学位論文（博士）

Critical role of central ghrelin in the modulation of Parkinson's disease

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Abbreviations

ACTB : Beta-actin

BDNF : Brain-derived neurotrophic factor

bFGF : Basic-FGF

BMP : Bone morphogenic protein

CHIR : CHIR99021

CNS : Central nervous system

DA : Dopamine

DAnergic : Dopaminergic

DAT : Dopamine transporter

DM : Dorsomorphin

DTA : Diphtheria toxin A

ESCs : Embryonic stem cells

GAD 67 : Glutamic acid decarboxylase 67

GAPDH : Glyceraldehyde-3-phosphate dehydrogenase

GDNF : Glial cell-derived neurotrophic factor

GH : Growth hormone

GHSR : Growth hormone secretagogue receptor

GOAT : Ghrelin O-acyltransferase

iPSC : Induced pluripotent stem cell

KIKO : Knock-in/knock-out

MOR : μ-Opioid receptors

MPTP : 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

PD : Parkinson's disease

PFA : Paraformaldehyde

SB : SB431542

Shh : Sonic hedgehog

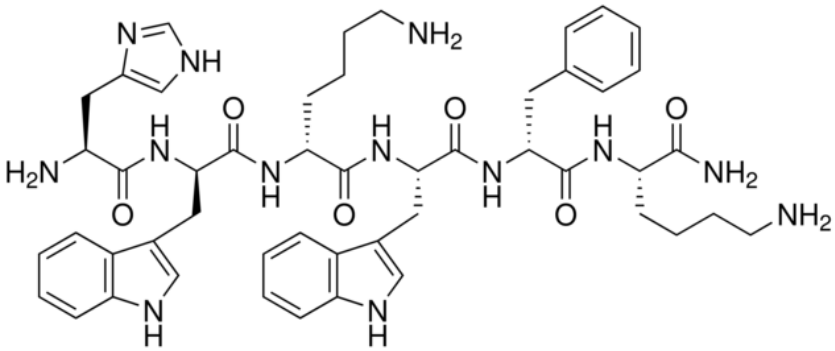
SNc : Substantia nigra pars compacta

TGFβ : Transforming growth factor-beta

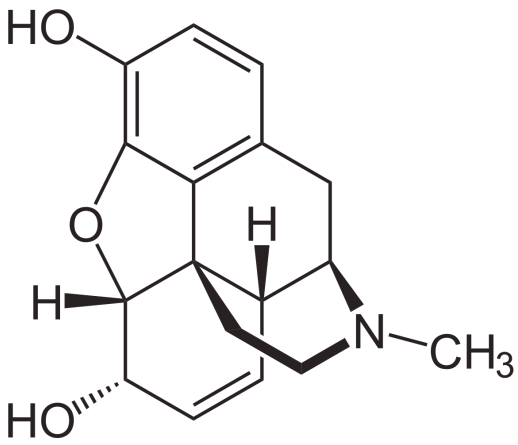
TH : Tyrosine hydroxylase

Structures of drugs used in the present study

[D-Lys3]-GHRP-6 : His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2

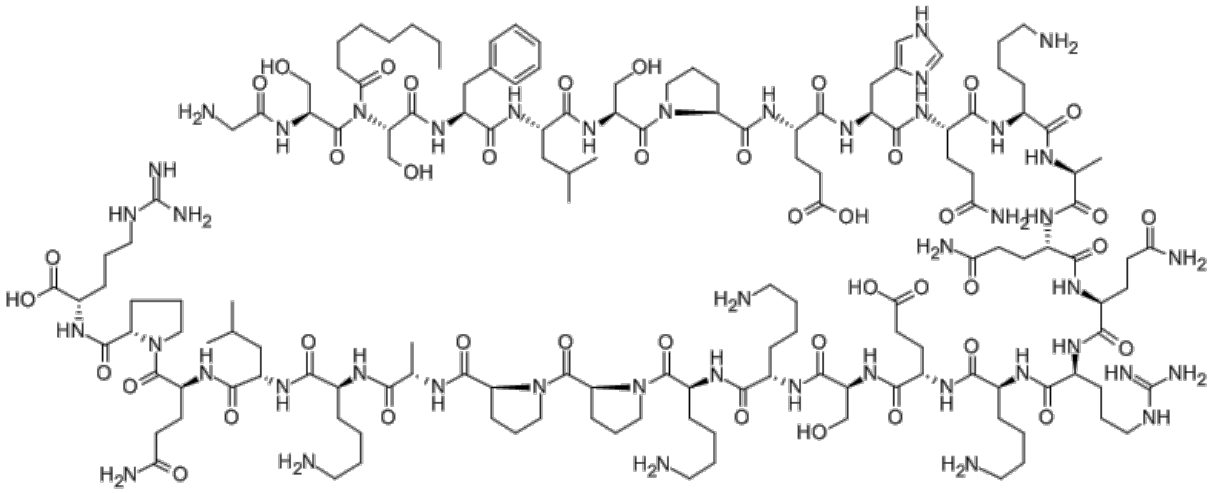


Morphine : (5α,6α)-7,8-didehydro- 4,5-epoxy-17-methylmorphinan-3,6-diol

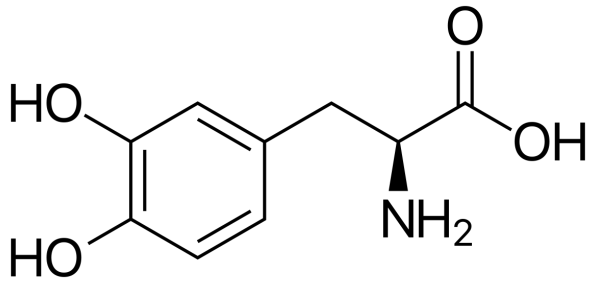


Ghrelin : Gly-Ser-Ser(*n*-Octanoyl)-Phe-Leu-Ser-Pro-Glu-His-Gln-Lys-Ala-

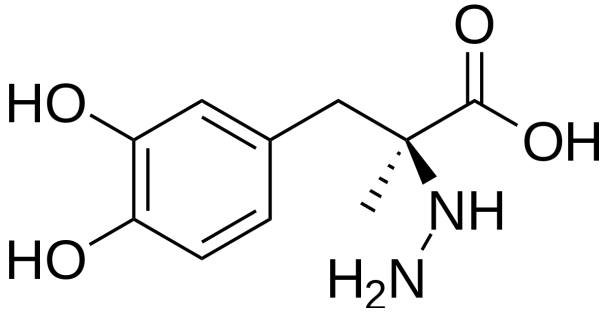
Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg



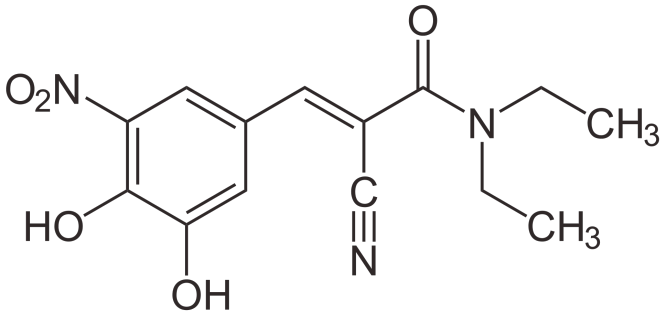
L-DOPA : (2*S*)-2-amino-3-(3,4-dihydroxyphenyl) propanoic acid



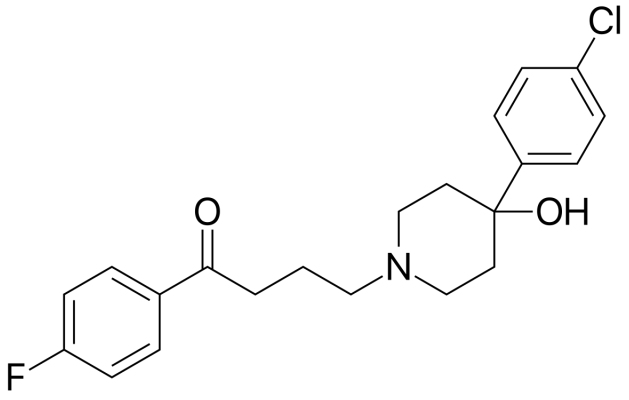
Carbidopa : (*S*)-3-(3,4-Dihydroxyphenyl)-2-hydrazino-2-methylpropionic acid



Entacapone **:** (2E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethylprop-2-enamide



Haloperidol : 4-[4-(4-Chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl) butan-1-one



General Introduction

Significance of induced pluripotent stem cell (iPSC) research

iPSCs are useful models for studying a variety of human diseases and a promising tool in cell therapy for human diseases. In 2006, Takahashi and Yamanaka introduced the concept of iPSCs by generating stem cells that had the properties of embryonic stem cells (ESCs) [(1)]. iPSCs were generated by using a combination of four reprogramming factors, including Oct4, Sox2, Klf4 and c-Myc, and exhibited both self-renewal and differentiation like ESCs. There have been tremendous advances in iPSC technology over the past 10 years. iPSCs from patients with various conditions, including diseases of the nervous, hematopoietic and metabolic systems, are now available, and their pathologies are the subject of intense investigation [(2-5)]. iPSCs make it possible to not only address important issues such as the functional relevance of molecular findings, the contribution of individual genetic variations, and patient-specific responses to specific interventions, but also help to recapitulate the long-term time-course of diseases. In fact, many groups have reported that the apparent cellular phenotypes of genetic disorders can be recapitulated in patient-specific iPSC-derived cells *in vitro* [(6-8)]. Some reports have involved drug screening using iPSCs, and these have led to the proposal of novel drug candidates [(9)].

Usefulness of multiple analyses

Meta-analyses are popular tools in which the results of several experiments and studies are combined to reach an overall decision. Recent technological advances have led to the development of high-throughput genomic assays, which make it possible to analyze thousands of genes simultaneously. Genetic association studies are a powerful approach for identifying susceptibility genes that underlie common diseases and provide linkage analyses in investigations of complex diseases.

Recent approach to Parkinson's disease

Parkinson's disease (PD) is the second-most common neurodegenerative disorder after Alzheimer's disease. Clinically, PD is characterized by bradykinesia, resting tremors, and rigidity due to loss of nigrostriatal dopaminergic (DAnergic) neurons. PD is a complex disease that is associated with a combination of environmental exposures and genetic factors. About 10% of PD patients have a positive family history, and a series of genes, including *SNCA, Parkin, PINK1, DJ-1* and *LRRK2*, have been cloned in familial PD patients [(10)]. Although α-synuclein accumulation, mitochondrial dysfunction, mitophagy and oxidative stress have been thought to play important roles in the development of PD [(11)], the mechanism by which DAnergic neurons are selectively lost in PD is still under investigation. One of the greatest limitations in the study of PD has been the lack of an animal model that mimics the cellular and behavioral abnormalities of the disease. In addition, previous explorations of

human neurological and psychiatric disorders have been hampered by the difficulty of obtaining patient-derived neural cells or tissues because of limited access to the brain, except for autopsy samples. The use of patient-specific iPSCs has great potential to provide new insights into the pathogenesis of PD, which may in turn lead to new clinical interventions.

Role of ghrelin in the substantia nigra pars compacta (SNc)

Ghrelin is a 28-amino acid peptide hormone that is derived from the proteolytic cleavage of pre-pro-ghrelin and pro-ghrelin. It is synthesized predominantly in the stomach, where it is secreted into the circulation. The binding of ghrelin to growth hormone secretagogue receptor (GHSR) 1a leads to the release of growth hormone [12, 13]. Numerous studies over the past decade have demonstrated that it plays important roles in food intake, body-weight regulation and glucose homeostasis. Although the abundant expression of GHSR1a in the hypothalamus highlights its importantce in energy metabolism, GHSR1a is also abundantly expressed in extra-hypothalamic sites including the SNc [14, 15]. In the SNc, ghrelin is considered to activate DAnergic neurons, and increases the dopamine (DA) concentration in the striatum by inhibiting native KCNQ/M-current through a GHS-R-PLC-PKC pathway [16, 17]. Ghrelin has been shown to stimulate proliferation and inhibit apoptosis in various cells through GHSR1a. In addition, ghrelin has also been described as an anti-inflammatory hormone, which inhibits the release of pro-inflammatory cytokines in response to treatment with the bacterial endotoxin lipopolysaccharide in peripheral macrophage and T-cells as well as from central

nervous system (CNS)-derived microglia [18]. Therefore, it should be worth examining the role of ghrelin in the modulation of PD.

