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Antimicrobial Peptide, Lumbricusin, Ameliorates Motor Dysfunction and Dopaminergic Neurodegeneration in a Mouse Model of Parkinson's Disease

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We recently reported that the antimicrobial peptide Lumbricusin (NH_2-RNRRWCIDQQA), isolated from the earthworm, increases cell proliferation in neuroblastoma SH-SY5Y cells. Here, we investigated whether Lumbricusin has neurotropic activity in mouse neural stem cells (MNSCs) and a protective effect in a mouse model of Parkinson's disease (PD). In MNSCs isolated from mouse brains, Lumbricusin treatment significantly increased cell proliferation (up to 12%) and reduced the protein expression of $p27^{Kip1}$ through proteasomal protein degradation but not transcriptional regulation. Lumbricusin inhibited the 6-OHDA-induced apoptosis of MNSCs, and also showed neuroprotective effects in a mouse PD model, ameliorating the motor impairments seen in the pole, elevated body swing, and rotation tests. These results suggest that the Lumbricusin-induced promotion of neural cell proliferation *via* $p27^{Kip1}$ degradation has a protective effect in an experimental PD model. Thus, the antimicrobial peptide Lumbricusin could possibly be developed as a potential therapeutic agent for the treatment of PD.

Keywords: Antimicrobial peptide, Parkinson's disease, neuroprotective activity, cell proliferation (neurotropine), $p27^{Kip1}$, proteasomal protein degradation, cell cycle arrest

Introduction

Parkinson's disease (PD) is a chronic, progressive neurodegenerative movement disorder [8, 21]. PD is due to a selective loss of dopaminergic neurons in the substantia nigra pars compacta, leading to decreased synthesis of dopamine [19, 20, 31]. The most common treatment is administration of the dopamine precursor, levodopa, which replaces lost dopamine. However, long-term levodopa therapy of 5 to 10 years can cause complications, such as motor fluctuations [4]. Agents targeting oxidative stress, mitochondrial dysfunction, and inflammation have been proposed as prime candidates for neuroprotection [7]. Since there is currently no effective therapy for PD, many researchers have sought to identify neuroprotective agents that are capable of slowing the disease progression.

Numerous studies have investigated neuroprotective strategies that use trophic factors to alleviate the symptoms of PD. Neurotrophic factors, such as glial cell-derived neurotropic factor (GDNF) [17], nerve growth factor (NGF) [25], and neurokines [2], are known to increase neuronal cell survival. These neurotrophic factors can potently protect against the degeneration of dopaminergic neurons while also promoting their regeneration [2, 25, 27]. The symptoms, development, and progression of PD have also shown beneficial responses to natural products, such as ginseng [3] and the NAP peptide derived from activity-dependent neuroprotective protein (ADNF) [29]. Minocycline, which is the most effective known neuroprotective agent for PD, is a broad-spectrum tetracycline antibiotic [1].

Similarly, beta-lactam antibiotics are also known to prevent neurotoxicity [28]. These reports indicate that antibiotics can have neuroprotective activity.

We recently demonstrated that Lumbricusin, a 11-mer antibacterial peptide (NH2-RNRRWCIDQQA) isolated from earthworms, possesses strong neurotropic properties [13]. Here, we tested the neuroprotective effect of the antimicrobial peptide Lumbricusin in neural stem cells isolated from neonatal mouse brains, and in the 6-OHDA-induced mouse model of PD.

Materials and Methods

Synthesis of Lumbricusin

As described in detail previously [12], the Lumbricusin peptide was synthesized by AnyGen (Gwangju, South Korea), purified by reverse-phase high-performance liquid chromatography using a Capcell Pak C18 column (Shiseido, Japan), and eluted with a linear gradient of water-acetonitrile (0–80%) containing 0.1% trifluoroacetic acid (45% recovery).

