veh) was evaluated by comparing those with the normal forelimb use in non-treated (NT) rats (Figure 1). The NT rats actively moved along the sidewall of the cylinder, touching the wall with their forepaws, alternately or simultaneously, with TWT of 144.0 ± 31.0 per 15 minutes. On the other hand, the hemi-PD rats moved less actively along the sidewall of the cylinder, with TWT of 21.6 ± 6.3 per 15 minutes ($P < 0.01$). The RFU of the NT rats consisted of ambidextrous (78.0 ± 3.0%), ipsilateral (11.1 ± 1.5%), and contralateral forelimb use (11.0 ± 2.3%). Meanwhile, the RFU of hemi-PD rats consisted of a decreased ambidextrous use (17.5 ± 5.6%), predominant ipsilateral forelimb use (81.8 ± 5.8%), and some contralateral forelimb use (0.7 ± 0.4%). Thus, the motor abnormality in hemi-PD rats was characterized by the reduced TWT and the imbalanced RFU in the cylinder test.

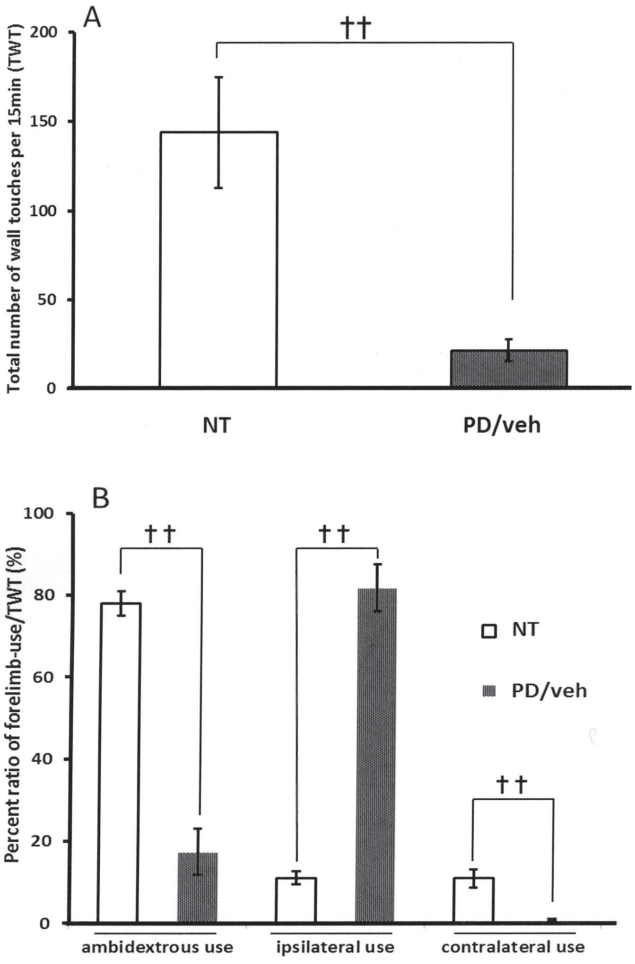



**Figure 1.** Characteristics of motor deficits in hemi-PD rats. NT rats (n = 10) and hemi-PD rats (n = 14) were subjected to the cylinder test.

