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Oral 'hydrogen water' induces neuroprotective ghrelin secretion in mice

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The therapeutic potential of molecular hydrogen (H_2) is emerging in a number of human diseases and in their animal models, including in particular Parkinson's disease (PD). H_2 supplementation of drinking water has been shown to exert disease-modifying effects in PD patients and neuroprotective effects in experimental PD model mice. However, H_2 supplementation does not result in detectable changes in striatal H_2 levels, indicating an indirect effect. Here we show that H_2 supplementation increases gastric expression of mRNA encoding ghrelin, a growth hormone secretagogue, and ghrelin secretion, which are antagonized by the β_1-adrenoceptor blocker, atenolol. Strikingly, the neuroprotective effect of H_2 water was abolished by either administration of the ghrelin receptor-antagonist, D-Lys[3] GHRP-6, or atenolol. Thus, the neuroprotective effect of H_2 in PD is mediated by enhanced production of ghrelin. Our findings point to potential, novel strategies for ameliorating pathophysiology in which a protective effect of H_2 supplementation has been demonstrated.

T herapeutic applications of molecular hydrogen (H_2) have been reported in a variety of human diseases and their animal models[1], including ischemia-reperfusion injury[2–4], metabolic syndrome[5], diabetes mellitus type 2[6], organ transplantation[7–9], reduction of adverse effects of anti-tumor drug therapy[10,11] and radiation therapy[12,13]. Although the mechanism of action of H_2 has not been clearly demonstrated, it is assumed that its anti-oxidative properties, particularly against hydroxyl radical ($\cdot$OH) and peroxinitrite ($ONOO^-$), are likely to underlie therapeutic efficacy[2]. Unlike other medical-gas therapy, H_2 can be applied in air for inhalation or in solution for drinking, intravenous injection or dialysis. Whereas intravenous injection or dialysis delivers H_2 directly into the blood stream, oral hydrogen-supplemented water (hydrogen water, H_2-water) must be absorbed into the circulation resulting in limited H_2 concentrations in the blood and in target organs[7,14].

Parkinson's disease (PD) has been a major focus in the field of oxidative stress and disease, because it is thought that degeneration of dopaminergic neurons can be triggered and aggravated by the accumulation of oxidative damage. However, although antioxidant therapies have been assessed in PD patients, clinical efficacy has not been established[15,16]. In contrast, a pilot study of hydrogen water therapy in PD patients has shown promising results[17], and it has been reported that hydrogen water exhibits neuroprotective effects[14] in the murine MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced PD model[18]. H_2 levels were below measurable thresholds in the substantia nigra in PD model mice[14], and hydrogen water, but not continuous inhalation of 2% H_2, prevented the development of PD in a rat model[19]. These findings suggest that the therapeutic effects of hydrogen water may not require its anti-oxidant activity in the brain, and further that its efficacy requires processing that is consequent upon oral administration.

The purpose of the present study was to employ PD model mice to elucidate the underlying mechanism of the neuroprotective effects of oral H_2-water. In particular, we hypothesized that oral H_2 induces a messenger molecule, which travels to the brain and exerts neuroprotective activity.

Results

Oral hydrogen water induces ghrelin gene expression in the stomach. The stomach functions as an endocrine organ that secretes various peptide hormones with a broad range of physiological effects. We first focused on the stomach to investigate possible effects of oral H_2-water at the level of gene induction. In a previous study[14], it was reported that drinking H_2-water for a period of 7 days prior to MPTP injection protected against MPTP toxicity. We administered oral H_2-water for 4 consecutive days and analyzed expression in stomach tissue of gastrin,
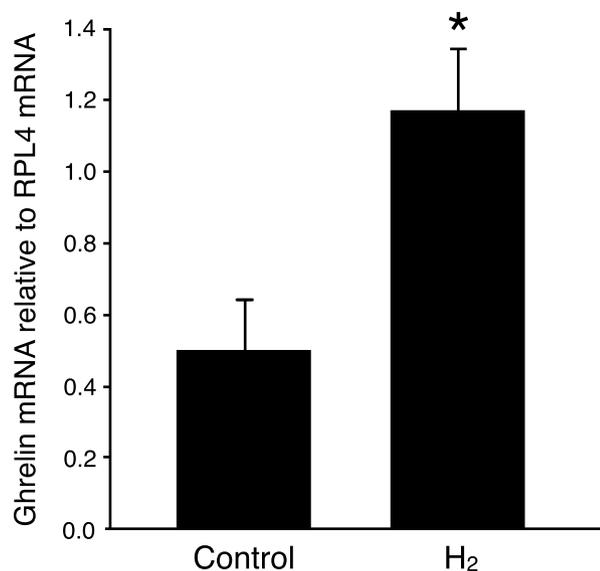
Figure 1 | Oral H$_2$ water increases ghrelin gene expression in the mouse stomach. Control water, or H$_2$ water (0.8 ml/mouse) made with a stick of magnesium (about 0.04 mM H$_2$; see Methods), was administered to mice (41–48 weeks of age; n = 4 per group) once a day for 4 days. Three hr after the final ingestion, the stomach was removed and prepared for qPCR. Data were normalized with respect to expression of RPL4 mRNA and are represented as mean ± SEM.