Mouse Neural Stem Cell (MNSC) Isolation and Reagents

MNSCs were isolated from the cerebral striata of neonatal (postnatal day 2) mouse brains as previously described [32]. This study was approved by the Animal Care and Use Committee of Daejin University (Pocheon, South Korea). To maintain the cells in an undifferentiated proliferative state, we cultured them as free-floating neurospheres in serum-free DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 1× B27 (Invitrogen), FGF2 (20 ng/ml; Peprotech, Rocky Hill, NJ, USA), and EGF (20 ng/ml; Peprotech). The polyclonal antibody against caspase-3 was obtained from Cell Signaling Technology (Beverly, MA, USA). Polyclonal antibodies against $p27^{Kip1}$, c-Src, phosphatase, and tensin homolog (PTEN), growth factor receptor-bound protein-2 (GRB-2), tyrosine hydroxylase, and H-Ras were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The β-actin antibody, propidium iodide (PI), 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) dye, MG132, bafilomycin A1, 6-hydroxydopamine (6-OHDA), and cycloheximide were purchased from Sigma Aldrich (St. Louis, MO, USA).

6-OHDA-Induced Dopamine Lesioning and Evaluation of Behavioral Abnormalities

As described in detail previously [6], mice were anesthetized and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA, USA) fitted with a mouse adaptor. The head skin was cut longitudinally and an injector was lowered bilaterally into the dorsal striatum. The following stereotaxic coordinates were used: +0.3 mm anterior to bregma, ±2 mm lateral to midline, and −3.0 mm ventral from the skull surface. Striatal dopamine lesions were induced using a 6-OHDA hydrochloride solution (1.5 μg/0.1 μl of saline containing Na-metabisulfite (0.1 M)) or 6-OHDA hydrochloride solution together with Lumbricusin (10 μg), both in an injection volume of 0.3 μl per side. For the control sham lesions, the skull was perforated at sites corresponding to the structures, and then injected with 0.3 μl of saline only. To protect the noradrenergic terminals, mice were given desipramine (35 mg/kg, i.p.; Sigma). Twenty days post-treatment, the elevated body swing test, the pole test, and the rotation test were conducted to evaluate behavioral abnormalities. To evaluate the content of tyrosine hydroxylase (TH; a marker for the respective numbers of dopaminergic terminals and cell bodies [6]), striata were isolated, washed in cold PBS, and then homogenized in cold PBS. After centrifugation (11,000 ×*g*, 10 min at 4°C), supernatants were collected and subjected to immunoblot analysis with an antibody against TH.

Cell Viability

MNSCs (3×10^3 cells/well) were pretreated with Lumbricusin (10 μg/ml) for 1 h, exposed to medium (control) or 6-OHDA (100 μM) for 12 h, and then incubated with MTT dye for 2 h [10]. The solubilization reagent was added, and absorbance was determined at 570 nm in a microplate reader (Model 3550; Bio-Rad, Mississauga, ON, Canada).

BrdU Cell Proliferation Assay

As described in detail previously [5], the proliferation of Lumbricusin-treated cells was measured by assessing the rate of DNA synthesis using a BrdU Cell Proliferation Assay (Roche, Indianapolis, IN, USA), according to the manufacturer's instructions. Briefly, MNSCs (1×10^4 cells/well) were seeded to a 96-well microplate, treated with or without Lumbricusin (10 μg/ml) for 36 h, and further cultured with the provided BrdU mixture for 12 h. The cells were then fixed, incubated with the anti-BrdU antibody for 1 h, and incubated with horseradish-peroxidase-conjugated goat anti-mouse IgG for 30 min. Absorbance at 450 nm was determined using a microplate reader.

RNA Isolation and Semi-Quantitative RT-PCR

RNA was prepared using the TRIzol reagent (Life Technologies, Gaithersburg, MD, USA), and reverse transcription was performed as previously described [15]. The resulting product (1 μl) was amplified with primers specific to mouse $p27^{Kip1}$ (sense, 5-CTGGAGCGGATGGACGCCAGA-3 and antisense, 5-CGTCTG CTCCACAGTGCCAGC-3). The polymerase chain reaction (PCR) was conducted with an optimal number of cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min.