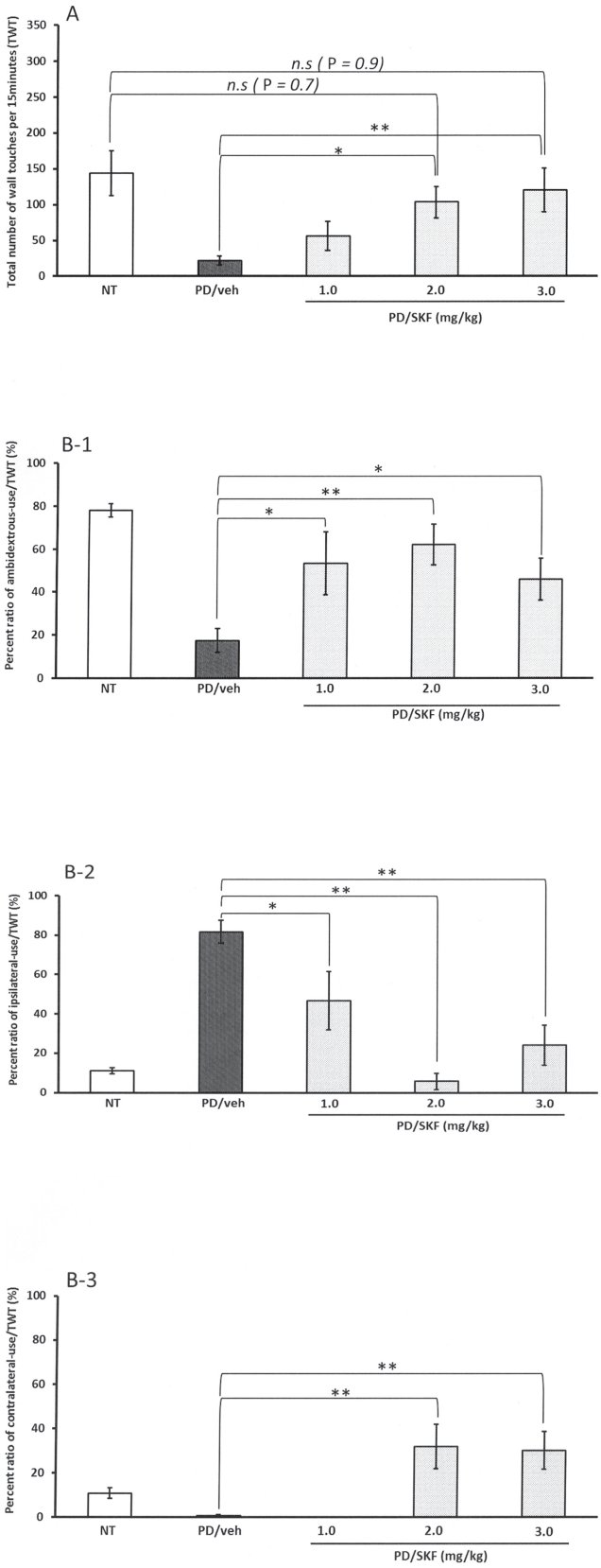
(**A**) A marked reduction of TWT for 15 minutes was observed in the hemi-PD rats, compared with NT rats. (**B**) The RFU consisted of a decreased ambidextrous-use, predominant ipsilateral-use and a few contralateral-use were observed in hemi-PD rats. ††$P < 0.01$ relative to NT

*Effects of various doses of SKF on the abnormality of forelimb use in hemi-PD rats*

Effects of a single administration of SKF on the motor abnormalities in hemi-PD rats were examined. The TWT of cylinder test was increased in all groups of administration of SKF compared to the PD/veh ($F_{(4,36)} = 4.4$, $P = 0.0094$). The administration of 2.0 mg/kg or 3.0 mg/kg of SKF significantly improved the reduced TWT in PD/veh in a dose-dependent manner ($P < 0.05$, PD/veh vs. 2.0 mg/kg of SKF; $P < 0.01$, PD/veh vs. 3.0 mg/kg of SKF; Figure 2A). Specifically, a complete recovery from the reduced TWT was observed, when 3.0 mg/kg SKF was administrated (144.0 ± 31.0 for NT rats, 120.2 ± 30.5 for 3.0 mg/kg SKF; *n.s*, $P = 0.9$ vs. NT rats, Figure 2A). On the other hand, the effects of SKF on the RFU was complicated ($F_{(3,36)} = 4.6$, $P = 0.0077$ for ambidexterity; $F_{(3,36)} = 14.3$, $P = 0.00001$ for ipsilateral; $F_{(3,36)} = 6.3$, $P = 0.0015$ for contralateral; Figure 2B-1－3); the significant increase of ambidextrous-use was observed with all doses of SKF as compared to PD/veh ($P < 0.05$, Figure 2B-1). The predominant use of ipsilateral forelimb in PD/veh (81.8 ± 5.8%) was significantly reversed in a dose-dependent manner (Figure 2B-2), while the markedly reduced contralateral forelimb use in PD/veh (0.7 ± 0.4%) was significantly but excessively increased when 2.0 mg/kg or 3.0 mg/kg SKF was administered ($P < 0.01$, Figure 2B-3). Thus, the improvement of the imbalanced RFU in PD/veh by 1.0 mg/kg SKF was damaged when 2.0 mg/kg or 3.0 mg/kg SKF was administered, mostly due to the SKF-induced excessive use of the contralateral forelimb (Parkinsonian paw).