Aim and Scope

The aim of the present study was to investigate the role of ghrelin in nigrostriatal dopaminergic (DAnergic) neurons in Parkinson's disease (PD). To achieve this end, this study included behavioral, biochemical and molecular biological experiments.

The specific aims of the proposed research are as follows:

In Chapter 1:

To identify susceptibility genes related to the vulnerability of DAnergic cells that underlies PD, I examined the expression of endogenous ghrelin receptors (GHSRs) in PD-specific induced pluripotent stem cell (iPSC)-derived DAnergic neurons generated from patients carrying *parkin* gene mutations (PARK2) compared to those from healthy controls, and found a dramatic reduction in the former. To further evaluate whether down-regulation of GHSR contributes to DAnergic cell vulnerability, I investigated whether the direct inhibition of GHSR function in the SN of mice could affect motor coordination *in vivo*.

In Chapter 2:

To evaluate the effect of ghrelin on motor deficits at the end stage of PD, I performed the stereotaxic injection of AAV-CMV-FLEX-diphtheria toxin A (DTA) into the SN of

DAT-Cre (DAT^{SN}::DTA) mice using a Flex-switch system to expunge DA neurons of the SNc, which could mimic end-stage PD. Furthermore, I used the mice treated with a classic D_2 receptor antagonist haloperidol as an initial PD model with the temporal dysregulation of DA transmission. I investigated whether treatment with ghrelin could improve the phenotypes induced by either the selective ablation of nigrostriatal DA neurons using DTA or the administration of haloperidol.

Ethics

All of the experimental procedures for cell differentiation and analysis were approved by the respective Ethics Committees of Keio University School of Medicine and Hoshi University.

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Ministry of Education, Culture, Sports, Science, and Technology of Japan. Every effort was made to minimize the numbers and any suffering of animals used in the following experiments. Animals were used only once in the present study.

Chapter 1

Down-regulation of ghrelin receptors on dopaminergic neurons in the substantia nigra contributes to Parkinson's disease-like motor dysfunction

Introduction

Parkinson's disease (PD) is a common, debilitating, neurodegenerative disorder that is associated with progressive motor dysfunction. PD is characterized by the progressive loss of dopamine (DA) neurons, and the DA neurons that degenerate in PD primarily project to the substantia nigra pars compacta (SNc). The motor symptoms of PD manifest only after a significant loss of striatal (70-80%) DA concentration in the brain and are seen relatively late in disease progression. PARKIN (PARK2), an E3 ubiquitin ligase, is the most frequently mutated gene that has been causally linked to autosomal recessive early-onset familial PD [19, 20]. Abnormalities of PARK2 have also been described in sporadic PD [21].

Induced pluripotent stem cells (iPSCs) give rise to all cells in an organism. A potential solution to the difficulty of modeling PD is to use reprogramming technology to generate disease-specific iPSCs. PD-specific iPSCs-derived DA neurons could recapitulate the pathological features of PD. The exact mechanisms by which mutant PARK2 causes PD-like syndromes and why DA neurons are primarily affected by a ubiquitously expressed mutation remain unknown [22, 23]. A monogenic form of PD-specific iPSCs-derived DA neurons could provide important clues for elucidating the pathogenesis of PD.

Ghrelin, an endogenous ligand for growth hormone secretagogue receptor (GHSR), which is classified as a G-protein coupled receptor, is a 28-amino acid peptide that regulates growth hormone secretion, food intake, reward-seeking behavior and memory performance [12, 24-26].

While it is mainly secreted from the stomach [12], small amounts are produced in the brain [27]. GHSR is expressed in various brain areas including the SNc, hypothalamus, ventral tegmental area and hippocampus, where ghrelin directly modulates neuronal activity [17, 24, 25, 28]. In the SNc, ghrelin electrically activates dopaminergic neurons, and increases the dopamine concentration in the striatum via the specific blockade of KCNQ channel function [16]. It has also been reported that ghrelin has a neuroprotective effect to prevent the greater loss of SNc dopaminergic neurons in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD [17].

In the present study, I evaluated the changes in GHSR expression in dopaminergic neurons derived from PD-specific iPSCs. Furthermore, I confirmed that the inhibition of GHSR in DA neurons of the substantia nigra, using the microinjection of a selective GHSR inhibitor into the SNc, leads to motor dysfunction.

Materials and methods

Human iPS cells

For control lines, I used two human iPS cell lines: 201B7 iPSCs were purchased from RIKEN BRC and kindly provided by Dr. Shinya Yamanaka [29] at Kyoto University and WD39 iPSCs were established [6] at Keio University. For PD (PARK2) lines, the patient A (PA9 and PA22) and patient B (PB2 and PB20) iPSCs were established by Dr. Imaizumi [6]. All of the iPSCs were maintained on feeder cells in iPSC culture media, as described previously [6]. All of the experimental procedures for iPS cell production were approved by the Ethics Committee of Keio University School of Medicine. All of the experimental procedures for cell differentiation and analysis were approved by the respective Ethics Committees of Keio University School of Medicine (Approval Number: 20-16-28) and Hoshi University School of Medicine (Approval Number: 28-008).

In vitro differentiation of human iPSCs (hiPSCs)

DA neuron differentiation from iPSCs was performed according to a previously reported protocol [30-32]. Neural induction was initiated through the inhibition of both bone morphogenic protein (BMP) and transforming growth factor-beta (TGFβ) signaling using the small molecules Dorsomorphin (DM, Sigma-Aldrich, St. Louis, MO, USA) and SB431542 (SB, Tocris Bioscience, Bristol, UK). The small molecule CHIR99021 (CHIR, Stemgent,

Lexington, MA, USA), a GSK3β inhibitor, was added to stimulate the canonical WNT signaling pathway. For neural induction from single hiPSCs, hiPSCs were incubated with TrypLE™ Select (Gibco, Life Technologies, CA, USA) for 5-10 minutes and dissociated into single cells by pipetting. Cells were plated into a T75 flask and cultured in KBM (KOHJINBIO, Saitama, Japan) supplemented with B27 (Gibco, Life Technologies), 20 ng/mL basic-FGF (bFGF, PeproTech. Inc., Rocky Hill, NJ, USA), 10 μM Y-27632 (Wako, Tokyo, Japan), 10 ng/mL hLIF (Millipore, Billerica, MA, USA), 1 μM Purmorphamine (Calbiochem, San Diego, CA, USA), 2 μM SB (Tocris Bioscience), 100 ng/ml CHIR, 100 ng/ml Sonic hedgehog (Shh, R&D Systems Inc., Minneapolis, MN, USA) and 100 ng/ml FGF8b (PeproTech) in 4% oxygen for 7 or 12 days. Neurospheres were repeatedly passaged by dissociation into single cells, and then cultured in the same manner. Neurospheres at passage 3 were typically used for analysis. For terminal differentiation, dissociated neurospheres were allowed to adhere to poly-L-ornithine (Sigma-Aldrich)- and fibronectin (Sigma-Aldrich)-coated coverslips and cultured in KBM (KOHJINBIO) containing B27 (Gibco, Life Technologies), 20 ng/mL brain-derived neurotrophic factor (BDNF, R&D Systems), 20 ng/mL glial cell-derived neurotrophic factor (GDNF, R&D Systems), 200 μM ascorbic acid (Sigma-Aldrich), and 500 μM dibutyryl-cAMP (Sigma-Aldrich) for 10 days.

Immunocytochemical analysis

Cells were fixed with 4% paraformaldehyde (PFA) and then washed three times with PBS. After cells were incubated with blocking buffer (PBS containing 5% normal fetal bovine serum and 0.3% Triton X-100) for 1 h at room temperature, they were incubated overnight at 4°C with anti-TH (Millipore), anti-βIII-tubulin (Sigma-Aldrich) and anti-Ghrelin receptor (Abcam, Cambridge, MA, USA) as primary antibodies diluted with blocking buffer. The cells were again washed three times with PBS and incubated with secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 546 for 1 h at room temperature. After cells were washed three times with PBS, samples were mounted on slides with DAPI-Fluoromount-G™ (SouthernBiotech, Birmingham, AL, USA). Fluorescence of immunolabeling was detected using a light microscope BZ-X710 (KEYENCE, Osaka, Japan) or IX-73 (Olympus, Tokyo, Japan) and photographed with a digital camera using BZ-X Analyze software (KEYENCE) or cellSens software (Olympus).

qRT-PCR

Total RNA was isolated from cells using an RNeasy mini kit (QIAGEN, Hilden, Germany) with DNase I treatment, and cDNA was prepared by using a SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Waltham, MA, USA). The qRT-PCR analysis was performed with Fast SYBR Green Master Mix (Thermo Fisher Scientific, Waltham, MA, USA) on a StepOne Plus™ System (Applied Biosystems Inc., Foster City, CA, USA). Values were normalized to β actin (ACTB). Primer sequences are shown below: GHSR1a (forword: 5'-

TCTACCTCAGTGCTGCCATCA-3'; reverse: 5'-TGGGAGAAGGGTTCGAATCC-3'); GHSR1b (forword: 5'-GACCAGAACCACAAGCAAACC-3'; reverse: 5'-AGGATAGGACCCGCGAGAGA-3'); Parkin (forword: 5'-AGGTGGTTGCTAAGCGACA G-3'; reverse: 5'-CTCCACGGTCTCTGCACAAT-3'); ACTB (forword: 5'-GATC AAGATCATTGCTCCTCCT-3'; reverse: 5'- GGGTGTAACGCAACTAAGTCA-3').

Generation of *PARK2* gene knock-in/knock-out (PARK2-KIKO line) by CRISPR-Cas9

I previously generated CRISPR/Cas9-dependent *PARK2*-KIKO line using 201B7 as control iPSCs, to evaluate *parkin* loss of function on DA neurons-derived from iPSCs (Kuzumaki et al., in submission). In brief, a targeting donor DNA plasmid (pUC-5'3'PARK2- PurTK) was used to disrupt exon 2 of *PARK2* gene by homologous recombination. The CSIV-U6-*PARK2* (Ex2)-sgRNA-L&R-EF-Csy4-2A-Cas9 was used as a house-made all-in-one vector. The 201B7 was suspended in Opti-MEM (Thermo Fisher) containing Y-27632, house-made all-in-one vector and targeting donor DNA vector plasmid. Electroporation of plasmid DNA was performed using a NEPA21 electroporator (Nepa Gene Co., Ichikawa, Japan).

Animals

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal

Research of Hoshi University, which is accredited by the Animal Research Committee of Hoshi University. Male C57BL/6J mice (Jackson Laboratory) were used in this study. All mice were housed at up to 6 mice per cage and kept in a temperature-controlled room (24 ± 1°C) maintained on a 12 h light-dark cycle (light on at 8 a.m.). Food and water were available *ad libitum*.

Drugs

[D-Lys-3]-GHRP-6 (Tocris, Bristol, United Kingdom) and morphine hydrochloride (Daiichi-Sankyo Co., Ltd., Tokyo, Japan) were used in this study.

Intracerebroventricular administration

Intracerebroventricular (i.c.v.) administration was performed according to the method described previously [33]. A 2 mm double needle (Natsume Seisakusho) attached to a 25 μl Hamilton microsyringe was inserted into the unilateral injection site using a V-shaped holder to hold the head of the mouse. On the day of the assay, [D-Lys-3]-GHRP-6 (0.3 to 10 nmol/ mouse) was injected into the hole. The injection volume was 4 μl for each mouse.

Cannula implantation into the SNc

Stereotaxic injections were performed under isoflurane (3%) anesthesia and using small-animal stereotaxic instruments (RWD Life Science, Shenzhen, China). Mice were

placed in a stereotaxic apparatus and the skull was exposed. A small hole was then made in the skull using a dental drill. A guide cannula (EIM-54; Eicom Co., Kyoto, Japan) was implanted into the SNc (from bregma: AP -3.0 mm, ML ±1.2 mm, DV -4.3 mm). [D-Lys-3]-GHRP-6 (1 to 5 nmol/side) was microinjected at a rate of 0.25 μl min^{-1} for 4 min. At the end of injection, the injection cannula was kept in the SNc for an additional 2 min before removal and then replaced by a stylet.