Statistical Analysis

The results are presented as the mean ± SEM. Data were analyzed using the SIGMA-STAT software package (Jandel Scientific Software, San Rafael, CA, USA). Analyses of variance with protected *t*-tests were used for intergroup comparisons.

Results and Discussion

Lumbricusin Has a Proliferative Effect on MNSCs

Since we previously demonstrated that the antimicrobial peptide Lumbricusin increased cell proliferation in human neuroblastoma SH-SY5Y cells [13], we first assessed whether Lumbricusin enhances the proliferation of neural stem cells isolated from the cerebral striata of neonatal mouse brains. MNSCs were exposed to 10 μg/ml Lumbricusin for 48 h, and BrdU uptake experiments were used to measure cell proliferation. Lumbricusin treatment of MNSCs increased cell proliferation by 12 ± 3% compared with the medium control (Fig. 1A). We previously showed that a high concentration (~150 μg/ml) of CopA3 peptide (isolated from the Korea dung beetle) can trigger apoptosis [10]. However, our MTT assays revealed that 10 μg/ml Lumbricusin did not show toxicity on MNSCs (Fig. 1B). Microscopic image analysis also confirmed that neurospheres grew as free-floating clusters [23] in both control and Lumbricusin-treated cultures (Fig. 1C). These results suggested that the antimicrobial peptide Lumbricusin encourages the proliferation of MNSCs without apparent cellular toxicity at the tested dose.

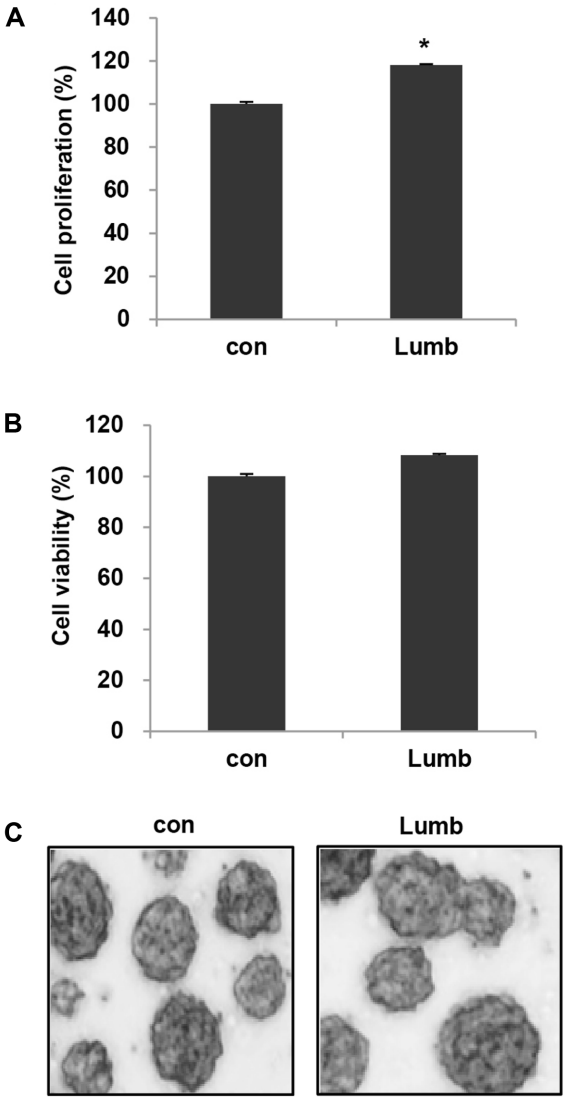

Fig. 1. Lumbricusin enhances cell proliferation in mouse brain neural stem cells (MNSCs).

