Short Communication

Video-based assessments of the hind limb stepping in a mouse model of hemi-parkinsonism

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HIGHLIGHTS

- Asymmetrical use of hind limb was assessed in unilateral 6-OHDA-lesioned mice.
- A significantly decreased number of the contralateral stepping was observed.
- Levodopa reversed the asymmetry of hind limb in a dose dependent manner.
- Counting hind limb steps could be utilized for screening PD therapeutics.

ABSTRACT

Unilateral injection of 6-hydroxydopamine (6-OHDA) is commonly used to generate a rodent model of Parkinson's disease (PD). Although motor deficits of the lower extremities represent one of the major clinical symptoms in PD patients, validated tests for assessing motor impairments of the hind limb in 6-OHDA mice are currently unavailable. We here report the video-based assessments of the asymmetric use of hind limbs in 6-OHDA mice. A significantly decreased number of spontaneous hind limb stepping was observed in the contralateral-to-lesioned side, and was dose dependently reversed by levodopa, suggesting that it could be utilized for screening PD therapeutics.

Key word: 6-OHDA; mouse; hind limb stepping; hemi-parkinsonism

Parkinson's disease (PD) is characterized by a wide variety of motor deficits due to striatal dopamine depletion, which is caused by a progressive loss of nigrostriatal dopamine (DA)-producing cells (Fahn et al., 1987; Samii et al., 2004). Administration of the DA precursor levodopa or dopamine receptor agonists is a major pharmacotherapy for PD. To investigate mechanisms of pathogenesis and pathophysiology, and to develop novel therapeutics, animal models of PD induced by chemicals are widely used (Bové and Perier, 2012). The 6-hydroxydopamine (6-OHDA)-lesioned unilateral PD model, originally established in rats (Ungerstedt, 1968), can also be applied to mice (Iancu et al., 2005). Injecting 6-OHDA unilaterally into the substantia nigra pars compacta (SNpc), dorsal striatum (ST), or medial forebrain bundle (MFB) causes robust reduction in striatal DA via loss of nigrostriatal dopaminergic neurons, and is accompanied by marked motor asymmetry known as hemi-parkinsonism (Ungerstedt, 1968; Iancu et al., 2005; Heuer et al., 2012). Behavioral indexes to evaluate motor deficiency related to PD and to levodopa-induced dyskinesia (LID), initially established in rats, have been developed in mice (Iancu et al., 2005; Heuer et al., 2012; Glajch et al., 2012). Among them, rotational activity is

60 commonly used to examine motor deficits and the efficacy of PD therapeutics. In this
61 model, animals receive a unilateral 6-OHDA lesion to increase spontaneous rotational
62 activity in the ipsilateral-to-lesioned side. Amphetamine increases this activity in
63 proportion to the number of surviving nigrostriatal dopaminergic neurons, whereas
64 apomorphine or levodopa reverses this effect in the contralateral-to-lesioned side in
65 proportion to the loss of nigrostriatal dopaminergic neurons (Ungerstedt and Arbuthnott,
66 1970; Heuer et al., 2012; Glajch et al., 2012). The motor impairments observed in the
67 contralateral-to-lesioned side of these animals are characterized by reduced limb use
68 (Miklyaeva and Whishaw, 1996; Glajch et al., 2012). Forelimb impairment can be
69 validated by the cylinder and stepping tests (Olsson et al., 1995; Iancu et al., 2005;
70 Blume et al., 2009; Heuer et al., 2012), which reveal decreased use of the contralateral
71 forelimb. This effect is ameliorated by PD therapeutic agents such as levodopa or
72 bromocriptine (Lunblad et al., 2002; Francardo et al., 2011). In contrast to the forelimb,
73 however, hind limb impairment in 6-OHDA-lesioned mice has not been sufficiently
74 characterized. Considering that motor deficits of the lower extremities represent a major
75 clinical symptom in patients with PD (Fahn et al., 1987), it is reasonable to validate

76 whether hind limb impairment could be an index that reflects motor deficiency in
77 6-OHDA-lesioned mice. Using video-based analysis, we characterized the asymmetrical
78 use of hind limb on spontaneous steps and the effect of levodopa on this behavior in
79 unilateral 6-OHDA-lesioned mice.