*Effects of combined administration of SKF and ifenprodil on motor deficits in hemi-PD rats*

To examine whether ifenprodil improves the inadequate effects of a single administration of 2.0 mg/kg or 3.0 mg/kg SKF on the motor deficits in hemi-PD rats, co-administration of 2.0 mg/kg SKF and 0.1 mg/kg ifenprodil or 3.0 mg/kg SKF and 0.1 mg/kg ifenprodil was performed ($F_{(2,46)} = 10.2$, $P = 0.0002$ factor of SKF; $F_{(1,46)} = 0.3$, $P = 0.6001$ factor of ifenprodil; $F_{(2,46)} = 3.8$, $P = 0.0304$ factor of SKF and ifenprodil; Figure 3A). As shown in Figure




3A, the recovery from the reduced TWT in PD/veh by 2.0 mg/kg SKF was completely prevented by the co-administered 0.1 mg/kg ifenprodil, thus reversing the ameliorative effect of 2.0 mg/kg SKF on the reduced TWT in hemi-PD rats. As shown in Figure 3B-3, when combined with 2.0 mg/kg SKF, 0.1 mg/kg ifenprodil completely improved the excessively increased contralateral forelimb use induced by 2.0 mg/kg SKF ($F_{(1,46)} = 7.4$, P = 0.0092; Figure 3B-3). Taken together, the co-administrated 0.1 mg/kg ifenprodil demonstrated a complicated modulation on the effects of 2.0 mg/kg SKF in hemi-PD rats.

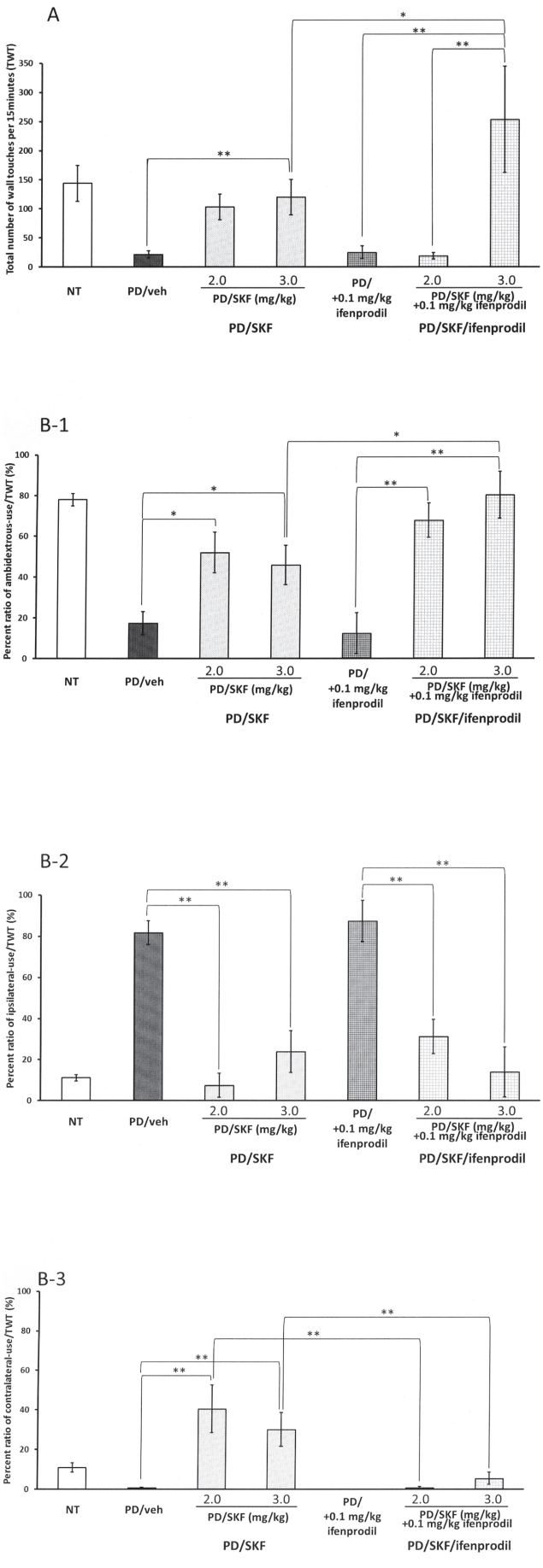
As shown in Figure 3A, the co-administrated 0.1 mg/kg ifenprodil potentiated the recovery of the reduced TWT in hemi-PD rats by 3.0 mg/kg SKF by further improving the TWT and not blocking the ameliorative effect of 3.0 mg/kg SKF ($F_{(1,46)} = 6.9$, P = 0.0115; Figure 3A). As shown in Figure 3B-1－3, the co-administrated 0.1 mg/kg ifenprodil completely improved the alternate type of imbalanced RFU induced by 3.0 mg/kg SKF, thus recovering the predominant use of both forelimbs ($F_{(1,46)} = 7.1$, P = 0.0102; Figure 3B-1).