(**A**) MNSCs (10^5 cells/well) were treated with medium (con) or Lumbricusin (Lumb, 10 μg/ml) for 48 h, and cell proliferation was assessed by measuring BrdU uptake. The results represent the mean ± SEM of three experiments performed in triplicate (*, $p < 0.005$). (**B**) MNSCs were incubated with Lumbricusin (10 μg/ml) for 48 h, and cell viability was measured by MTT assay (*, $p < 0.005$). (**C**) Light microscopic images (100×) of MNSCs after 48 h incubation with medium or Lumbricusin.

Lumbricusin Enhances Proteasome-Mediated $p27^{Kip1}$ Degradation in MNSCs

Given our observation that Lumbricusin enhances MNSC growth, we next sought to identify the intracellular molecules responsible for this effect. Since we previously revealed that Lumbricusin promotes the growth of a human neuroblastoma cell line (SH-SY5Y) by down-regulating the cyclin-dependent kinase inhibitor, $p27^{Kip1}$ [24], we herein tested whether this mechanism was conserved in MNSCs. We treated MNSCs with Lumbricusin for 24 h, and then used immunoblotting to assess the expression levels of signaling components known to regulate cell proliferation. As shown in Fig. 2A and 2B, Lumbricusin treatment down-regulated $p27^{Kip1}$ in a dose- and time-dependent manner, but did not alter the levels of the other tested proteins, including c-Src, phosphatase and tensin homolog (PTEN), H-Ras, and growth factor receptor-bound protein-2 (GRB-2). Lumbricusin treatment did not reduce the level of the $p27^{Kip1}$-encoding mRNA in MNSCs (Fig. 2C), indicating that the marked reduction in $p27^{Kip1}$ was not associated with an altered transcript level. To determine whether Lumbricusin altered $p27^{Kip1}$ protein degradation, we treated MNSCs with the translation inhibitor cycloheximide (100 μM; to prevent *de novo* protein synthesis [23]), in the presence or absence of Lumbricusin, and examined the levels of $p27^{Kip1}$ protein. As shown in Fig. 2D, the half-life of the $p27^{Kip1}$ protein was significantly shorter in Lumbricusin-treated MNSCs (<2 h) compared with control MNSCs (~8 h). Next, we assessed whether MG132, a proteasome inhibitor [11], could block the Lumbricusin-induced degradation of $p27^{Kip1}$. MNSCs were