Rotarod assay test

Motor coordination was assessed using the rotarod test. Mice were individually placed on a slowly rotating rod (4 rpm/minute), and subjected to continuous acceleration at 20 rpm/minute; the time at which the mouse fell off the rod was recorded. The test was performed 10 min after i.c.v. injection of either saline vehicle or [D-Lys-3]-GHRP-6 (0.3 to 10 nmol/mouse), or 15 min after microinjection of either saline vehicle or [D-Lys-3]-GHRP-6 (1 to 5 nmol/side).

Balance beam test

The apparatus consisted of a 1m-long bar (28 or 11 mm in diameter) with a black escape box on one end (O'HARA & Co., LTD., Tokyo, Japan). Mice were acclimated to enter the escape box on the 28 mm-diameter bar for 2 days before testing. The latency to reach the box on the 11 mm-diameter bar was measured (cut off time = 60s). The test was performed

10 min after microinjection of either saline vehicle or [D-Lys-3]-GHRP-6 (1 to 5 nmol/side).

Locomotor assay

After 30 min of habituation to the apparatus, the locomotor activity of mice was measured by a Three-point Meter (O'HARA & Co., LTD). With this device, the position of the mouse is detected when the infrared beams positioned along the X and Y axes around the cage are interrupted. This device detects the movement of the whole body of the target animal, without being misled by the movement of the tail or any other part of the mouse. Counts of hyperlocomotor activity were obtained at 1-min intervals for 120 min after the injection of morphine hydrochloride (Daiichi-Sankyo Co., Ltd., Tokyo, Japan).

Statistics

The data are presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed by an unpaired t-test or one-way analysis of variance (ANOVA) test followed by the Bonferroni's multiple comparison test. All statistical analyses were performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A p value of < 0.05 was considered to reflect significance.

Results

Differentiation of *PARK2*-specific iPSCs into DA neurons

Since PD is defined pathologically by the progressive degeneration of DA neurons, I modified the method used to generate a DA neuron-enriched culture by treating iPSC-derived cells with several small molecules that lead to the formation of ventral midbrain cells, including DA neurons (Fig. 1-1a). Most of the differentiated cells derived from control and *PARK2*-specific iPSCs were labeled by antibodies to βⅢ-tubulin (a neuron-specific marker) and TH (a dopaminergic neuronal marker) (Fig. 1-1b). There was no significant difference in the ratio of TH-positive DA neurons between control and *PARK2*-specific iPSCs-derived neurons (Fig. 1-1c). In the previous study, it was demonstrated that this iPSC-based model of PARK2 recapitulated the vulnerability of DA neurons [30, 34].

Decreased expression of GHSR in *PARK2*-specific iPSC-derived DA neurons

I observed a significant decrease in mRNA levels of *GHSR1a* and *GHSR1b* in *PARK2*-specific iPSC-derived DA neurons (Fig. 1-2a-b, **p<0.01, ***p<0.001 vs. control-iPSC derived DA neurons). Furthermore, the protein levels of GHSR were mostly abolished in DA neurons-derived from *PARK2*-specific iPSCs (Fig. 1-2c).

Recapitulation of *GHSR* expression in isogenic *PARK2*-KIKO iPSC-derived DA neurons

I next used isogenic iPSC lines mimicking loss of function of the *PARK2* gene through CRISPR Cas9 technology in the healthy control iPSC line 201B7 (Fig. 1-3a). I found that one of the *PARK2*-KIKO iPSC lines, B7PA21 differentiated to TH-positive neurons (Fig. 1-3b). I confirmed the knock-down of Parkin mRNA in DA neurons-derived from *PARK2*-KIKO isogenic iPSCs (Fig. 1-3c) Under these conditions, I found a significant decrease in the mRNA expression of GHSR1a and GHSR1b in the *PARK2*-KIKO isogenic line, similar to that seen in the familial *PARK2* lines (Fig. 1-3d-e).

Effects of intracerebroventricular injection of the selective GHSR1a antagonist [D-Lys3]-GHRP-6 on motor coordination

To evaluate the *in vivo* effect of the blockade of central GHSR1a, normal mice were subjected to intracerebroventricular (i.c.v.) injection of the selective GHSR1a antagonist [D-Lys3]-GHRP-6 (0.3 to 10 nmol/mouse). The i.c.v. injection of [D-Lys3]-GHRP-6 in mice induced a dose-dependent impairment of motor coordination based on the rotarod performance test (Fig. 1-4a). [D-Lys3]-GHRP-6 given i.c.v. at 0.3 nmol, which alone had no effect on motor coordination, significantly inhibited morphine-induced hyperlocomotion (Fig. 1-4b-c, $*p<0.05$ vs. SAL-MRP5).

Effects of intra-SNc injection of [D-Lys3]-GHRP-6 on motor coordination

To evaluate the *in vivo* effect of the blockade of GHSR1a, a guide cannula was implanted into the SNc for microinjection (Fig. 1-5a). One day after cannula implantation (Fig. 1-5b), microinjection of [D-Lys3]-GHRP-6 (1 to 5 nmol/side) into the SNc of normal mice produced a significant and dose-dependent impairment of motor coordination in the rota-rod test (Fig. 1-5c, $*p<0.05$ vs. saline). In the balance beam test to further evaluate catalepsy behaviors, microinjection of [D-Lys3]-GHRP-6 into the SNc significantly increased the latency to cross the beam and increased the number of mice that fell from the beam (Fig. 1-5d-e, $***p<0.001$ vs. saline). These results suggest that the blockade of GHSR activation on DA neurons of the SNc induced motor dysfunction.

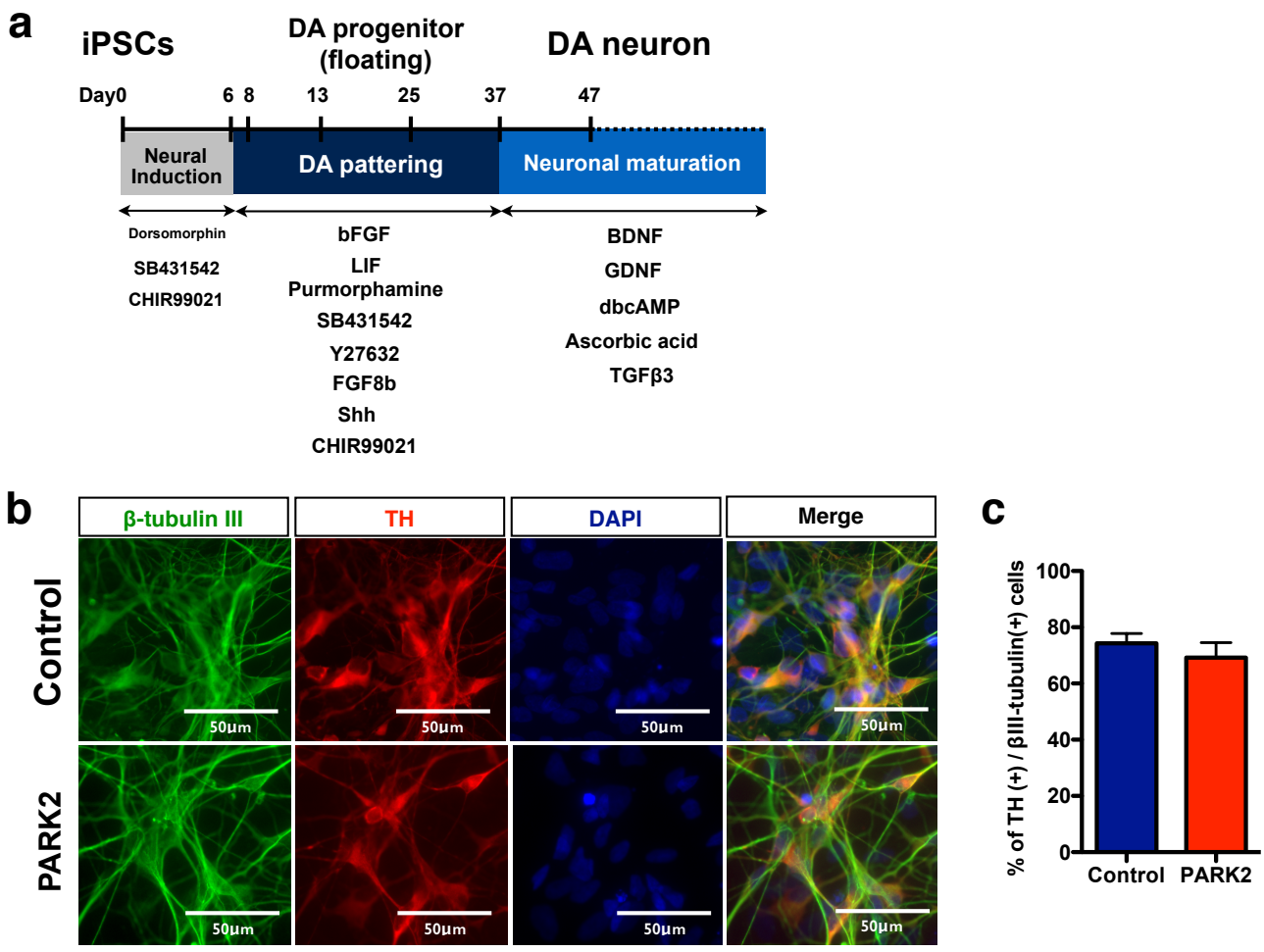


Figure 1-1 Ghrelin receptor (GHSR) expression in dopaminergic neurons derived from control and *PARK2*-specific iPSCs. (a) Schematic of the induction of a DA-enriched culture protocol. (b) Double-labeling for the dopaminergic neuron marker tyrosine hydroxylase (TH, red) and neurons (βIII-tubulin, green) of control and *PARK2*-specific iPSC-derived dopaminergic neurons. Scale bar = 50 μm. (c) Quantitative data of the percentage of TH positive cells per βIII-tubulin positive cells.