80 Subjects were male C57BL/6N mice aged 8 weeks (Japan SLC, Shizuoka,
81 Japan) housed in a controlled environment (25 ± 1°C, 50 ± 1% humidity) with food and
82 tap water available *ad libitum*. The room lights were on between 8:30 and 20:30. All
83 experiments were performed with the approval of the Institutional Animal Care and Use
84 Committees of Tokushima University, Japan, and in accordance with the NIH
85 guidelines for the care and use of animals.

86 The hemi-parkinsonian model mice by 6-OHDA were made according to the
87 protocol reported previously (Thiele et al., 2011). Mice received an intraperitoneal (i.p.)
88 injection of desipramine hydrochloride (1 mg/kg dissolved in 0.9% saline, Wako, Osaka,
89 Japan) and pargyline hydrochloride (0.2 mg/kg dissolved in 0.9% saline, Sigma-Aldrich,
90 St. Louis, MO, USA). After 15 min, they were anesthetized with isoflurane (MSD
91 Animal Health, Whitehouse Station, NJ, USA), 70% N_2O, and 30% O_2, and were

92 mounted on the stereotaxic frame (NARISHIGE, Tokyo, Japan). Each mouse received
93 an injection of 6-OHDA (3 μg in 0.9% saline containing 0.2% ascorbic acid,
94 Sigma-Aldrich, St. Louis, MO, USA) targeting the right MFB (from bregma: AP -1.2
95 mm, ML +1.1 mm, DV +5.0 mm). It was administered at 0.24 μl/min using a
96 microsyringe (Hamilton, Bonaduz, GR, Switzerland), which was removed 5 min after
97 injection.

98 Fourteen days post 6-OHDA injection, spontaneous rotations were measured
99 for 30 min in a 2-litre beaker. Mice with over 80% ipsilateral rotations were used in this
100 study. To measure apomorphine-induced rotation, mice further received a single i.p.
101 injection of apomorphine (0.5 mg/kg dissolved in 0.9% saline containing 0.2 mg/mL
102 ascorbic acid, Sigma-Aldrich, St. Louis, MO, USA). Control mice received an
103 equivalent volume of 0.9% saline. Levodopa-induced rotation was evaluated for 5 min
104 in the same experimental condition with the hind limb step analysis described below.

105 Mice received a single i.p. injection of levodopa (1 or 3 mg/kg of free base
106 dissolved in 0.9% saline containing 0.5% carboxymethyl cellulose; Sigma-Aldrich, St.
107 Louis, MO, USA) 15 days after the 6-OHDA injection. An equivalent volume of 0.9%

saline containing 0.5% carboxymethyl cellulose was injected in control mice. All mice were pre-treated with a single i.p. injection of benserazide (12.5 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) dissolved in 0.9% saline 20 min before the administration of levodopa or saline.

Ten minutes after administration of levodopa, spontaneous steps were video-recorded from the bottom of a 600 mL beaker for 5 min. Each hind limb step was counted manually using a playback speed slowed to 0.3–0.5× using VLC media player. Total hind limb steps were summed for both the ipsilateral (intact) and contralateral (impaired) sides, and the number of contralateral steps was calculated as a % of the count. Further, sequential use of the ipsilateral hind limb was independently counted, with two sequential steps defined as double and three steps as triple. The video-analysis was performed by examiners who unaware of the grouping information of mice.