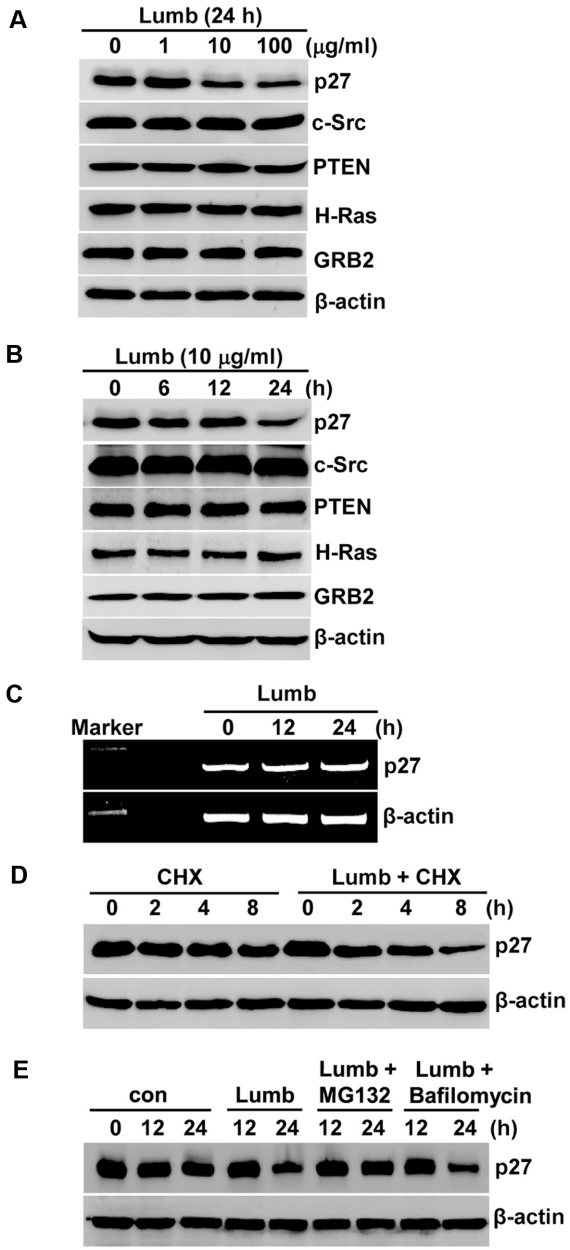

Fig. 2. Lumbricusin enhances the degradation of $p27^{Kip1}$ protein in MNSCs.

(**A**) MNSCs (10^5 cells/well) were treated with Lumbricusin (Lumb) for 24 h, and then lysed. Proteins were resolved by 10% SDS-PAGE, and blots were probed with antibodies against $p27^{Kip1}$ (p27), c-Src, PTEN, H-Ras, GRB2 and β-actin. (**B**) Lumbricusin (Lumb) treatment time-dependently decreases $p27^{Kip1}$ protein levels. (**C**) Total RNA was obtained, cDNA was synthesized, and $p27^{Kip1}$ and β-actin were amplified by PCR. (**D**) MNSCs (10^5 cells/well) were incubated with cycloheximide alone (CHX; 100 μM) or CHX plus Lumbricusin (Lumb) for the indicated time periods. (**E**) MNSCs were pretreated with MG132 (10 μM) or bafilomycin A1 (100 nM) for 1 h and then exposed to Lumbricusin (Lumb) for the indicated durations.

treated with 10 μM MG132 for 1 h and then exposed to Lumbricusin. As shown in Fig. 2E, the Lumbricusin-induced down-regulation of $p27^{Kip1}$ was completely blocked by MG132 treatment. In contrast, the lysosomal inhibitor [11] bafilomycin A1 had no effect. These results indicate that, consistent with our previous report that $p27^{Kip1}$ down-regulation is essential for the increased proliferation of human neuroblastoma cells, the marked decrease of 27^{Kip1} observed in Lumbricusin-treated MNSCs is due to proteasomal-dependent protein degradation.

Lumbricusin Protects MNSCs Against 6-OHDA-Induced Apoptosis

Since we found that the antimicrobial peptide Lumbricusin has neurotropic activity in MNSCs, we next assessed whether it can neuroprotect against 6-OHDA-induced apoptosis, which is used as an *in vitro* model of Parkinson's disease [16, 18]. As shown in Fig. 3A, 100 μM 6-OHDA markedly decreased the viability of MNSCs, but this was significantly blocked by pretreatment with Lumbricusin (10 μg/ml) for 1 h. Next, we assessed whether Lumbricusin blocked the 6-OHDA-induced apoptosis of MNSCs. We incubated MNSCs with Lumbricusin for 1 h, treated them with 6-OHDA for 48 h, and then measured the caspase-3 activity [12]. In MNSCs, caspase-3 activation was significantly induced by 6-OHDA alone, but this effect was markedly inhibited by Lumbricusin (Fig. 3B). Consistent with our previous findings in SH-SY5Y cells [13], the expression of $p27^{Kip1}$ was markedly reduced in Lumbricusin-treated MNSCs, suggesting that the specific down-regulation of $p27^{Kip1}$ is critical for the effects of Lumbricusin on cell proliferation and apoptosis in neuronal cells. Next, we assessed whether the adenovirus-mediated overexpression of $p27^{Kip1}$ could inhibit the neurotropic effects of Lumbricusin in MNSCs. We infected MNSCs with a $p27^{Kip1}$-encoding adenovirus (1×10^7 PFU/ml) or a control GFP-adenovirus for 24 h, treated the cells with medium, 6-OHDA alone, or 6-OHDA plus Lumbricusin for 48 h, and then measured the caspase-3 activity. In control MNSCs overexpressing GFP, the 6-OHDA-induced caspase-3 activation was strongly inhibited by Lumbricusin (Fig. 3C). In MNSCs overexpressing $p27^{Kip1}$, in contrast, Lumbricusin did not block 6-OHDA-induced caspase-3 activation. Interestingly, $p27^{Kip1}$ overexpression alone caused a marked activation of caspase-3 in the absence of 6-OHDA (Fig. 3C). These findings are consistent with the previous reports showing that: (i) the half-life of the $p27^{Kip1}$ protein is altered in some neurodegenerative diseases [22]; (ii) $p27^{Kip1}$ inhibits the proliferation of adult brain neural progenitor cells [26]; and