somatostatin, and ghrelin by real-time PCR method. Levels of ghrelin mRNA increased 1.9-fold in H$_2$-water-treated versus control mice (Figure 1), whereas no effects of hydrogen water were detected on expression of the somatostatin gene (expressed only at trace levels) or the gastrin gene (expression of which was highly variable from individual to individual) (not shown).

To examine the time-course of ghrelin induction by hydrogen water, mice were administered hydrogen water or control water once a day for 0 (control) 1, 2 or 4 days and ghrelin levels were measured by ELISA in plasma derived from blood obtained 4–5 hours after the final administration of H$_2$- or control water. Only mice that received H$_2$ water for 4-day exhibited a significant increase in plasma ghrelin level, although mice administered hydrogen water for 2-day showed a non-significant increase (Figure 2).

β$_1$-adrenergic receptor signaling mediates enhancement of ghrelin secretion by oral hydrogen water. It has been shown that gastric secretion of ghrelin is regulated by local environmental cues including blood glucose, estrogen, insulin and catecholamines[20–22]. In particular, it has been reported that β$_1$-adrenergic receptor stimulation increases ghrelin secretion *in vitro* and *in vivo*[22,23]. We verified the expression of β$_1$- and β$_2$- adrenergic receptors in stomach tissue samples by real-time PCR method, and determined that there were no significant changes in the levels of expression after administration of oral H$_2$-water for 4 days (data not shown). The increase in plasma ghrelin levels by oral hydrogen water was eliminated by administration of the β$_1$-adrenergic receptor-specific blocker, atenolol (10 mg/kg i.p.) injected 30 min prior to H$_2$ water administration on each of four days (Figure 3). Thus, activation of β$_1$-adrenergic receptors is required for hydrogen water-induced enhancement of circulating ghrelin.

Blockades of ghrelin action abrogates the protective effect of H$_2$ water in PD model mice. In a previous report, oral H$_2$-water exhibited significant protective effects in MPTP-induced PD model mice against the loss of dopaminergic neurons from the substantia nigra[14]. To test directly the role of ghrelin, either the growth hormone
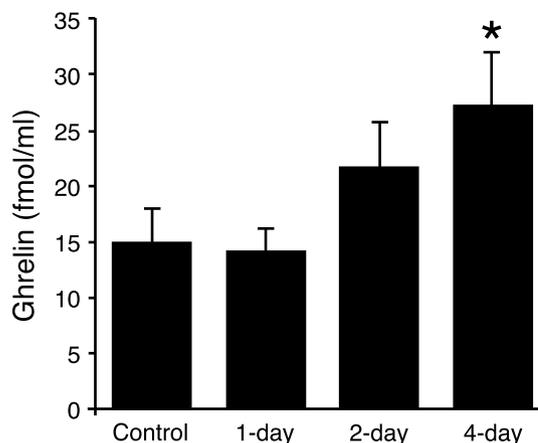

Figure 2 | Plasma ghrelin levels following administration of oral H$_2$ water. Mice (16–21 weeks, average 18.5 weeks, n = 5–10) received H$_2$ water (or control water) as in Fig. 1 for 1, 2 or 4 d. Blood was collected on the final day of the experiment, 4 hr after the last ingestion, and plasma levels of ghrelin were quantified by ELISA. Panel shows data from male mice. The ghrelin level was significantly higher only in the group of mice drinking H$_2$ water for 4 d. Data are represented as mean ± SEM.

secretagogue receptor antagonist, D-Lys³ GHRP-6 (100 nmol/day i.p.), or β$_1$-adrenoceptor blocker, atenolol (10 mg/kg i.p.), was administered along with control or hydrogen water for 7 days prior to administration of MPTP. Loss of dopaminergic neurons from the substantia nigra was evaluated seven days following administration of MPTP. As shown in Figure 4, systemic administration of MPTP caused a significant loss of dopaminergic neurons from the pars compacta of the substantia nigra (SNpc) as assessed by immunohistochemical detection of tyrosine hydroxylase (TH) (a), and further confirmed by immunological detection of TH protein in
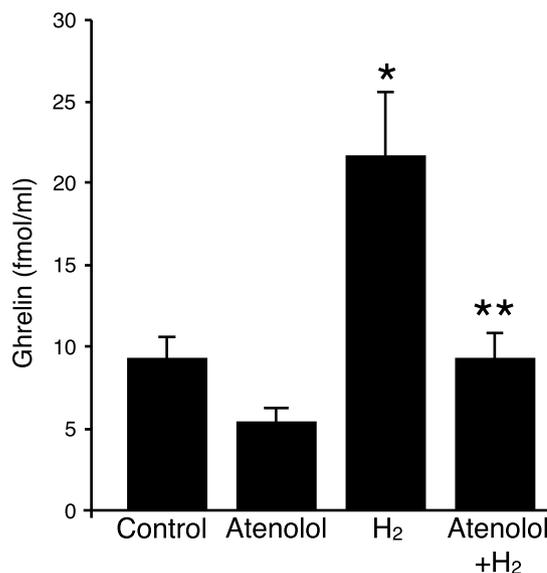