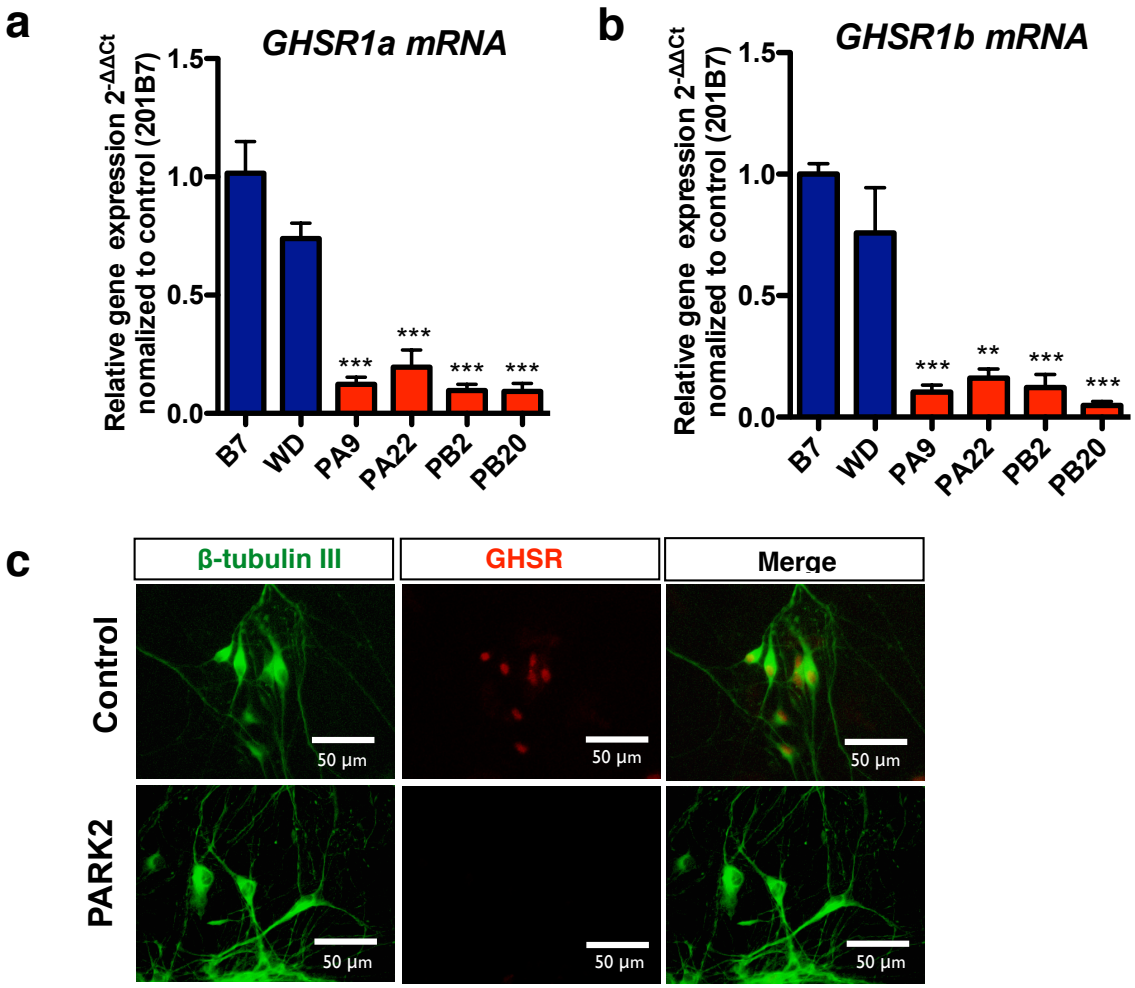


Figure 1-2 Ghrelin receptor (GHSR) expression in dopaminergic neurons derived from control and *PARK2*-specific iPSCs. (a-b) mRNA expression of GHSR1a (a) and GHSR1b (b) between control (B7 and WD) and Parkinson's disease-specific iPS cells (PA9, PA22, PB2 and PB20) derived-dopaminergic neurons. **$p<0.01$, ***$p<0.001$ vs. control iPS cell-derived dopaminergic neurons. (c) Immunocytochemical analysis for TH (green) and ghrelin receptor (red) in control and *PARK2* iPS cell-derived dopaminergic neurons.

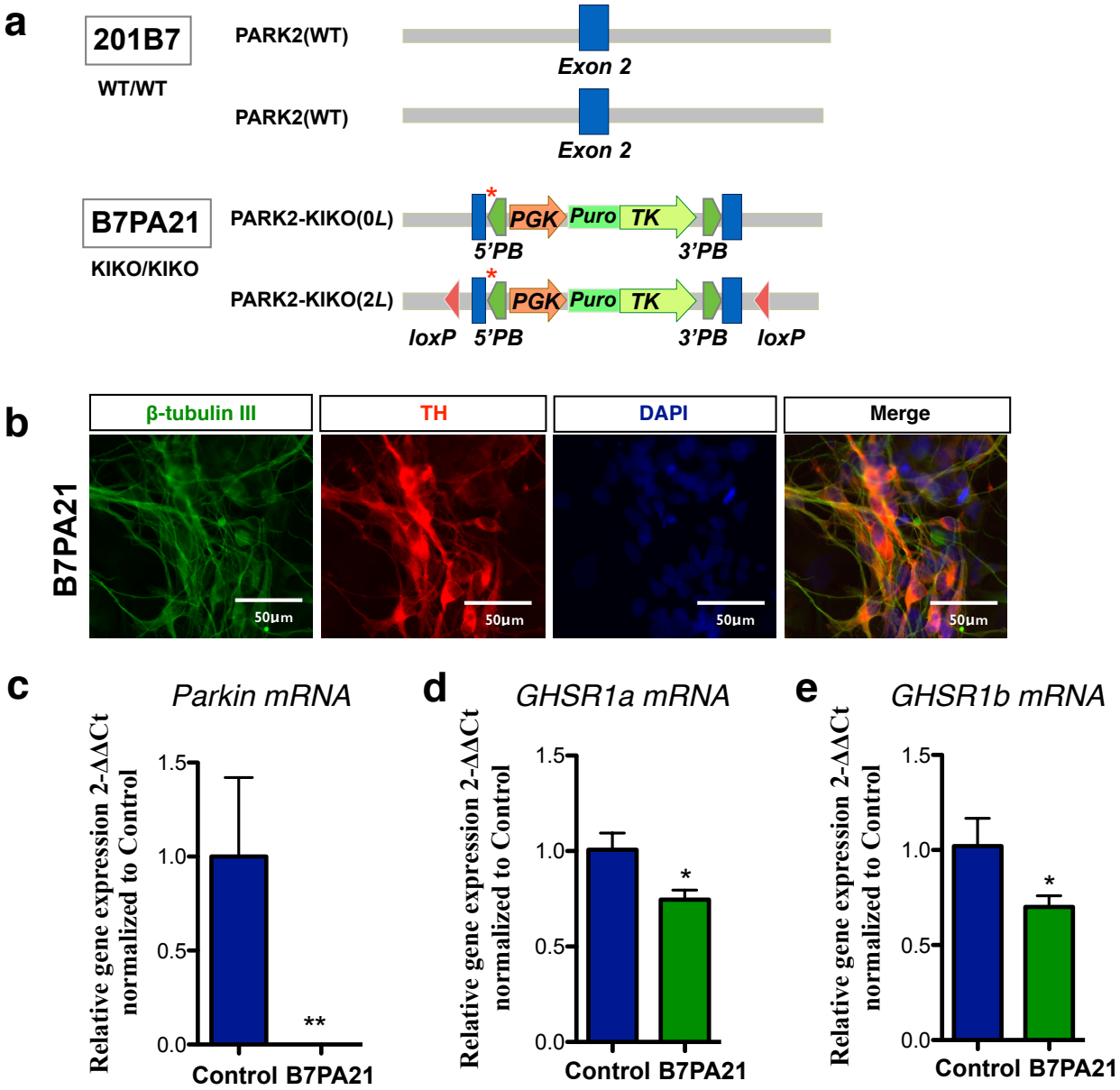


Figure 1-3 Decreased expression of GHSR in isogenic *PARK2*-KIKO iPSC-derived DA neurons.
(a) Generation of isogenic *PARK2*-KIKO iPSCs. Schematic illustration of the gene-editing strategy for knock-in of the stop codon and the puromycin resistance gene into control iPSCs (201B7). (b) Double-labeling for the DA neuron marker tyrosine hydroxylase (TH, red) and the neuronal marker β-tubulin III (TUJ1, green) of control and *PARK2*-KIKO iPSC-derived dopaminergic neurons. (c) Expression level of Parkin mRNA in differentiated DA neurons derived from the control and *PARK2*-KIKO iPSC groups. $^{**}p<0.01$ vs. control. (d-e) The expression levels of GHSR1a (d) and GHSR1b (e) in differentiated dopaminergic neurons derived from the control and *PARK2*-KIKO iPSC groups. $^{*}p<0.05$ vs. control.

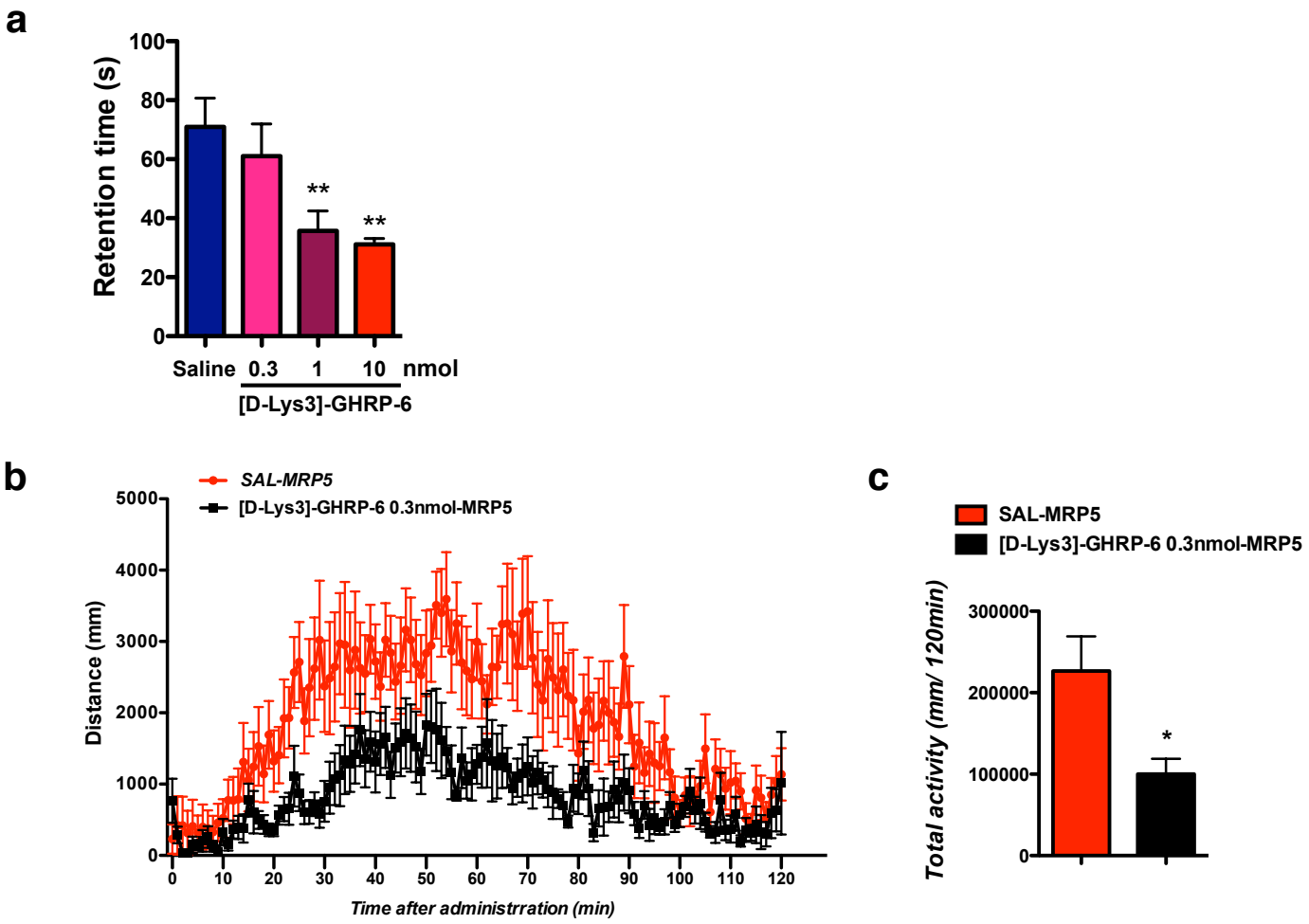


Figure 1-4 Effects of intracerebroventricular injection of the selective GHSR1a antagonist [D-Lys3]-GHRP-6 on motor coordination.

(a) Average latency to fall in the rotarod test. $^{**}p<0.01$ vs. SAL. (b) Time-course change in the locomotor-enhancing effect of morphine (5mg/kg, s.c.) after treatment with [D-Lys3]-GHRP-6 at 0.3 nmol (n=7) or saline (n=8). Each point represents the mean activity distance for 1 min with SEM. (c) Total locomotor activity induced by morphine (5mg/kg, s.c.) after treatment with [D-Lys3]-GHRP-6 at 0.3 nmol (n=7) or saline (n=8). Each column represents the mean total activity distance for 120 min with SEM. $^{*}p<0.05$ vs. SAL-MRP5.