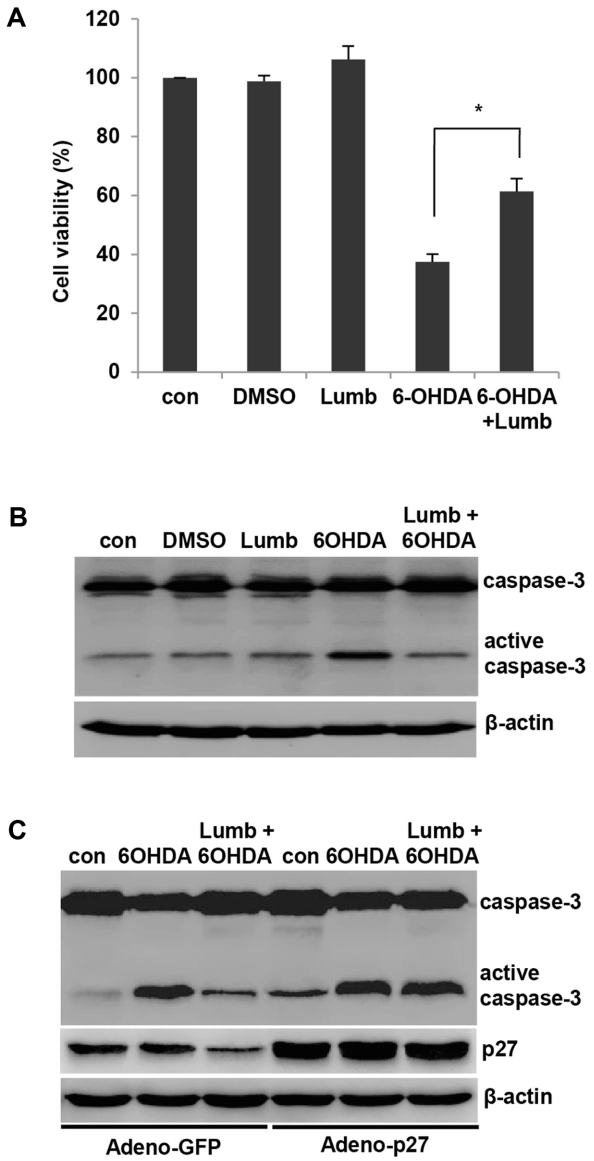

Fig. 3. Lumbricusin protects MNSCs against 6-OHDA-induced loss of viability and induction of apoptosis.

(**A**) MNSCs (10^5 cells/well) were pretreated with Lumbricusin (10 μg/ml) for 1 h and then incubated with medium (control), vehicle (dimethylsulfoxide (DMSO)), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb) for 12 h, and cell viability was measured by MTT assay (*, $p < 0.001$). (**B**) MNSCs were pretreated with Lumbricusin (10 μg/ml) for 1 h and then incubated with medium (con), 100 μM 6-OHDA alone, or 6-OHDA plus Lumbricusin (Lumb) for 48 h. Apoptosis was identified by immunoblot analysis for cleaved caspase-3. Shorter fragments (lower bands) represent active forms of caspase-3. (**C**) MNSCs (10^5 cells/well) were infected with a $p27^{Kip1}$-expressing adenovirus (1×10^7 PFU/ml) or a control GFP adenovirus (1×10^7 PFU/ml) for 24 h, pretreated with Lumbricusin (10 μg/ml) for 1 h, and then incubated with medium, 6-OHDA alone, or 6-OHDA plus Lumbricusin for 48 h.