Figure 3 | H$_2$ water increases plasma ghrelin levels in a β$_1$-adrenergic receptor-dependent manner. Plasma ghrelin levels were measured on the last day of experiment. Each group of mice (11–13 weeks of age; n = 4–5 for each group) was administered control water or H$_2$ water (made with an open-air water electrolysis system) once a day for 4 d with or without i.p. injection of atenolol (10 mg/kg) prior to water ingestion. The H$_2$ water group showed a significant increase in plasma ghrelin level compared to the control group (* p = 0.038), which was abrogated by pretreatment with atenolol (** p = 0.039). Datafare represented as mean ± SEM.
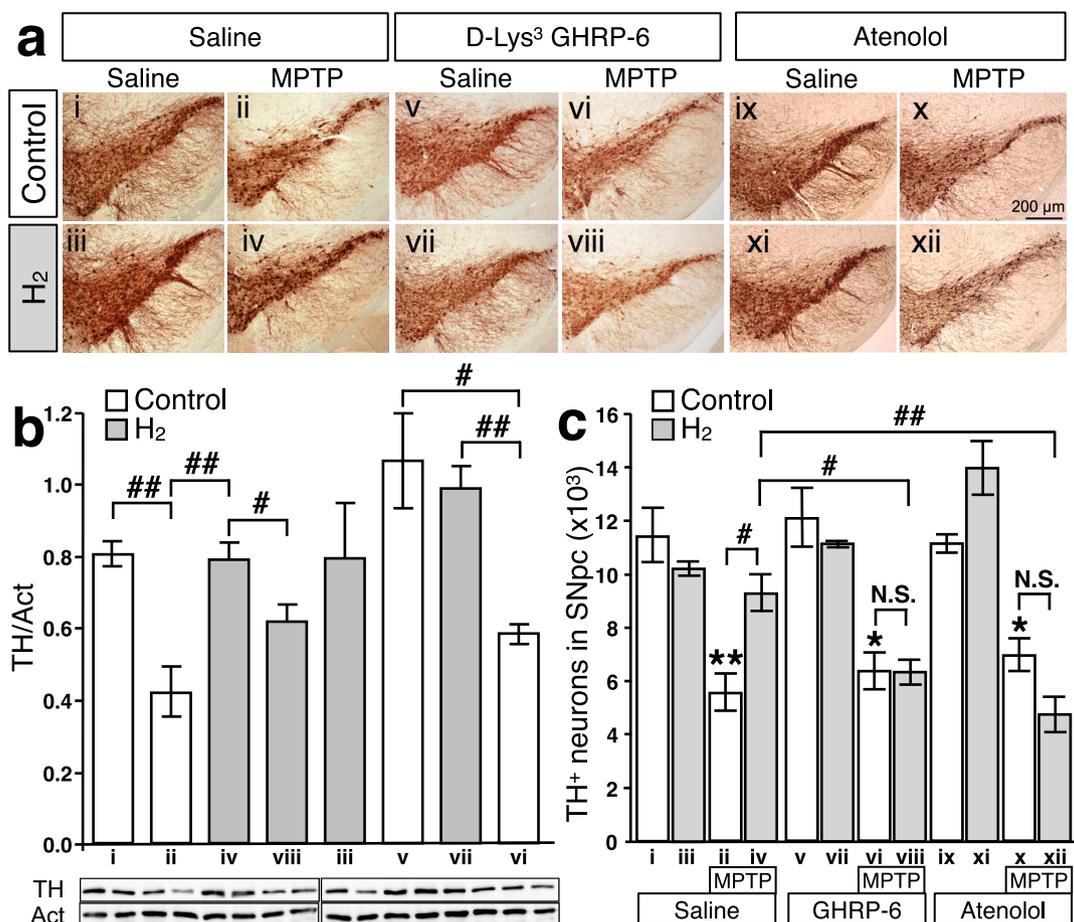
Figure 4 | Inhibition of ghrelin secretion or the ghrelin receptor antagonist cancels the neuroprotective effect of oral H$_2$ water in MPTP-induced Parkinson's disease model mice (male, 8–12 weeks, n = 2–5 for each group). Saturated H$_2$ water was made daily as described in materials and methods. (a): Tyrosine hydroxylase (TH) staining was performed in substantia nigra pars compacta from saline-injected (i–iv), D-Lys^3 GHRP-6-injected (v–viii), and atenolol-injected (ix–xii) mice, supplied with either normal tap water (control) or H$_2$ water for 7 days. MPTP was injected (i.p.) after 7 days of D-Lys^3 GHRP-6, atenolol, or saline-injection (ii, iv, vi, viii, x, and xii). (b): Summary of the immunoblotting analysis of TH protein in the substantia nigra tissue (n = 3–6 for each group) and the representative blot images were shown underneath (two samples per group). The TH band intensity was normalized to the band of actin on the same sample. (## p = 0.007, i vs. ii; 0.010, ii vs. iv; 0.004, vi vs. vii; # p = 0.043, iv vs. viii; 0.023, v vs. vi). Data in figures are represented as mean ± SEM. (c): Summary of the stereological analysis of nigral dopaminergic neurons. Although MPTP injections caused significant loss of TH-positive neurons (ii) (** p = 0.0001, i vs. ii), drinking H$_2$ water for 7 days prior to MPTP-treatment significantly attenuates the loss of TH-positive cells (iv) (# p = 0.008, ii vs. iv). D-Lys^3 GHRP-6, growth hormone secretagogue receptor antagonist, or β$_1$-adrenoceptor blocker, atenolol, canceled the preservation effect of oral H$_2$ water (viii and xii) (# p = 0.008; ii vs. iv; p = 0.003, iv vs. viii; p = 0.0003, iv vs. xii). Data in figures are represented as mean ± SEM. The statistical significance of data was assessed by one-way ANOVA followed by Benferroni test.