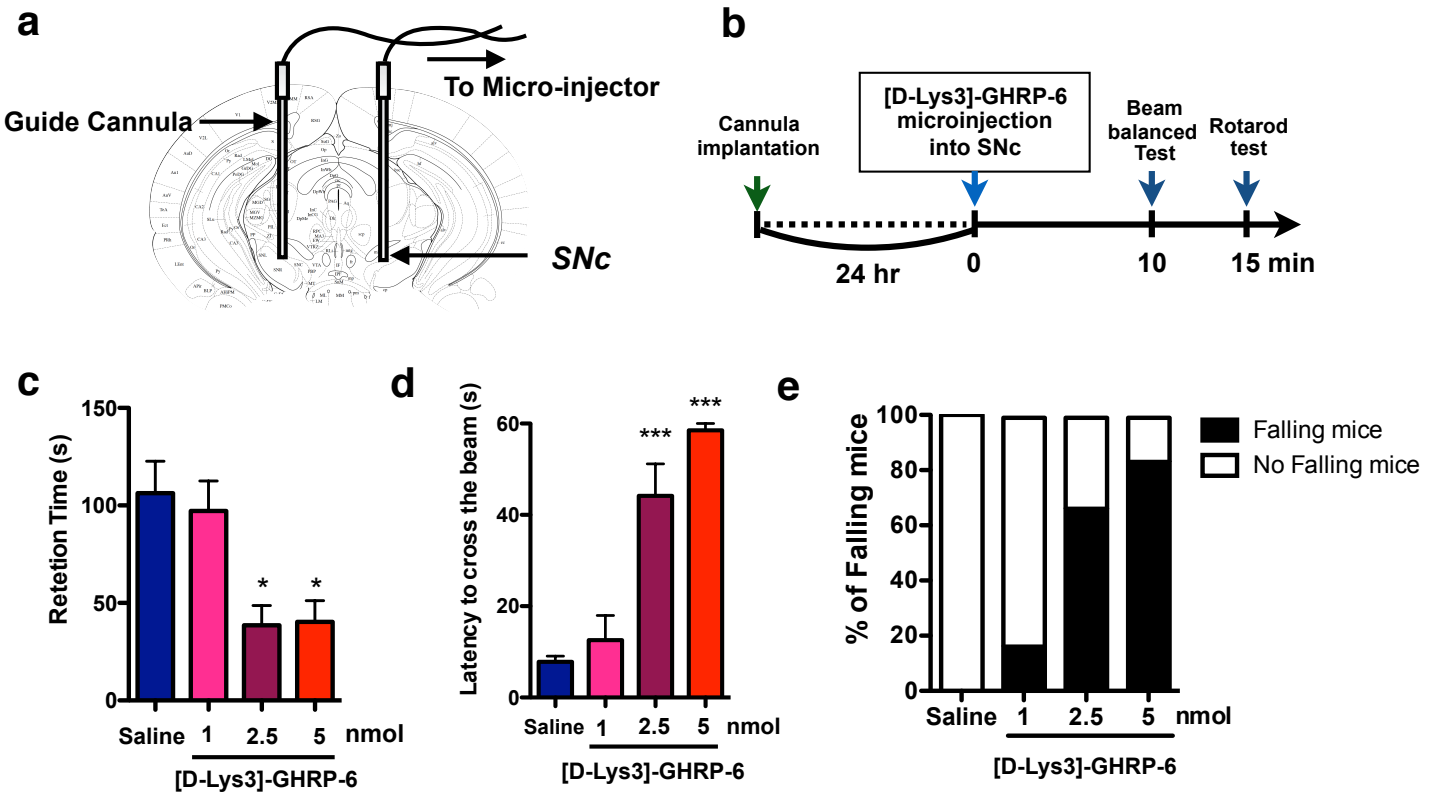


Figure 1-5 Effects of intra-SNc injection of [D-Lys3]-GHRP-6 on motor coordination.
(a) Microinjection sites of [D-Lys3]-GHRP-6 in the SNc. Plates show coronal sections of the mouse brain. (b) Schedule for the experiment. (c) Accelerated rotarod test (4-20rpm). The line graph shows the average latency to fall in the rotarod test for 15min after bilateral microinjection of [D-Lys3]-GHRP-6 (1, 2.5 or 5 nmol / each site) or saline (n=6/group) into the SNc. $^{*}p<0.05$ vs. Saline. (d-e) The balance beam test was performed 10min after the microinjection of [D-Lys3]-GHRP-6 (1, 2.5 or 5 nmol / each site) or saline (n=6/group) into the SNc. $^{***}p<0.001$ vs. Saline.

Discussion

The identification of cell biological or biochemical changes in the initial stages of PD, before the onset of symptoms, has been difficult through the use of analyses conducted on postmortem brains. With the recent development of iPS cell technologies, it has become possible to establish pluripotent stem cells from the somatic cells of anyone, regardless of race, genetic background, or the presence of disease symptoms. I have established iPS cells from cutaneous fibroblasts obtained from patients with the PARK2 form of familial PD (Patient A: female with an exon 2-4 deletion mutation; Patient B: male with an exon 6-7 deletion mutation) by performing retroviral gene transduction (*Oct4*, *Sox2, Klf4*, and *c-Myc*) [6, 35]. In the previous study, I demonstrated that this iPSC-based model of PARK2 recapitulated the vulnerability of DA neurons with a significant increase in the ROS production [30, 34]. In my preliminary DNA microarray study, a dramatic decrease in mRNA levels of *GHSR1a* and *GHSR1b* was found in *PARK2*-specific iPSC-derived DA neurons (unpublished observation). In the present q-PCR assay, I confirmed that a significant decrease in both mRNAs was detected compared to a control in *PARK2*-specific iPSC-derived DA neurons. Furthermore, the level of GHSR protein was clearly down-regulated in DA neurons in *PARK2*-specific iPSC-derived DA neurons. To evaluate whether *PARK2* mutation is sufficient to cause the down-regulation of GHSR, I generated isogenic iPSC lines mimicking loss of function of the *PARK2* gene in a healthy control iPSC line. Under these conditions, I consistently found the

significant, but not dramatic, decrease in the mRNA expression of both GHSR1a and GHSR1b in the *PARK2*-KIKO isogenic line. Although further analyses will be required to identify how PARK2 mutation could affect the expression of GHSR1a, PARK2 mutation may lead to the possible changes in the transfer of ubiquitin onto substrate proteins, which could affect the transcriptional level of GHSR1a. I thus hypothesized that although the deletion of *PARK2* gene would, at least in part, contribute to the decrease in the mRNA expression of both GHSR1a and GHSR1b, *PARK2*-specific iPSC-derived DA neurons from patients could be influenced by another genomic mutant factors to induce the dramatic knockdown of both mRNAs.

GHSR1a is the only functional ghrelin receptor that has been characterized to date. It is a G protein-coupled 7-transmembrane receptor that was first cloned from the pituitary and hypothalamus [13]. It has been reported that GHSR1a is localized in dopaminergic neurons of the SNc [15]. Higher numbers of TH and GHSR co-expressing neurons have been identified within the SNc [15]. Ghrelin has been shown to have neuroprotective effects in numerous animal models of neurological disorders, including PD. Studies using the mitochondrial toxin MPTP, which selectively kills DAnergic neurons in the SNc, have shown that i.p. injection of ghrelin restricts dopamine cell loss in the SNc and the loss of dopamine in the striatum in mice [17, 36, 37]. Ghrelin activates SNc DAnergic neurons, increases the expression of tyrosine hydroxylase (which is involved in the biosynthesis of dopamine) in the midbrain, and increases dopamine turnover in the dorsal striatum [17]. In the present study, I investigated

whether the direct inhibition of GHSR function in the brain including the SNc could affect motor coordination. Either i.c.v. or intra-SNc injection of the selective GHSR1a antagonist [D-Lys3]-GHRP-6 in normal mice induced dose-dependent cataleptic behaviors related to the dysfunction of motor coordination. Furthermore, [D-Lys3]-GHRP-6, given i.c.v. at a dose which alone had no effect on motor coordination, caused a significant inhibition of the DA-related hyperlocomotion induced by the systemic administration of morphine. These findings suggest that deficits in GHSR activity in SNc-dopamine neurons could cause marked motor impairment. Inconsistently, it has been reported that $GHSR^{-/-}$ mice did not show reduced performance in the rotarod test [38]. One reason for this discrepancy may be the possibility of significant compensation in the dopaminergic system when genes are deleted or overexpressed in the germline and during development of the dopaminergic system. In fact, germline deletion of parkin fails to lead to the loss of dopaminergic neurons, whereas adult conditional knockout of parkin leads to a progressive loss of DA neurons [39, 40]. Thus, it is likely that a conditional knockout technique in adults will be required to evaluate DA-related behaviors under PD.

In conclusion, I found that the expression level of GHSR was dramatically decreased in DA neurons under PD using disease-specific iPSCs. Furthermore, treatment by the injection of a selective GHSR1a inhibitor into the SNc of normal mice induced PD-like behaviors. Taken together, these results indicate that the down-regulation of GHSR in DA neurons may

correspond to the initial dysfunction of DA neurons, leading to extrapyramidal disorder under PD.

Chapter 2

Effect of ghrelin on the motor deficit caused by the ablation of nigrostriatal dopaminergic cells or the inhibition of striatal dopamine receptors

Introduction

Ghrelin is synthesized and acylated in the stomach by ghrelin O-acyltransferase (GOAT), which is required for activation of the growth hormone secretagogue receptor (GHSR) [12, 41]. The GHSR is located in both the central nervous system and the periphery [17, 24, 25, 28]. Central GHSRs play roles in a wide range of physiological functions including food intake, rewarding effect and memory performance [24-26, 42]. In particular, in the substantia nigra pars compacta (SNc), ghrelin could activate dopamine (DA) neurons and increase the DA concentration in the striatum via the specific blockade of KCNQ channel function [16]. Furthermore, ghrelin is considered to act on SNc neurons to increase the concentration of tyrosine hydroxylase (TH) in the midbrain as well as DA turnover in the dorsal striatum [17, 43]. In addition, it has been reported that ghrelin provides neuroprotective effects in DA neurons [17, 43, 44].

Progressive degeneration of DA neurons in the SNc and the resultant depletion of DA from the striatum underlie the motor behavior deficits observed in Parkinson's disease (PD) patients and in animals that have sustained damage to the nigrostriatal pathway [45]. In my present studies, I found that the expression level of endogenous ghrelin receptors was dramatically decreased in DA neuron-differentiated from PD-specific iPSCs (Chapter 1). Furthermore, the inhibition of GHSRs in DA neurons induced the initial dysfunction of DA neurons, leading to extrapyramidal disorder under PD (Chapter 1).

In this study, I therefore examined whether phasic activation of the SN-ghrelin system could improve the motor deficits. For this purpose, I performed the stereotaxic injection of AAV-CMV-FLEX-diphtheria toxin A (DTA) into the SN of dopamine transporter (DAT)-Cre (DAT^{SN}::DTA) mice using a Flex-switch system to expunge DA neurons of the SNc. Furthermore, I used the mice treated with a classic D_2 receptor antagonist haloperidol as an initial PD model with the temporal dysregulation of DA transmission. I investigated the effect of phasic activation of the endogenous ghrelin system in the SN by microinjection of ghrelin into the SN on the recovery from the motor deficits under the conditions with the ablation of the nigrostriatal DA network using DAT^{SN}::DTA mice and the haloperidol-induced transient reduction in DA transmission.

Materials and methods

Animals

The present study was conducted in accordance with the Guiding Principles for the Care and Use of Laboratory Animals, Hoshi University, as adopted by the Committee on Animal Research of Hoshi University, which is accredited by the Animal Research Committee of Hoshi University. Male B6. SJL-*Slc6a3*$^{tm1.1(cre)Bkmm}$*/J* (DAT-Cre) mice (8-10 weeks old, Jackson Laboratory, Bar Harbor, ME, USA) and C57BL/6J mice (8-10 weeks old, Jackson Laboratory), weighing 20 to 23 g were used in this study. All mice were housed at up to 6 mice per cage and kept in a temperature-controlled room (24 ± 1°C) maintained on a 12 h light-dark cycle (light on at 8 a.m.). Food and water were available *ad libitum*.

Drugs

Ghrelin (Peptide Institute, Inc., Osaka, Japan) and haloperidol (Serenace, Dainippon Pharmaceutical. Co., Ltd) were used in this study.

Stereotaxic intranigral virus injection

Stereotaxic injections were performed under isoflurane (3%) anesthesia using small-animal stereotaxic instruments (RWD Life Science, San Diego, CA, USA). Virus

(AAV-CMV-FLEX-DTA) was bilaterally injected into the SN (from bregma: AP -3.0 mm, ML ±1.2 mm, DV -4.3 mm), as previously described [39] at a rate of 0.25 μL/min for 4 min.