*Modulation of SKF-induced c-Fos expression by ifenprodil in the 4 brain regions*

Neurons with c-Fos-positive nucleus were assessed in the c-Fos-immunostained coronal sections obtained from hemi-PD and NT rats, 2 hours after the administration of 3.0 mg/kg SKF or the co-administration of 3.0 mg/kg SKF and 0.1 mg/kg ifenprodil. In the ipsilateral hemisphere (lesioned side) of hemi-PD rats, neurons with the 3.0 mg/kg SKF-induced c-Fos expression were densely observed in the striatum (Figure 4A2) and STN (Figure 4C2), and moderately observed in the motor cortex M1 (Figure 4B1-2) and M2 (Figure 4B2-2). On the other hand, with the co-administered 0.1 mg/kg

**Figure 2.** Effects of the single administration of various doses of SKF on motor deficits in hemi-PD rats

Treatment groups were: NT rats (n = 10), non-treated controls; PD/veh (n = 14), hemi-PD rats administered with vehicle; and PD/SKF, hemi-PD rats administered with various doses of SKF (1.0 mg/kg, n = 5; 2.0 mg/kg, n = 7; 3.0 mg/kg, n = 14). (**A**) The effect of various doses of SKF on the reduced TWT. The reduced TWT in hemi-PD rats was significantly increased when administered with 2.0 mg/kg or 3.0 mg/kg of SKF. (**B1-3**) The effect of various doses of SKF on the imbalanced RFU. (**B-3**) The imbalanced RFU in hemi-PD rats was reversed by SKF, but another type of the imbalanced RFU due to excessive use of Parkinsonian paw was developed with SKF. The statistical significance of difference between the groups was assessed with one-way ANOVA followed by Dunnett's test. *P < 0.05, ** P < 0.01




ifenprodil, the 3.0 mg/kg SKF-induced c-Fos expression observed in the striatum (Figure 4A3) and M1 motor cortex (Figure 4B1-3) on the lesioned side was markedly increased, and that in the STN (Figure 4C3) was attenuated. Figure 5 shows a quantitative analysis of the density of neurons with 3.0 mg/kg SKF-induced c-Fos expression in the four restricted regions in both the lesioned side and the intact side of hemi-PD rats, following 3.0 mg/kg SKF administration with/without 0.1 mg/kg ifenprodil. As shown in Figure 5A and 5B, the co-administrated 0.1 mg/kg ifenprodil significantly potentiated the 3.0 mg/kg SKF-induced c-Fos expression in the striatum and M1 on the lesioned side ($P < 0.05$), while it significantly attenuated the 3.0 mg/kg SKF-induced c-Fos expression in the STN (Figure 5D) on the lesioned side ($P < 0.01$). In the M2 of the lesioned side, the 3.0 mg/kg SKF-induced c-Fos expression was somewhat increased by the co-administered 0.1 mg/kg ifenprodil, although not statistically significant.

## Discussion

The motor abnormality in hemi-PD rats was characterized by the reduced TWT and the imbalanced RFU in the cylinder test. The reduced TWT has been correlated to motor hypoactivity like akinesia or bradykinesia in PD.[25,26] On the other hand, the imbalanced RFU due to the predominant use of the ipsilateral forelimb corresponds to the deficit in the bilaterally coordinated movement of forelimb.[21] The bilaterally coordinated movement of the

**Figure 3.** Effects of the combined administration of SKF and 0.1 mg/kg ifenprodil on motor deficits in hemi-PD rats

Treatment groups were: NT (n = 10), non-treated controls; PD/veh (n = 14), hemi-PD rats administered with vehicle; PD/SKF, hemi-PD rats administered with various doses of SKF (2.0 mg/kg, n = 7; 3.0 mg/kg, n = 14); PD/+0.1 mg/kg ifenprodil, hemi-PD rats administered with 0.1 mg/kg of ifenprodil, n = 5; PD/SKF/ifenprodil, hemi-PD rats co-administered with various doses of SKF and ifenprodil (2.0 mg/kg of SKF and 0.1 mg/kg ifenprodil, n = 5; 3.0 mg/kg of SKF and 0.1 mg/kg ifenprodil, n = 7). (**A**) The ameliorative effect of SKF on the reduced TWT was prevented by co-administered ifenprodil with 2.0 mg/kg of SKF and 0.1 mg/kg ifenprodil. The ameliorative effect of SKF on the reduced TWT was significantly enhanced by co-administered with 3.0 mg/kg of SKF and 0.1 mg/kg ifenprodil ($P < 0.05$). (**B**) The recovery from another type of the imbalanced RFU induced by SKF was achieved by co-administered ifenprodil (Figure B-3). The recovery from another type of the imbalanced RFU induced by SKF was completed by co-administered with 3.0 mg/kg of SKF and 0.1 mg/kg ifenprodil ($P < 0.05$, Figure B-1, $P < 0.01$, Figure B-3). The statistical significance of difference between the groups was assessed with two-way ANOVA followed by Bonferroni/Dunnett's test. *$P < 0.05$, **$P < 0.01$