Mouse tissue was fixed by circulating 4% paraformaldehyde (Wako, Osaka, Japan) dissolved in phosphate-buffered saline (PBS) 16 days after 6-OHDA injection. Immunostaining was conducted using free-floating sections and the tyramide signal amplification (TSA) method. After blocking endogenous peroxidase activity, the

124 sections were incubated in PBS containing 3% bovine serum albumin for 60 min, and
125 then incubated with primary antibody against tyrosine hydroxylase (TH, rabbit
126 polyclonal, 1:200,000, Chemicon, CA, USA) for 18 h. Antigens were detected using the
127 Histofine Simple Stain kit (Nichirei, Tokyo, Japan) and TSA-system with Fluorescein
128 (Perkin Elmer, Shelton, CT, USA). Immunofluorescence-stained images were captured
129 using a fluorescence microscope (BZ-9000, Keyence, Tokyo, Japan). Measurement of
130 TH-immunopositive density in the ST was performed using an image analyzer
131 (WinRoof Ver.5, Mitani, Fukui, Japan), and the averaged pixel intensities (16-bit) were
132 expressed with arbitrary unit.

133 All experimental values are expressed as means ± SEM. For two-group
134 comparisons, a paired two-tailed *t*-test was used. For multiple comparisons, one-way
135 analysis of variance (ANOVA) followed by Scheffe's *post hoc* test was used unless
136 otherwise stated. Statistical analysis was performed using Stat View 5.0 (SAS Institute,
137 Cary, USA).

138 Fourteen days after mice received a unilateral injection of 6-OHDA into the
139 MFB, spontaneous and apomorphine-induced rotations were assessed (Figure 1A).

Spontaneous rotations in the contralateral-to-lesioned side, indicated as % of total (sum of ipsi- and contralateral rotations), were significantly less common in 6-OHDA-lesioned mice than in control mice (control, 55.1 ± 2.5%; 6-OHDA, 5.9 ± 3.6%; n = 5, $P < 0.005$). Conversely, 6-OHDA-lesioned mice that received apomorphine showed reversal of this laterality, with significantly more frequent rotation in the contralateral side than control mice (100 ± 0%, n = 5, $P < 0.005$ vs. control). Immunohistochemical analysis revealed robustly lower TH immunoreactivity (IR) in the ipsilateral-to-lesioned side of the ST than in the contralateral non-lesioned side (Figure 1B). Quantitative analysis showed approximately 70% significantly lower TH IR in the lesioned side than in the non-lesioned side (lesioned side, 7503.2 ± 385.8; non-lesioned side, 2199.6 ± 107.7; n = 5, $P < 0.005$), as shown in Figure 1C (arbitrary unit).

We next analyzed spontaneous hind limb steps 1 day after examination of rotational behavior. Figure 2A shows the topological configuration of the hind limb analyzed. The number of steps taken by the hind limb is shown in Figure 2B. The total number of hind limb steps was not significantly different between control (121.8 ± 13.3;

156 n = 5) and 6-OHDA-lesioned mice (137.6 ± 22.2; n = 5, $P > 0.05$). Levodopa
157 dose-dependently increased total hind limb steps in 6-OHDA-lesioned mice (1 mg/kg of
158 levodopa, 226.4 ± 82.3, $P > 0.05$; 3 mg/kg of levodopa, 516.4 ± 118.5, n = 5, $P < 0.05$
159 vs. control or 6-OHDA).

160 As shown in Figure 2C, the percent use of the contralateral-to-lesioned side of
161 the hind limb relative to total steps was significantly decreased by 6-OHDA-induced
162 lesions (control, 54.0 ± 1.64; 6-OHDA, 38.5 ± 1.09; n = 5, $P < 0.05$). Levodopa was
163 shown to dose-dependently reverse this decrease (1 mg/kg of levodopa, 53.7 ± 5.08, $P <$
164 0.05 vs. 6-OHDA; 3 mg/ kg of levodopa, 65.2 ± 4.33, $P < 0.005$ vs. 6-OHDA).
165 Additionally, we analyzed the sequential use of the ipsilateral-to-lesioned side of hind
166 limb by counting double and triple steps. In 6-OHDA-lesioned mice, it tended to
167 increase, probably due to the compensatory response to the disabled movement in the
168 contralateral side, and was also reversed by levodopa with a dose-dependent manner
169 (Supplemental Figure).