Cannula implantation into the SN and microinjection

For cannula implantation into the SN of DAT^{SN}::DTA or C57BL/6J mice for ghrelin treatment, mice were placed in a stereotaxic apparatus and the skull was exposed the day before bilateral microinjection. A small hole was then made in the skull using a dental drill. A guide cannula (EIM-54; Eicom Co., Kyoto, Japan) was implanted into both sides of the SN (from bregma: AP -3.0 mm, ML ±1.2 mm, DV -4.3 mm). The bilateral injection was performed with two sets of a glass micropipette and an air pressure injector system (Micro-syringe Pump-Model ESP-32; Eicom Co.) at a rate of 0.25 μL/min for 4 min and was started at the same time.

Balance beam test

The apparatus consisted of a 1m-long bar (28 or 11 mm in diameter) with a black escape box on one end (O'HARA & Co., LTD., Tokyo, Japan). Following habituation trials, the mice were acclimated to enter the escape box on the 28mm-diameter bar for 2 days before testing. The latency to reach the box on the 11mm-diameter bar was then measured (cut off time = 60s). The test was performed 2-10 days after the injection of AAV-CMV-FLEX-DTA in DA neurons of the SN.

Rotarod test

Ten days after bilateral virus (AAV-CMV-FLEX-DTA) injection into the SN of DAT-Cre mice, rotarod test (4 rpm for 120 sec maximum) was performed. The test was performed 30 min after bilateral intra-SN injection of ghrelin (1 nmol/site) in DAT^{SN}::DTA mice. In another test condition, mice treated with haloperidol were individually placed on a slowly rotating rod (4 rpm/min), and subjected to continuous acceleration at 20 rpm/min; the time at which the mouse fell off the rod was recorded (for 128 sec maximum). Mice were treated with haloperidol (0.5 mg/kg, s.c.) or saline given 30 min before ghrelin treatment. The test was performed 30 min after bilateral intra-SN injection of either saline vehicle or ghrelin (1 nmol/site, 0.25 μl min^{-1} for 4 min) using the auto-injector connected with guide cannula. Time spent on the rod was measured.

Horizontal Bar Test for the Evaluation of Catalepsy

Catalepsy was evaluated using the horizontal bar test as described previously [46]. Briefly, animals were placed so that both forepaws were over a horizontal bar 5 cm above the floor, and the amount of time (s) the animal maintained this position was recorded for up to 60 s. Catalepsy was considered to have finished when a forepaw touched the floor or when the mouse climbed on the bar. Mice were treated with haloperidol (0.5 mg/kg, s.c.) or saline given 30 min before ghrelin treatment. The test was performed 30 min after bilateral intra-SN

injection of either saline vehicle or ghrelin (1 nmol/site, 0.25 μl min^{-1} for 4 min) using guide cannula.

Immunohistochemistry

Mice were deeply anaesthetized with 3% isoflurane and intracardially perfusion-fixed with freshly prepared 4% paraformaldehyde. Coronal brain sections (8 μm) were incubated in blocking solution for 1 h at room temperature, and then incubated for 48 h at 4°C with primary antibodies; anti-TH (ImmunoStar, WI, USA) or anti-DAT (Millipore, MA, USA). The antibody was then rinsed and incubated with an appropriate secondary antibody for 2 h at room temperature. Fluorescence of immunolabelling was detected using a light microscope (BX-61; Olympus, Tokyo, Japan) and digitized images (Metamorph 7.8 software, Molecular Devices).

***In situ* hybridization**

The *in situ* hybridization of brain sections was performed as described previously [47]. The riboprobes of mouse GHSR1a and TH were designed according to the Allen Brain Atlas database (RP_060220_03_A02 and RP_Baylor_103073), and riboprobe of mouse GAD67 (provided by A. Watakabe ([RIKEN Brain Science Institute, Saitama, Japan]) was prepared from the nucleotide positions 43-641. Digoxigenin (DIG)-, fluorescein (FITC)-, and biotin-labeled riboprobes were synthesized by *in vitro* transcription. C57BL/6J mice (Japan

SLC, Inc., Hamamatsu, Japan) were anesthetized and perfused with 4% paraformaldehyde. The sections (30 μm) were treated with proteinase K (2 μg/ml), acetylated, prehybridized in hybridization buffer (50% formamide, 5 × saline sodium citrate buffer [SSC], 0.01% sodium dodecyl sulfate, 0.1% N-lauroylsarcosine [NLS], and 2% blocking reagent [Roche Diagnostics, Mannheim, Germany]), and then hybridized overnight at 65 °C in hybridization buffer containing DIG-, FITC-, and biotin-labeled riboprobes (0.5 μg/ml). The sections were washed in 2 × SSC, 50% formamide, and 0.1% NLS at 65 °C, and treated with 20 μg/ml RNase A at 37 °C. After blocking, the sections were incubated with an anti-FITC antibody conjugated with horse radish peroxidase (HRP; 1:4,000; Roche Diagnostics) followed by TSA Plus DNP kit reagents (PerkinElmer, MA, USA). After quenching of FITC-HRP activity, the sections were incubated with a streptavidin conjugated with HRP (1:2,000; Roche Diagnostics). After washing, the sections were incubated with TSA Plus Biotin kit reagents (PerkinElmer), followed by anti-DNP antibody conjugated with Alexa 488 (1:500; Molecular Probes, OR, USA), anti-DIG antibody conjugated with alkaline phosphatase (1:1,000; Roche Diagnostics), and anti-streptavidin antibody conjugated with Alexa 647 (1:1,000; Molecular Probes), and processed for HNPP/Fast Red (Roche Diagnostics) reaction. The sections were counterstained with DAPI, mounted, and then coverslipped with CC/Mount (Diagnostic BioSystems, CA, USA).

qRT-PCR

Total RNA obtained from the mouse SN was extracted and 0.5 μg of purified RNA was then reverse-transcribed into cDNA. qPCR was performed using *an* Applied Biosystems StepOne Plus Real-Time PCR system (Applied Biosystems Inc., Foster City, CA, USA) with a *Fast SYBR® Green Master Mix* (Applied Biosystems Inc.). The amount of target RNA was determined from the appropriate standard curve and normalized relative to the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Primer sequences are shown below: DAT (forword: 5'-CTTCTCCTCCGGCTTCGTCGT-3'; reverse: 5'-CCGGGTAGATGATGAAGATCAGCC-3') ; TH (forword: 5'-TTCGAGGAGAGGGA TGGAAA-3'; reverse: 5'-GGTGGATTTT GGCTTCAAATG-3') ; GAD67 (forword: 5'-CAAGTTCTGGCTGATGTGGA-3'; reverse: 5'-GCCACCCTGTGTAGCTTTTC-3') ; MOR (forword: 5'-GTGTGTAGTGGGCCT CTTTGG-3'; reverse: 5'-TGCCAGAGC AAGGTTGAAAA-3') ; GHSR1a (forword: 5'-GGACCAGAACCACAAACAGACA-3'; reverse: 5'-CAGCAGAGGATGAAAGCAAA CA-3') ; GAPDH (forword: 5'-CATGG CCTTCCGTGTTCCTA-3'; reverse: 5'-GATGCCT GCTTCACCACCTT-3').

Statistics

The data are presented as the mean ± S.E.M. The statistical significance of differences between groups was assessed by an unpaired *t*-test or one-way analysis of variance (ANOVA) test followed by Bonferroni's multiple comparison test. All statistical analyses were

performed with GraphPad Prism (GraphPad Software, La Jolla, CA, USA). A p value of < 0.05 was considered to reflect significance.

Results

Localization of GHSR1a mRNA in the SN

I examined the co-expression of GHSR1a with TH in the SN of control mice following *in situ* hybridization. In the SN, the SNc in a cell-rich zone that comprises densely aggregated pigmented neurons along the dorsal part of the structure, while neurons of the substantia nigra pars reticulata (SNr) are less abundant, more diffusely distributed along the ventral part of the structure and do not contain pigmentation. Control experiments to confirm the specificity of this protocol involved hybridization with sense probes (Fig. 2-1). I demonstrated a high degree of GHSR1a mRNA expression within dopaminergic neurons in the SNc (Fig. 2-1).

Selective ablation of nigrostriatal DA neurons by DTA leads to motor deficits

To generate mice with the conditional expression of DTA in DA neurons, Cre-dependent AAV vectors that selectively induce DTA expression only in cells expressing Cre recombinase (AAV-CMV-FLEX-DTA) were used in the present study. DTA catalyzes the ADP-ribosylation of eukaryotic elongation factor 2, and causes cell death by inhibiting protein synthesis. DAT-Cre mice were stereotaxically injected with AAV-CMV-FLEX-DTA (DAT^{SN}::DTA mice, Fig. 2-2a,b). In these DAT^{SN}::DTA mice, I evaluated motor coordination following several behavioral tests. In the balance beam test to

evaluate limb coordination and balance, 9 days after intra-SN injection of AAV-CMV-FLEX-DTA virus, DAT^{SN}::DTA mice exhibited a significant increase in the time required to cross the beam compared to WT mice that had been microinjected with AAV-CMV-FLEX-DTA into the SN (Fig. 2-2c, $^{**}p<0.01$, $^{***}p<0.001$ vs. WT^{SN}::DTA). Fig. 2-2d and 2-2e show a significant decrease in the rotarod retention time and a significant increase in catalepsy time in DAT^{SN}::DTA mice at 10 days after AAV injection ($^{***}p<0.001$ vs. WT^{SN}::DTA). Under these conditions, mRNA levels of TH and DAT were greatly reduced in the SN of DAT^{SN}::DTA mice (Fig. 2-2f,g, $^{***}p<0.001$ vs. WT^{SN}::DTA). Furthermore, immunohistochemistry with TH and DAT antibodies showed that there was a dramatic loss of TH-positive DA cells in the SNc and DAT-labeled axon terminals in the striatum of DAT^{SN}::DTA mice (Fig. 2-2h,i).

Changes in mRNA of GHSR1a in the SN by the selective ablation of nigrostriatal DA neurons

I measured the changes in mRNA of GHSR1a in the SN by the selective ablation of nigrostriatal DA neurons. Schematic illustration shows the dissected SN region including both SNc and SNr (Fig. 2-3a). I found that mRNA of GHSR1a were significantly decreased in the SN of DAT^{SN}::DTA mice (Fig. 2-3b, $^{***}p<0.001$ vs. WT mice). In contrast, there were no changes in mRNA levels of glutamic acid decarboxylase 67 (GAD67) and μ-opioid receptors (MOR) in the SN of DAT^{SN}::DTA mice (Fig. 2-3c,d). Interestingly, the

expression of GHSR1a mRNA was found within GAD67-positive GABAergic neurons in the SNr of control mice following *in situ* hybridazation (Fig. 2-3e).

Effect of intra-SN injection of ghrelin on motor function in mice

To evaluate whether ghrelin could affect motor function, DAT^{SN}::DTA mice were bilaterally microinjected with ghrelin (1 nmol/site, Fig. 2-4a). Behavioral tests were performed 10 days after microinjection of DTA into bilateral SN of WT and DAT-Cre mice (Fig. 2-4b). As a result, microinjection of ghrelin into the SN did not improve the rotarod retention time (Fig. 2-4c) and catalepsy time (Fig. 2-4d). Next, I used a mouse model for catalepsy induced by haloperidol, a classic D_2 receptor antagonist. In the rotarod test, subcutaneous administration of haloperidol (0.5 mg/kg) clearly induced motor dysfunction. Thirty min after bilateral microinjection of either saline or ghrelin (1 nmol/site) into the SN (Fig. 2-4e), haloperidol-treated mice showed an improved rotarod retention time (Fig. 2-4f, $^{**}p<0.01$, $^{***}p<0.001$ vs. saline-saline, $\#p<0.05$ vs. haloperidol-saline) and catalepsy time (Fig. 2-4g, $^{***}p<0.001$ vs. saline-saline, $\#\#p<0.01$ vs. haloperidol-saline).