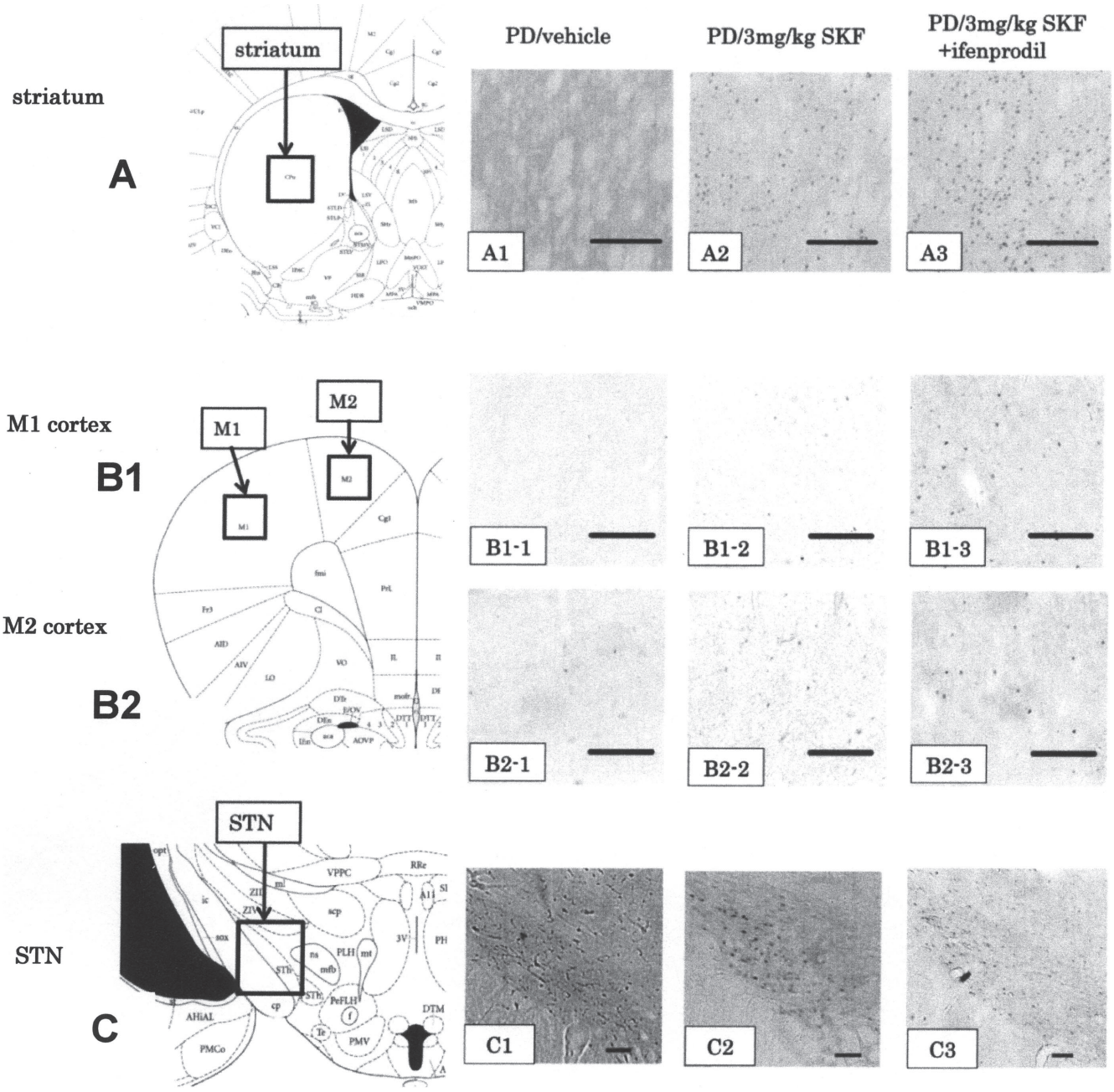



**Figure 4.** c-Fos-immunostained sections of the striatum and the STN in both lesioned- and intact-hemisphere of hemi-PD rats, demonstrating neurons with SKF-induced c-Fos expression