170 Representative examples of hind limb stepping presented in here are available
171 in the supplemental video materials (Supplemental Video 1-3).

We further analyzed levodopa-induced rotation of 6-OHDA-lesioned mice in the same experimental condition with the hind limb step analysis. As shown in Figure 3, a significantly reduced rotation to contralateral-to-lesion side in 6-OHDA mice compared to control, indicated as % of total (control, 52.9 ± 7.16 ; 6-OHDA, 16.4 ± 8.59; n = 5, $P < 0.05$ vs. control), was reversed significantly by levodopa in a dose-dependent manner (1 mg/kg of levodopa, 73.6 ± 13.4; 3 mg/kg of levodopa, 90.9 ± 10.2; n = 5, $P < 0.005$ vs. 6-OHDA).

Targeting the MFB with 6-OHDA lesions is considered to mimic late stage PD since it causes a rapid and massive loss of dopaminergic neurons, accompanied by severe motor deficiency (Ungerstedt, 1968; Iancu et al., 2005; Bové and Perier, 2012; Heuer et al., 2012; Glajch et al., 2012; Tronci and Francardo, 2018). In fact, as shown in Figure 1, we confirmed that the mice used in this study expressed features, namely rotational activity and TH IR, of a hemi-parkinsonism model. These effects can be easily reproduced regardless of animal species. However, the behavioral tests initially established in rats are not always directly applicable in mice; rather, modifications are sometimes needed to optimize experimental conditions and apparatus (Iancu et al.,

188 2005; Heuer et al., 2012; Glajch et al., 2012). Since the current study design did not
189 require any additional/modified procedures, we simply video-recorded the spontaneous
190 hind limb steps of 6-OHDA-lesioned mice. Thus, the current model can be applicable to
191 both rats and mice under similar conditions.

192 Although asymmetric use of the hind limbs in unilateral 6-OHDA-lesioned
193 mice has previously been reported (Glajch et al., 2012), the study was not validated with
194 a PD therapeutic agent such as levodopa. In the present study, we demonstrated a
195 reduced use of the contralateral-to-lesioned side of hind limb in unilateral
196 6-OHDA-lesioned mice. This effect was dose-dependently ameliorated by levodopa
197 administration (Figure 2), strongly suggesting that the 6-OHDA-induced hind limb step
198 changes were due to striatal DA deficiency. Thus, in the unilateral 6-OHDA-lesioned
199 mouse model, abnormal hind limb stepping might reflect motor deficits of the lower
200 extremities such as bradykinesia, akinesia, and gait disturbance observed in PD patients
201 (Fahn et al., 1987), and could be utilized to develop and evaluate novel therapeutics of
202 PD with satisfying face, constructive, and predictive validities, together with the
203 rotational behavior (Figures 1 and 3). Different dosing of 6-OHDA will further

characterize the sensitivity of abnormal hind limb stepping with correlation to neuronal loss in SNpc and striatal denervation, as was previously studied in other behavioral tests (Boix et al., 2015). It would also be expected to develop a software-based automated system for counting steps.

Repeated administration of levodopa elicits LID, which has also been investigated in unilateral 6-OHDA-lesioned animals (Lunblad et al., 2002; Tronci and Francardo, 2018). Further examination will reveal how LID affects abnormal hind limb stepping and the scoring of mice according to abnormal involuntary movement scales.

Conflicts of Interests

The authors declare that they have no conflict of interests for this study.

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291 **Legends to Figures**

292 **Figure 1**: Generation of 6-OHDA-induced hemi-parkinsonism mice. (A) Rotation to
293 contralateral side indicated as % of total counts in a 30-min test. Values are expressed
294 as mean ± SEM (n = 5, $F_{2,12}$ = 363.6, ***P < 0.005 vs. control). One-way ANOVA
295 followed by Scheffe's *post hoc* test. (B) Representative image of TH immunostaining in
296 the striatum of a unilateral 6-OHDA-lesioned mouse. Scale bar indicates 500 μm. (C)
297 Quantitative analysis of striatal TH immunoreactivity (IR) indicated by density (A.U.,
298 arbitrary unit). Values are expressed as mean ± SEM (n = 5, ***P < 0.005 vs.
299 non-lesioned side). Two-tailed *t*-test.