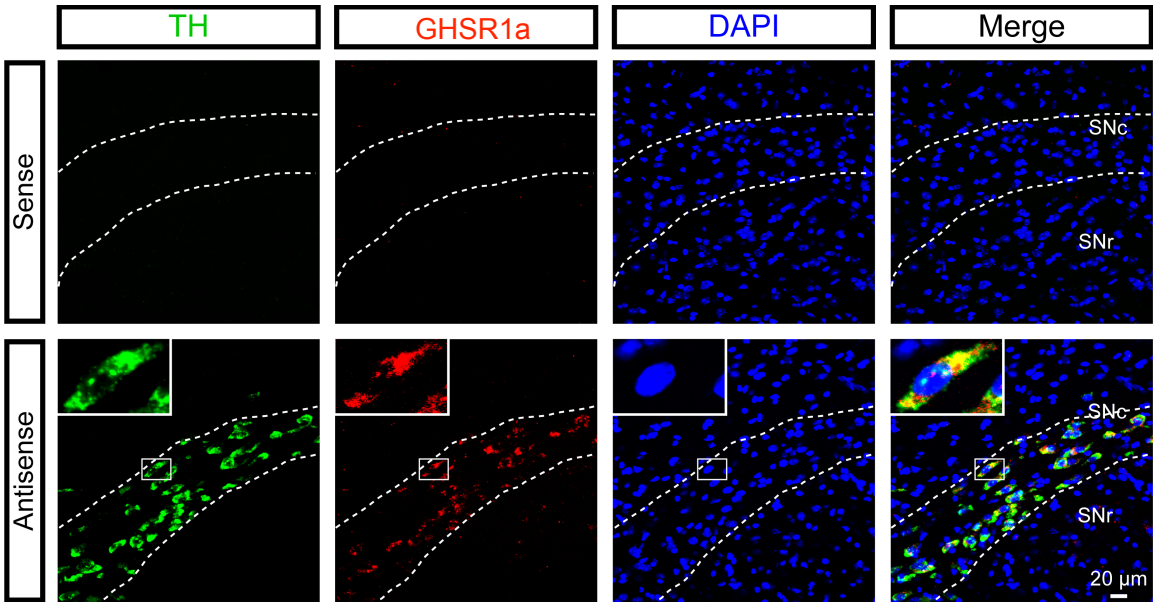


Figure 2-1. Expression of GHSR1a mRNA in the SN of mice.
Coronal sections were hybridized with either the sense (upper panel) or antisense (lower panel) probes of TH (green) and GHSR1a (red). Almost GHSR1a mRNA signals overlap with TH mRNA signals. Nuclei were visualized by counterstaining with DAPI (blue). Each inset shows high magnification image marked by square. Scale bar = 20 μm.

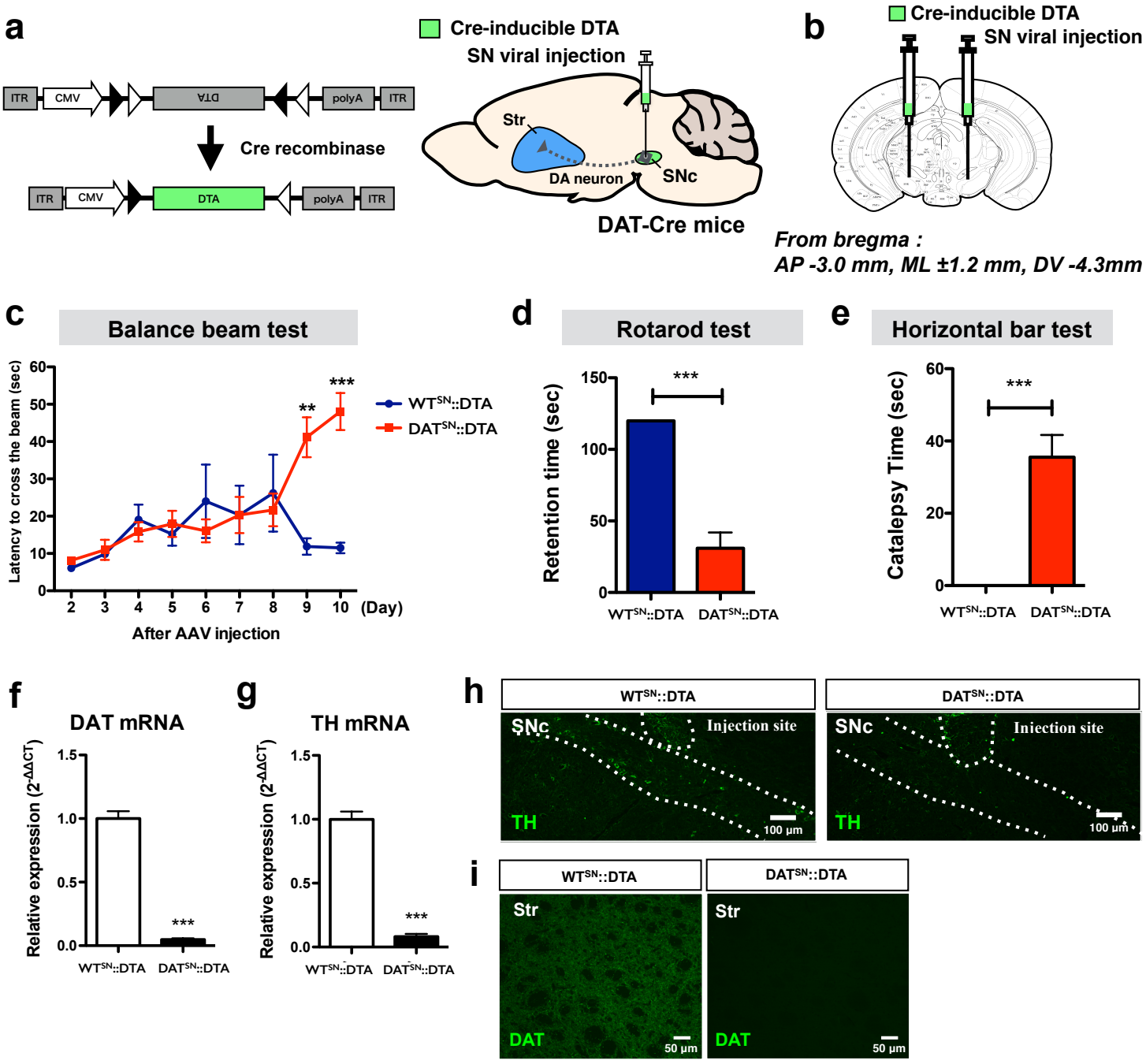


Figure 2-2. Motor deficits induced by selective ablation of nigrostriatal DA neurons.
(a) Schematic representation of double-floxed Cre-dependent AAV vector expressing DTA and stereotaxic intranigral injection of virus. (b) Microinjection site of AAV-CMV-FLEX-DTA in the SN of the mouse brain. (c) Time-course of the latency to cross the beam for WT^{SN}::DTA (n=5) or DAT^{SN}::DTA mice. (d) The average latency to falling in WT^{SN}::DTA and DAT^{SN}::DTA mice. (e) Catalepsy was assessed by measuring the latency until the first movement in seconds in WT^{SN}::DTA and DAT^{SN}::DTA mice. (F-G) Expression levels of mRNA for DAT (f) and TH (g) in the SN of WT^{SN}::DTA and DAT^{SN}::DTA mice. $^{**}p<0.01$,$^{***}p<0.001$ vs. WT^{SN}::DTA mice. (h) Immunoreactivity for TH in the SNc in WT^{SN}::DTA and DAT^{SN}::DTA mice. Scale bar = 100 μm. (i) Immunoreactivity for DAT in the striatum in WT^{SN}::DTA and DAT^{SN}::DTA mice. Scale bar = 50 μm.

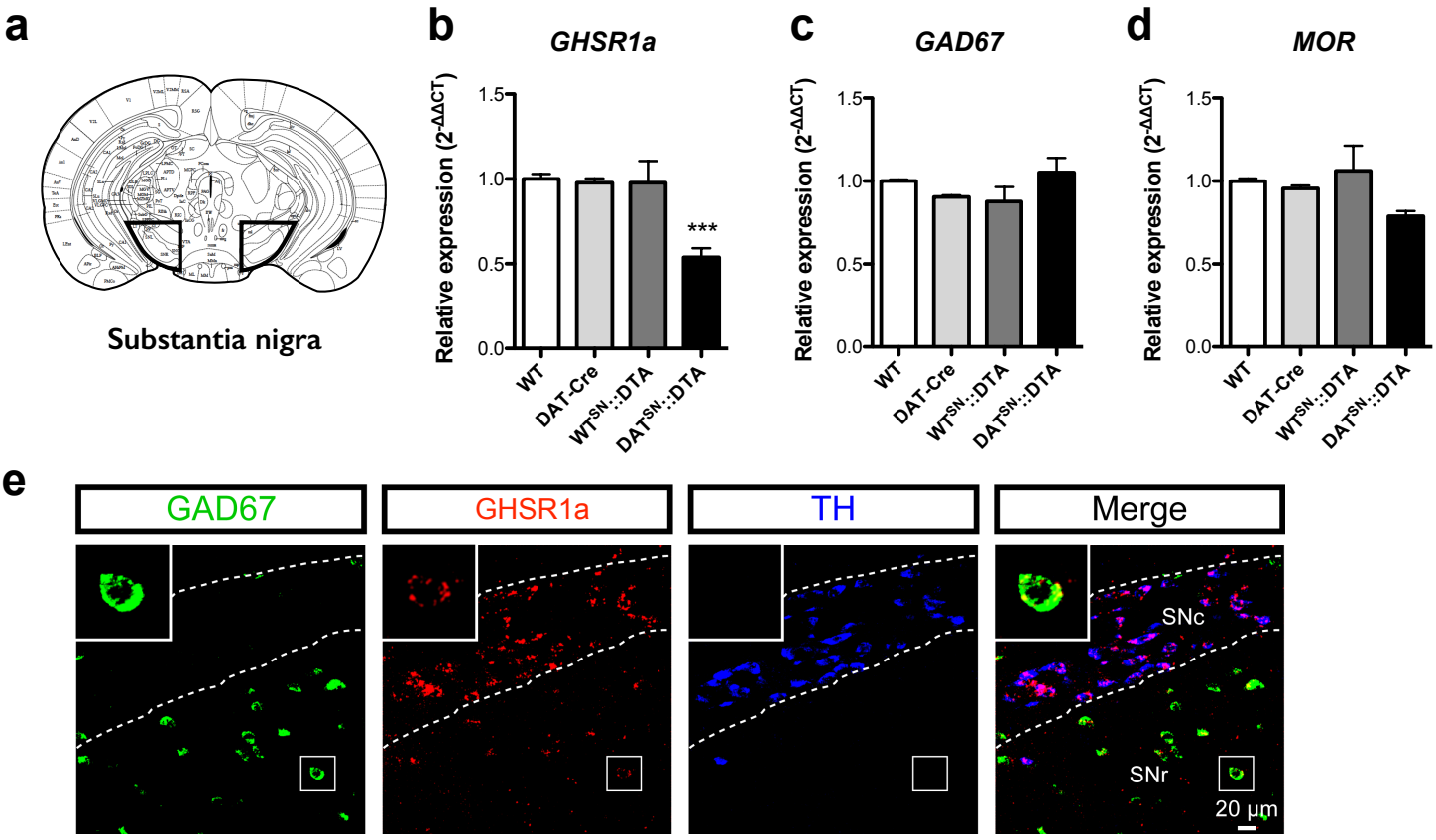


Figure 2-3. Changes in GHSR1a mRNA in the SN by the selective ablation of nigrostriatal DA neurons.
(a) Schematic illustration of the dissected site for the present study. The black frames indicate the dissected substantia nigra region. (B-D) Expression levels of mRNAs for GHSR1a (b), GAD 67 (c) and MOR (d) in the SN of WT, DAT-Cre, WT^{SN}::DTA and DAT^{SN}::DTA mice. $^{***}p<0.001$ vs. WT mice. (e) Representative images of triple *in situ* hybridization of GAD67 (green), GHSR1a (red), and TH (blue) mRNAs. GHSR1a mRNA is expressed in a subset of GAD67-expressed neurons. Each inset shows high magnification image marked by square. Scale bar = 20 μm.