(**A**, **B1**, **B2**, **C**) Schematic drawings of coronal section of the striatum (0.24 mm rostral to the bregma), the M1 and M2 (3.72 mm rostral to the bregma) and the STN (3.84 mm caudal to the bregma) (Paxinos G, Watson C, The Rat Brain Stereotaxic Coordinates, 6th edition, San Diego; Elsevier Academic Press; 2007). The black-frame indicates the area where photomicrographs of c-Fos positive neurons are taken. A1, B1-1, B2-1, C1 sections of the striatum, the cortex (M1, M2) and the STN of hemi-PD rats treated with vehicle (veh) demonstrating few c-Fos positive neurons. A2, B1-2, B2-2, C2 sections of the striatum, the cortex (M1, M2) and the STN of hemi-PD rats treated with 3.0 mg/kg SKF. Sections (A2, C2) were showing densely packed c-Fos positive neurons. A3, B1-3, B2-3, and C3, sections of the striatum, the cortex (M1, M2) and the STN of hemi-PD rats treated with the combined administration of 3.0 mg/kg SKF and 0.1 mg/kg ifenprodil. A section of the striatum (A3) with densely packed c-Fos positive neurons and a section of the STN (C3) with moderately packed c-Fos positive neurons treated by co-administered ifenprodil in hemi-PD rats. The scale bars represent 0.1mm.