300

301 **Figure 2**: Evaluation of hind limb steps. (A) Topological configuration of the analyzed
302 hind limb. (B) Total count of spontaneous hind limb steps in a 5-min test. Values are
303 expressed as mean ± SEM (n = 5, $F_{3,16}$ = 6.24, *P < 0.05 vs. control, $^{\#}P$ < 0.05 vs.
304 6-OHDA). (C) Steps of the contralateral hind limb, indicated as % of total counts.
305 Values are expressed as mean ± SEM (n = 5, $F_{3,16}$ = 10.2, *P < 0.05 vs. control, $^{\#}P$ <
306 0.05, $^{\#\#\#}P$ < 0.005 vs. 6-OHDA). One-way ANOVA followed by Scheffe's *post hoc*

test.

Figure 3: Evaluation of levodopa-induced rotation. Rotation to contralateral-to-lesion side indicated as % of total counts in a 5-min test. Values are expressed as mean ± SEM (n = 5, $F_{3,16}$ = 10.67, *P < 0.05 vs. control, $^{\#\#\#}P$ < 0.005 vs. 6-OHDA). One-way ANOVA followed by Fisher's PLSD test.

Legends to Supplemental Figure

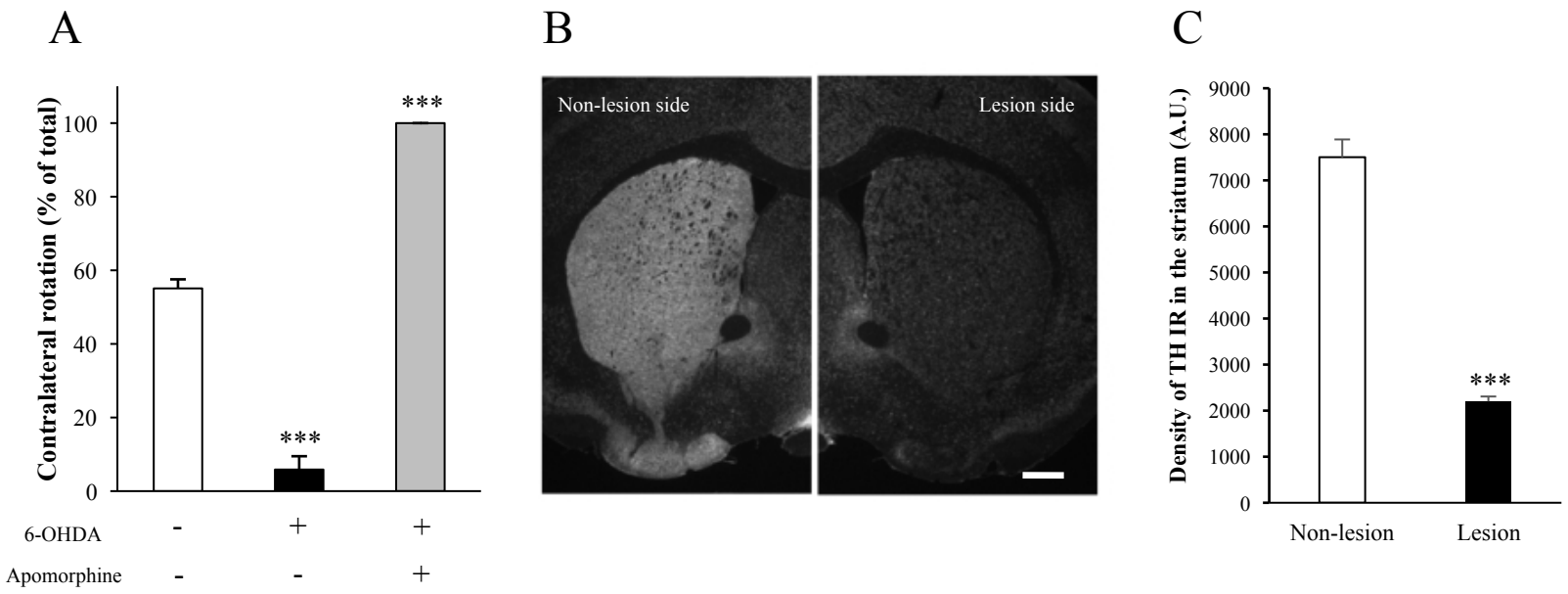
Supplemental Figure: Evaluation of sequential ipsilateral hind limb steps in a 5-min test. (A) Counts of sequential double steps in the ipsilateral hind limb. Values are expressed as mean ± SEM (n = 5, $F_{3,16}$ = 2.83). (B) Counts of sequential triple steps in the ipsilateral hind limb. Values are expressed as mean ± SEM (n = 5, $F_{3,16}$ = 7.35, *P < 0.05 vs. control, $^{\#}P$ < 0.05 vs. 6-OHDA). One-way ANOVA followed by Scheffe's *post hoc* test.