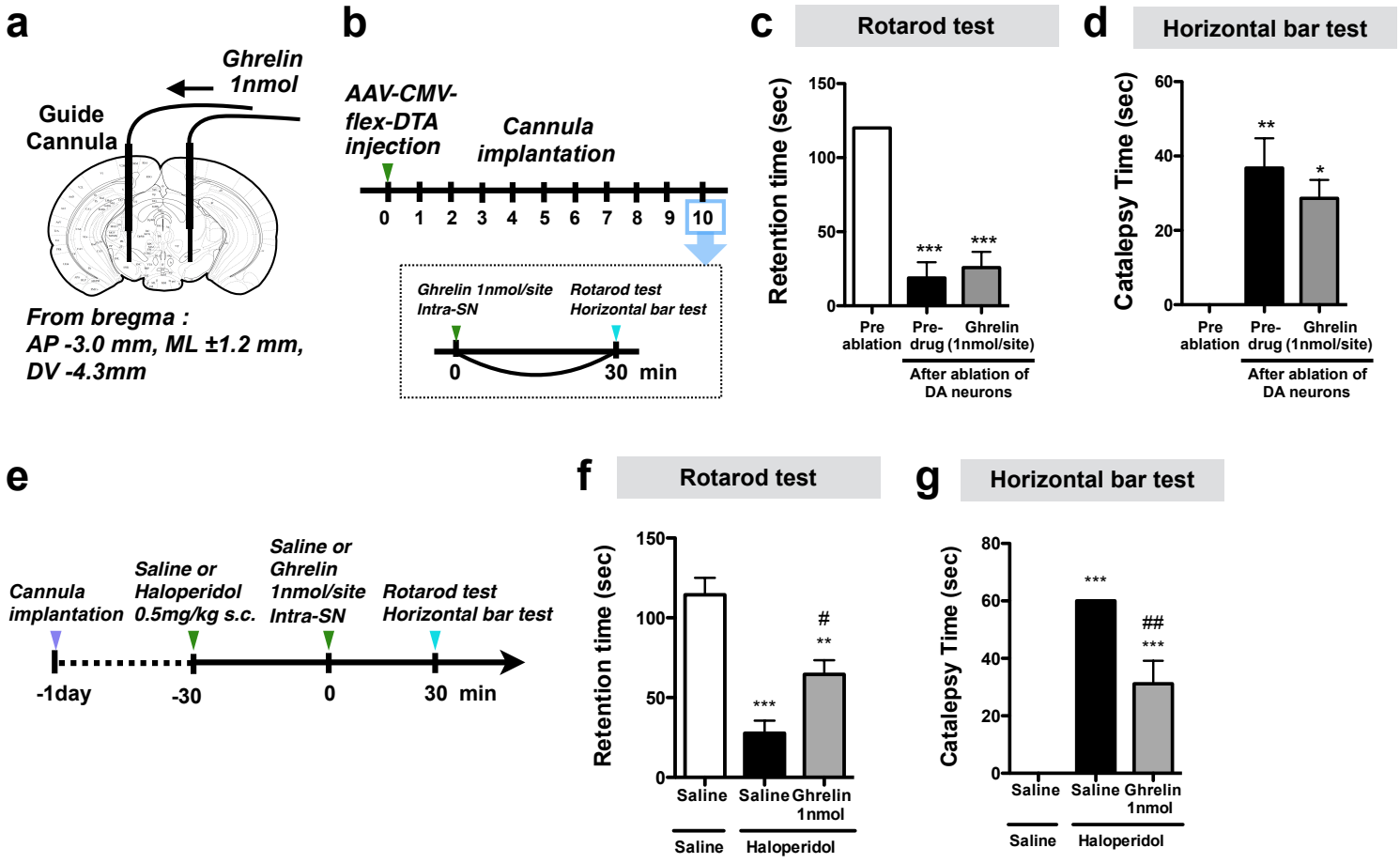


Figure 2-4. Effect of ghrelin on motor coordination.
(a) Microinjection site of ghrelin in the SN. (b) Schedule for the experiment in a model induced by the selective ablation of nigrostriatal DA neurons. (c) Effects of ghrelin (1 nmol/site) on performance in the rotarod test in DAT^{SN}::DTA mice. The bar graph shows the average latency to falling. $^{***}p<0.001$ vs. Pre-ablation. (d) Effects of ghrelin (1 nmol/site) on catalepsy in DAT^{SN}::DTA mice. The bar graph shows the average catalepsy time. $^{*}p<0.05$, $^{**}p<0.01$ vs. Pre-ablation. (e) Schedule for the experiment in a haloperidol-induced PD model. (f) Effects of saline or ghrelin (1 nmol/site) on performance in the rotarod test in haloperidol (0.5 mg/kg, s.c.)-treated mice. The bar graph shows the average latency to falling after the injection of ghrelin. $^{**}p<0.01$ and $^{***}p<0.001$ vs. saline-saline. $^{\#}p<0.05$ vs. haloperidol-saline. (g) Effects of saline or ghrelin (1 nmol/site) on catalepsy in haloperidol (0.5mg/kg, s.c.)-treated mice. The bar graph shows the average catalepsy time. $^{***}p<0.001$ vs. saline-saline. $^{\#\#}p<0.01$ vs. haloperidol-saline.

Discussion

The hypothalamic effects of ghrelin are well recognized, and include appetite regulation, glucose homeostasis and energy metabolism, growth hormone (GH) release and body-weight regulation [48]. Ghrelin is also known to affect many brain regions other than the hypothalamus [24, 25, 42]. On the other hand, GHSR1a is the only functional ghrelin receptor; it is a G protein-coupled 7-transmembrane receptor that was first cloned from the pituitary and hypothalamus [13]. GHSR1a mRNA is expressed at the highest levels in the hypothalamus and pituitary, which is consistent with the known role of ghrelin in GH release and the regulation of body weight and metabolism [14, 48]. High expression levels are also found in the dentate gyrus of the hippocampus, the SN, the ventral tegmental area, and the dorsal raphe nucleus [14, 15]. Collectively, these findings indicate that significant levels of GHSR1a are expressed within the CNS outside of the hypothalamus, and suggest that ghrelin has important physiological functions besides those associated with GH release and body-weight regulation.

In the present study, I investigated whether treatment with ghrelin could improve the motor dysfunction under either nigrostriatal DA cell ablation or the inhibition of striatal DA receptors. First, I generated a model of end-stage PD using DAT-Cre mice by the stereotaxic injection of AAV-CMV-FLEX-DTA into the SN, which led to nigrostriatal DA cell ablation. These DAT^{SN}::DTA mice exhibited cataleptic behaviors related to the dysfunction of motor coordination in the balance beam test, rotarod test and horizontal bar test. In an

immunohistochemical study, these mice showed a dramatic loss of TH-positive DA cells in the SNc and DAT-labeled axon terminals in the striatum. The mRNA levels of DAT and TH were very low in the SN of DAT^{SN}::DTA mice, indicating the ablation of nigrostriatal DA neurons. The key finding of this study was that about a 50% reduction in the mRNA expression of GHSR1a was found in the SN of DAT^{SN}::DTA mice in the absence of TH and DAT mRNAs. The number of neurons in the SNc that co-express TH and GHSR has been identified [15]. In the present study, a high degree of GHSR1a mRNA expression within DAergic neurons was detected in the SNc. However, the expression of GHSR mRNA was also found within GABAergic neurons in the SNr. These findings suggest that functional GHSRs are dominantly located on SNc-DA cells, whereas some non-DA cells including GABAergic cells in the SN may possess GHSRs.

Next, I investigated whether activation of remaining GHSR1a expressed on nigrostriatal non-DA cells of DAT^{SN}::DTA mice could improve motor deficits. Although about 50% of GHSRs in the SN remained in DAT^{SN}::DTA mice, phasic stimulation of GHSRs expressed on nigrostriatal non-DA cells by a single microinjection of ghrelin into the SN failed to improve motor deficits. These findings provide evidence that GHSRs located on DA neurons in the SNc play a critical role in motor function. Although further studies are needed, including on the effect of tonic or chronic activation of GHSRs by chronic treatment with ghrelin are needed, the present results suggest that phasic activation of

GHSRs in the SN does not recover motor dysfunction under the ablation of nigrostriatal DA neurons, which may mimic end-stage PD.

Finally, I performed a behavioral assay to investigate the pharmacological interaction between the classic D_2 receptor antagonist haloperidol and ghrelin. In this study, I considered the haloperidol-treated mouse as an initial PD model with non-competitive neuronal dysregulation of DA transmission. After bilateral microinjection of ghrelin into the SN, haloperidol-induced catalepsy was dramatically suppressed. These results suggest that treatment with ghrelin could recover the reduction in DA transmission following systemic treatment with haloperidol through a non-competitive mechanism. This contention can be partly supported by my preliminary finding that administration of ghrelin produced a significant increase in DA-related locomotor activity in normal mice (unpublished observation).

In conclusion, phasic activation of the SN-ghrelin system may improve the initial dysregulation of nigrostriatal DA transmission, but not the motor deficits seen under the depletion of nigrostriatal DA. Further studies are needed to investigate the utility of tonic or chronic activation of GHSRs by chronic treatment with ghrelin for recovery from the motor deficits seen under the ablation of nigrostriatal DA neurons.

General Conclusion

The above findings led to the following conclusions:

In Chapter 1:

I report for the first time that the expression level of GHSR was dramatically decreased in PD-specific iPSC-derived DA neurons. Consistent with this finding, a significant decrease in the expression of GHSR was also found in DA neurons of isogenic PARK2-iPSC lines that mimicked loss of function of the PARK2 gene through CRISPR Cas9 technology. Furthermore, either intracerebroventricular injection or microinjection of the selective GHSR1a antagonist [D-Lys3]-GHRP-6 into the SNc of normal mice produced cataleptic behaviors related to the dysfunction of motor coordination. These findings suggest that the down-regulation of GHSR in DA neurons may correspond to the initial dysfunction of DA neurons, leading to extrapyramidal disorders under PD.

In Chapter 2:

I performed the stereotaxic injection of AAV-CMV-FLEX-DTA into the SN of DAT-Cre (DAT^{SN}::DTA) mice to expunge DA neurons of the SNc, which may mimic end-stage PD. In these DAT^{SN}::DTA mice, I observed a dramatic loss of DAT-labeled axon terminals in the striatum and down-regulation of GHSR1a in the SN. Under these conditions, injection

of ghrelin into the SN failed to improve the motor deficits in DAT^{SN}::DTA mice, whereas intra-SN injection of ghrelin suppressed the motor dysfunction caused by the administration of haloperidol. These findings suggest that activation of the SNc-ghrelin system could improve the dysregulation of nigrostriatal DA transmission, and ghrelin may be more effective for improving neuronal dysregulation in the initial stage of PD than in its end stage.

In conclusion, the present study demonstrated that the down-regulation or blockade of nigral GHSR in DA neurons may correspond to the initial dysfunction of DA neurons in PD. Additionaly, activation of the SNc-ghrelin system may be effective for improving neuronal dysregulation related to the initial stage of PD. I believe that this information will be of particular interest to people who have been working in the fields of neuroscience, neurology and clinical pharmacology.

List of Publications

1. Yukari Suda, Naoko Kuzumaki, Takefumi Sone, Michiko Narita, Kenichi Tanaka, Yusuke Hamada, Chizuru Iwasawa, Masahiro Shibasaki, Aya Maekawa, Miri Matsuo, Wado Akamatsu, Nobutaka Hattori, Hideyuki Okano and Minoru Narita: Down-regulation of ghrelin receptors on dopaminergic neurons in the substantia nigra contributes to Parkinson's disease-like motor dysfunction. *Mol. Brain* (*in press*): **Chapter 1**

2. Yukari Suda, Naoko Kuzumaki, Michiko Narita, Yusuke Hamada, Masahiro Shibasaki, Kenichi Tanaka, Hideki Tamura, Takashi Kawamura, Takashige Kondo, Akihiro Yamanaka and Minoru Narita: Effect of ghrelin on the motor deficit caused by the ablation of nigrostriatal dopaminergic cells or the inhibition of striatal dopamine receptors. *Biochemical and Biophysical Research Communications* (*in press*): **Chapter 2**

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