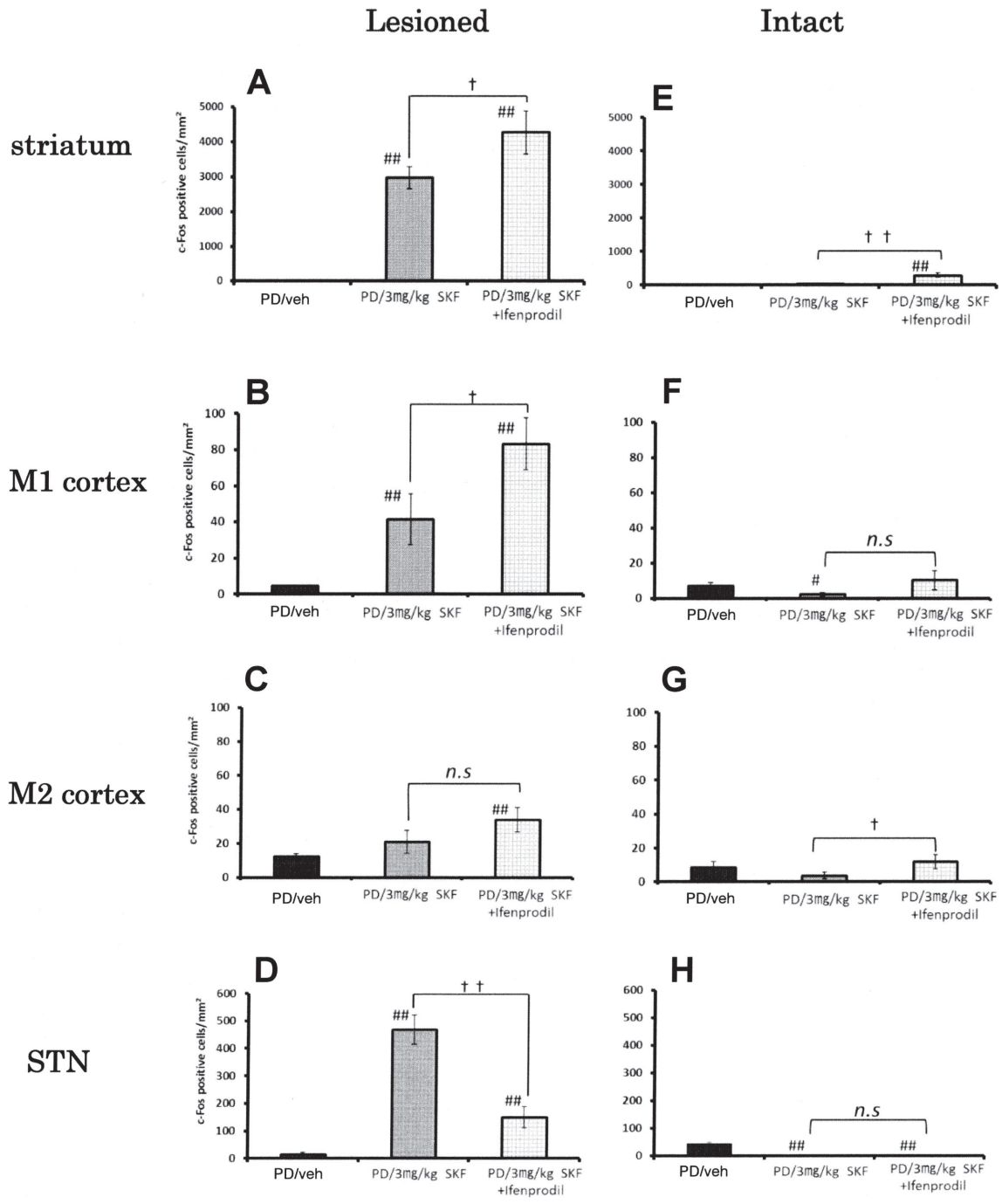



**Figure 5.** Quantitative analysis of c-Fos positive neurons in various brain regions

(**A**－**H**) The density of c-Fos positive neurons (cells/mm$^2$) was calculated in the bilateral (lesioned and intact) sides of various brain regions of hemi-PD rats such as the strtiatum. (**A**, **E**), the M1 cortex (**B**, **F**), the M2 cortex (**C**, **G**) and the STN (**D**, **H**). Treatment groups were: PD/veh (n = 4), hemi-PD administered with vehicle; PD/3.0 mg/kg SKF (n = 5), hemi-PD rats administered with 3 mg/kg of SKF; PD/3.0 mg/kg SKF + ifenprodil (n = 5), hemi-PD rats co-administered with 3 mg/kg of SKF and 0.1 mg/kg ifenprodil. #$P < 0.05$, ##$P < 0.01$ relative to PD/veh. †$P < 0.05$, †$P < 0.01$ compared with PD/3.0 mg/kg SKF and PD/3.0 mg/kg SKF + ifenprodil (0.1 mg/kg). n.s represents no significant difference.

forelimb was represented by the mutual or simultaneous use of both forelimbs in the cylinder test. Therefore, the recovery from the motor deficits in hemi-PD rats by therapeutic drugs should be estimated by the total recovery from both motor abnormalities in the forelimb during the cylinder test. Indeed, Shi et al.[25] have reported, using cylinder test, that the improvement of motor deficits in hemi-PD rats was achieved by deep brain stimulation in the STN (STN-DBS), demonstrating a complete recovery from the imbalanced use of ambidextrous forelimbs and hypoactivity during the STN-DBS.

The reduced TWT in hemi-PD rats (akinesia or bradykinesia) was improved by SKF in a dose-dependent manner. On the other hand, the imbalanced RFU in hemi-PD rats was not reversed by SKF. In particular, the imbalanced forelimb use in hemi-PD rats was converted into another type of imbalanced forelimb use by SKF due to the development of the predominant use of the contralateral forelimb (Parkinsonian paw). Thus, the total recovery from motor abnormalities in hemi-PD rats achieved by 1.0 mg/kg SKF was damaged mostly by the SKF-induced excessive use of the Parkinsonian paw, when 2.0 mg/kg SKF or 3.0 mg/kg SKF was administered. Here the question why SKF induces the excessive use of the Parkinsonian paw in hemi-PD rats emerges. It has been reported that dopamine or D1R agonists activate the sensitized D1-like receptors and over activate the motor system with cortical input via the supersensitive D1Rs in the dopamine-depleted striatum in hemi-PD rats, resulting in the deficit of bilateral movement and excessive use of Parkinsonian paw.[27,28] Therefore, it is considerable that high-dose of SKF over activates the motor system in the lesioned striatum via the sensitized D1Rs, producing the imbalanced RFU due to the excessive use of Parkinsonian paw.