Legends to Supplemental Video Materials

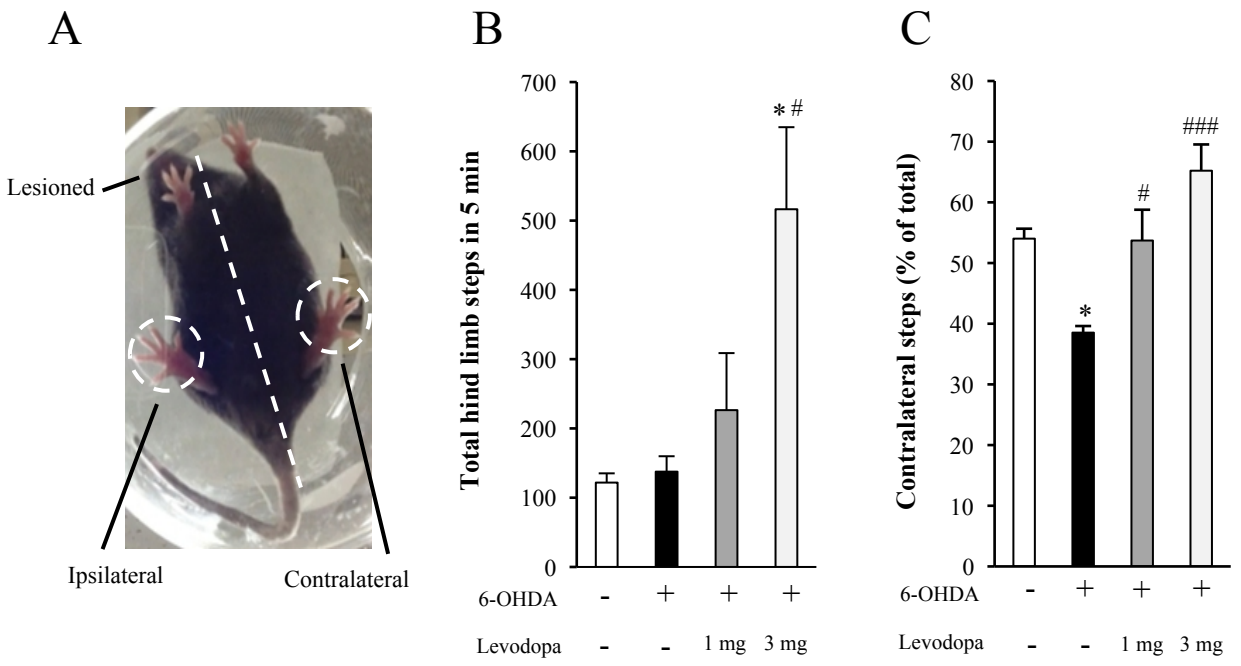
Video 1: Spontaneous steps of a control mouse.

Video 2: Spontaneous steps of a unilateral 6-OHDA-lesioned mouse. The contralateral-to-lesioned side of the hind limb is indicated by an arrow.