(iii) down-regulation of $p21^{Kip1}$ (another cyclin-dependent kinase inhibitor [14]) is critical for the neuroprotective activity of the insect peptide CopA3 [24]. These results together with our present findings suggest that the expression of $p27^{Kip1}$ may be essential for the proliferation of neuronal stem cells and/or the cytotoxicity seen during the progression of neural dysfunction.

The Lumbricusin peptide, which is a natural isolate from the earthworm *Lumbricus terrestris*, is an antimicrobial peptide that has bactericidal activity [13]. Here, we report for the first time that Lumbricusin can confer neurotropic and neuroprotective effects in MNSCs, indicating that it is an antimicrobial peptide that also displays biological activity against neuronal cells. In this, it is comparable to minocycline, which is a broad-spectrum tetracycline antibiotic that also has neuroprotective properties against Parkinson's disease [1]. Similarly, beta-lactams and some of their semi-synthetic derivatives are potent antibiotics that are also known to increase the brain expression and activity of glutamate transporter-1, thereby preventing glutamate neurotoxicity [28]. Our work suggests that Lumbricusin can be added to the list of neuroprotective agents that have bactericidal effects against microorganisms.

Lumbricusin Treatment Attenuates Motor Impairments After 6-OHDA Treatment

To examine whether the neuroprotective effect of Lumbricusin could confer behavioral improvements, we carried out three motor tests post-treatment [9, 30]. As shown in Fig. 4A, a marked decrease was seen in the results of the elevated body swing test [30] in mice treated with 6-OHDA alone, but this was significantly recovered by the co-administration of Lumbricusin. We also used a modified pole test [9], in which the pole was set at a 45° incline with respect to the floor. Mice were placed facing upwards at the floor end of the pole, and the total distance climbed was recorded. As expected, mice treated with 6-OHDA alone traveled a significantly shorter distance compared with the saline-treated control group (Fig. 4B). However, mice co-treated with Lumbricusin plus 6-OHDA showed a significant increase in the distance they migrated up the pole compared with those treated with 6-OHDA alone (Fig. 4B). In the amphetamine-induced rotation test [30], mice co-treated with Lumbricusin plus 6-OHDA showed significantly fewer ipsilateral turns than those treated with 6-OHDA alone (Fig. 4C). We also measured the TH content (a marker for the numbers of dopaminergic terminals and cell bodies in the striatum and midbrain, respectively [6]) by immunoblot analysis. The TH level in