The combined administration of 2.0 mg/kg SKF and 0.1 mg/kg ifenprodil reversed the SKF-induced alternate type of imbalanced forelimb movement, while the co-treatment with ifenprodil damaged the ameliorative effect of SKF on motor hypoactivity. However, considering that the ameliorative effect of 2.0 mg/kg SKF is resulted from the development of the frequent use of Parkinsonian paw, the damage of the ameliorative effect of 2.0 mg/kg SKF on akinesia by ifenprodil seems to be a part of the improvement of the excessive use of parkinsonian paw by ifenprodil. On the other hand, the combined administration of 3.0 mg/kg SKF and 0.1 mg/kg ifenprodil achieved a total recovery from both abnormalities of the forelimb movement in hemi-PD rats, completely reversing the alternate imbalanced forelimb movement while preserving the ameliorative effect of SKF on motor hypoactivity. Thus, the reversal of the imbalanced forelimb movement by ifenprodil is mostly due to the improvement of the SKF-induced excessive use of Parkinsonian paw. As mentioned above, the SKF-induced excessive use of Parkinsonian paw is related to the over-activated motor system via the hypersensitive D1Rs in the lesioned striatum. It is well known that D1R-mediated long-term potentiation of synaptic response in the neurons develops following dopamine or D1R agonist treatments.[29,30] Therefore, it is possible that ifenprodil normalizes the SKF-induced potentiation of glutamatergic transmission in the DA-depleted striatum by a partial blockade of NMDA receptors and thereby reverses the SKF-induced excessive use of Parkinsonian paw in hemi-PD rats.

In the present study, neurons expressing SKF-induced c-Fos were observed in four restricted brain areas in the DA-depleted hemisphere—the dorsal striatum, STN, and motor cortex (M1 and M2). The level of SKF-induced c-Fos expression showed a tendency to increase in a dose-dependent manner (data not shown). On the other hand, the behavioral test revealed that the improvement of the reduced use of the forelimb in hemi-PD rats by SKF occurred in a dose-dependent manner. Taken together with the SKF-induced motor and cellular events, the SKF-induced c-Fos expression must be related with the SKF-induced recovery from the motor hypoactivity or akinesia in hemi-PD rats. It has been shown that the expression of the immediate early genes, like *c-fos*, is a biological marker for hyperactivity or excessive excitation of neurons in the central nervous system.[31,32] Therefore, the prolonged tonic hyperactivity of neurons in a specific region correlating with robust c-Fos expression is considered to be induced by either a chronic intense stimulation via excitatory afferents, or prolonged disinhibition via a chronic blockade of inhibitory afferents.[33] Interestingly, the four restricted regions showing SKF-induced c-Fos expression in the DA-depleted hemisphere belong to the cortico-basal ganglia-thalamocortical loop for motor regulation. Interestingly, the neurons with the c-Fos expression were not observed in the substantia nigra reticulata (SNr) and the globus pallidus external (GPe) belonging to the same motor circuit, which receive intense GABAergic afferents from the striatum with robust c-Fos expression. This region-specificity in the localization of neurons with or without c-Fos expression in the motor circuit in hemi-PD rats suggests that the SKF-induced motor abnormality is related to the prolonged hyperactivity or excessive excitation of neurons, which occurs in a cascade within the motor circuit of the DA-depleted hemisphere. Since

the area with neurons showing the most intense c-Fos expression was restricted to the striatum and STN, it is likely that the origin of the tonic hyperactivity cascade within the motor circuit is the striatal neurons, which probably act as the trigger mechanism for motor activity. The second site in the cascade must be the STN where the prolonged tonic hyperactivity of neurons occurs due to disinhibition by a chronic blockade of GABAergic afferents from the GPe via the abnormally activated striatum. The STN is hypothesized to function in the regulation of bilateral movement as follows. The phasic activity of the STN neurons elicited via an indirect pathway from the activated striatum inhibits the ongoing motor system via the GABAergic SNr efferents to terminate the ongoing motor system driven previously via a direct pathway from the activated striatum, and, thereby, permitting the conversion of one-sided motion to opposite-sided motion in the bilateral movement. This hypothesis needs to be directly tested in future investigations. However, according to this interpretation of the c-Fos expression cascade, the tonic hyperactivity of the STN neurons prevents the regulatory mechanism for bilateral movement leading to its deficit, while the prolonged hyperactivity of the striatal neurons promotes the trigger mechanism that enhances the motor activity of Parkinsonian paw.

The co-administrated ifenprodil significantly enhanced the SKF-induced c-Fos expression in the striatum and M1, while it markedly attenuated the robust c-Fos expression in the STN. These results suggest that the improvement of the SKF-induced hyperactivity of the STN neurons by ifenprodil, corresponding to the attenuation of robust c-Fos expression, results in the complete recovery of the deficit in the bilateral forelimb movement. Moreover, the potentiation of the SKF-induced hyperactivity by ifenprodil, corresponding to the enhancement of c-Fos expression, enables to maintain the SKF-induced recovery from motor hypoactivity under the condition of enhanced suppression of motor activity via the efferents from the still hyperactive STN.

In the future, it will be necessary for us to investigate the action-mechanism of NR2B antagonist more accurately than merely the systemic administration of the drug in this study, by using the microinjection of the drug into the striatum or the STN.

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