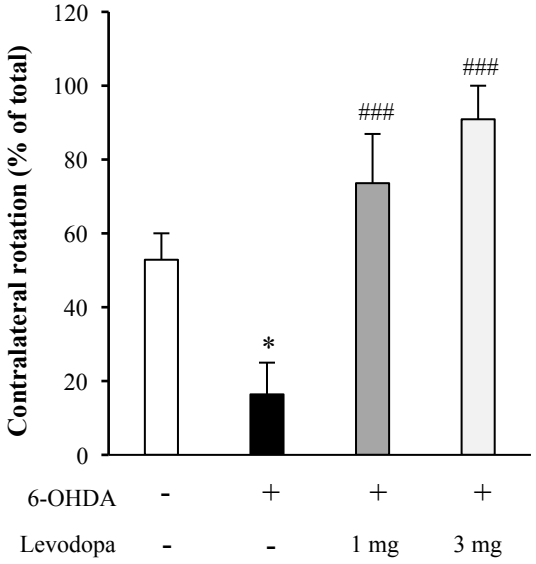
Video 3: Spontaneous steps of a unilateral 6-OHDA-lesioned mouse treated with 1 mg/kg of levodopa. Playback speed was 0.5x.



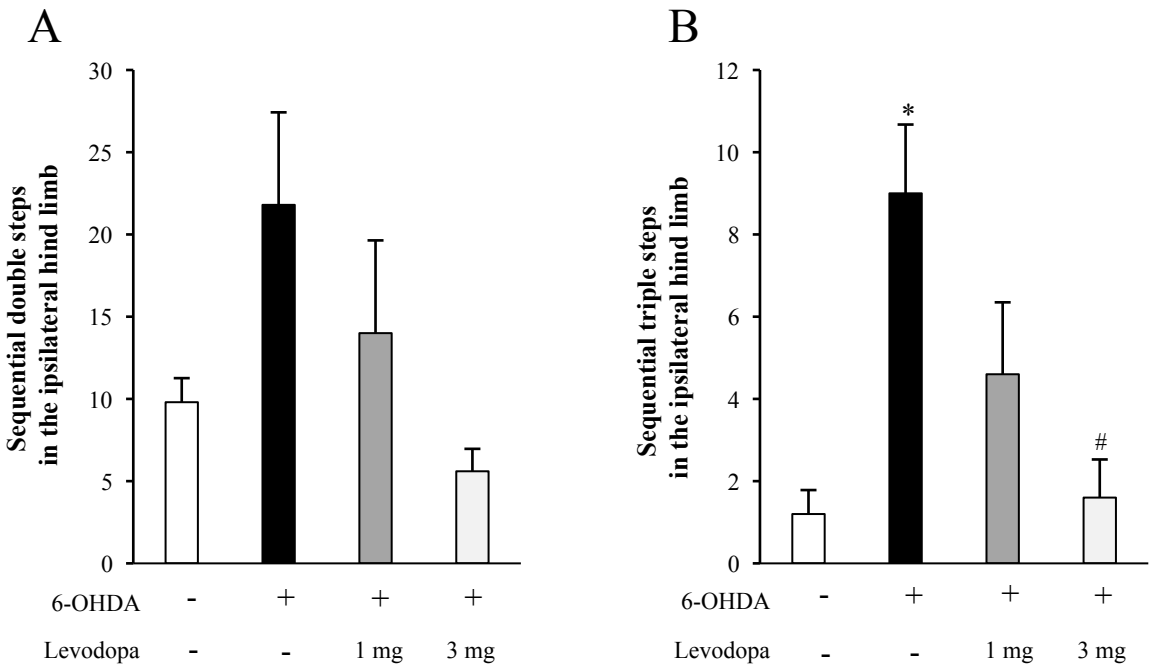
Ogawa et al., Figure 1



Ogawa et al., Figure 2



Ogawa et al., Figure 3



Ogawa et al., Supplemental Figure