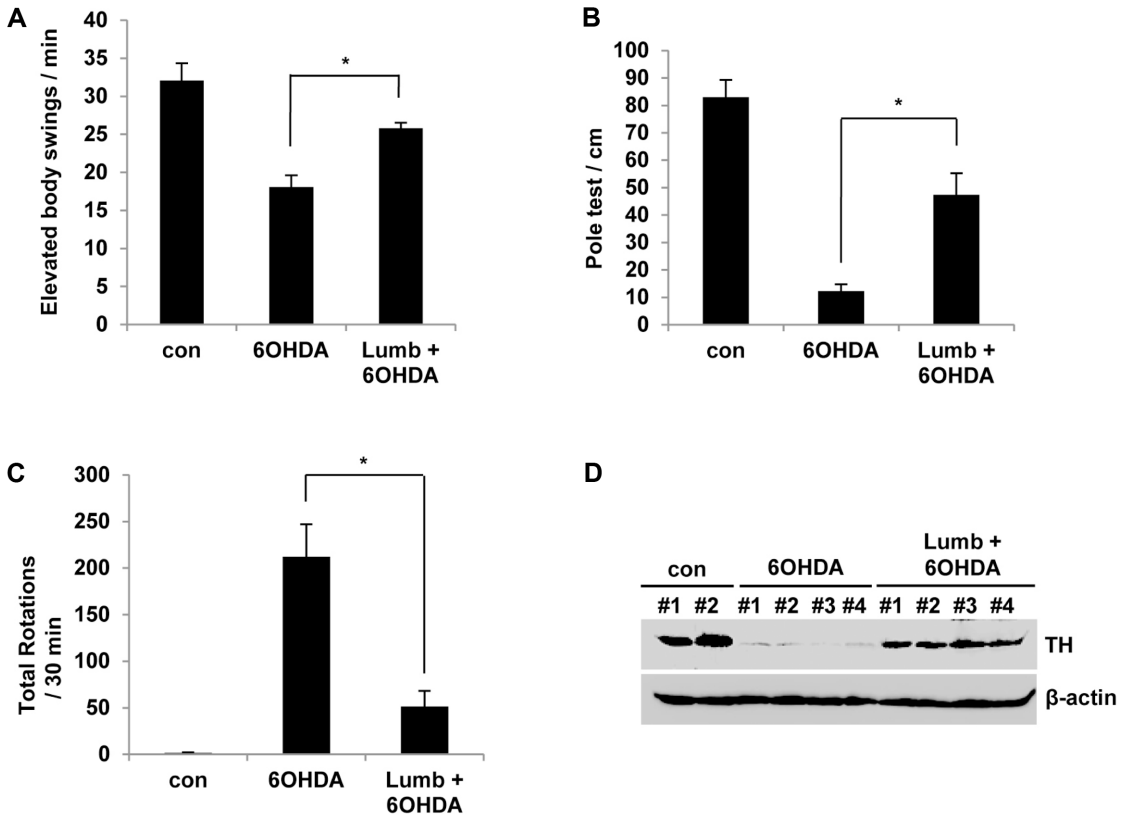

Fig. 4. Lumbricusin recovers behavioral abnormalities in 6-OHDA-treated mice.

Mice (n = 10 per group) were placed in a stereotaxic apparatus and striatal dopamine lesions were produced by injection of 6-OHDA hydrochloride solution alone (6OHDA), or 6-OHDA together with Lumbricusin (10 μg) (Lumb + 6OHDA). Twenty days post-treatment, behavioral abnormalities were evaluated. Sham controls were injected with 0.3 μl of saline only (con). (**A**) The elevated body swing test. The results represent the means ± SEM of three experiments performed in triplicate (*, $p < 0.001$). (**B**) The pole test (*, $p < 0.005$). (**C**) The rotation test (*, $p < 0.001$). (**D**) Striata were isolated from the brains of conditioned mice and homogenized in cold PBS; the supernatants were subjected to 10% PAGE and blots were probed with an antibody against TH.

the striatum was markedly reduced by administration of 6-OHDA alone, but this decrease was significantly reversed in mice treated with Lumbricusin plus 6-OHDA.

These results collectively suggest that in addition to enhancing the cell proliferation of MNSCs, Lumbricusin can also recover behavioral impairment in a mouse model of PD.

Additionally, based on the notion that low-molecular-weight agents have reduced antigenicity, Lumbricusin (an 11 mer peptide) may have reduced antigenicity compared with higher-molecular-weight agents. Thus, the Lumbricusin peptide may be a potential drug candidate for the treatment of Parkinson's disease.

Acknowledgments

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Abbreviations

MNSCs, mouse neural stem cells; 6-OHDA, 6-hydroxy dopamine; p27, $p27^{Kip1}$; MTT, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; DMSO, dimethyl sulfoxide; $p27^{Kip1}$; GFP, green flruorescence protein; CHX, cycloheximide; BrdU, bromodeoxyuridine.

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