the substantia nigra with actin as control (b), and stereological analysis (c). Whereas administration of H$_2$ water alone in control mice had no effect, the loss of dopaminergic neurons in MPTP-treated mice was significantly decreased by administration of hydrogen water, as previously reported[14]. Strikingly, although administration of D-Lys^3 GHRP-6 or atenolol in control mice had no effect alone and D-Lys^3 GHRP-6 or atenolol did not affect MPTP-induced loss of dopaminergic neurons, the protective effects of hydrogen water were eliminated by either one of D-Lys^3 GHRP-6 or atenolol. Thus, induction of gastric ghrelin production and subsequent activation of ghrelin-initiated signal transduction underlies the protective effects of hydrogen water in the MPTP model of PD.

Discussion

Our findings demonstrate that the neuroprotective effects of oral hydrogen water, which produces negligible levels of H$_2$ in the brain, result from gastric induction of the neuroprotective peptide hormone ghrelin and the subsequent activation of ghrelin receptors. In addition, we have shown an obligate role for β$_1$-adrenergic receptors in hydrogen water-induced ghrelin up-regulation in plasma, consistent with previous reports that adrenergic stimulation regulates ghrelin release *in vitro* and *in vivo*[22,24,25].

The neuroprotective effects of ghrelin in PD are well-established[26], and it has been demonstrated that the receptor for ghrelin, the growth hormone secretagogue receptor (GHSR), is highly expressed by dopaminergic neurons of the substantia nigra[27]. It has been suggested that ghrelin protects nigrostriatal dopamine neurons via an uncoupling protein 2 (UCP2)-dependent mitochondrial mechanism[28,29]. However, we found that the expression of neither UCP2 mRNA nor protein was significantly upregulated by administration of H$_2$ water drinking for 7 days (data not shown). This finding suggests an alternative signaling mechanism downstream of GHSR activation, perhaps involving PI3K/Akt[30].

It was reported that administration of saturated hydrogen water (approx. 0.8 mM) led to symptomatic improvement in PD patients[17]. Administration of hydrogen water at about 0.05% saturation successfully maintained dopaminergic neurons in MPTP-induced PD model mice[14]. We employed three different methods to prepare

Table 1 | Primer sets used for real-time PCR analyses

RPL4.For	GCCAAGACTATGCGCAGGAAT
RPL4.Rev	GTAGCTGCTGCTTCCAGCTT
Ghrelin.For	TCAAGCTGTCAGGAGCTCAGTA
Ghrelin.Rev	TTGTCAGCTGGCGGCCTCTT
Somatostatin.For	TGGCTGCGCTCTGCATCGT
Somatostatin.Rev	AGTACTTGGCCAGTTCCTGTT
Gastrin.For	CAGCGCCAGTTCAACAAGCT
Gastrin.Rev	ATTCGTGGCCTCTGCTTCTT
b1AR.For	CACTGTGGACAGCGATTCGA
b1AR.Rev	ACCTTGGACTCCGAGGAGAA
b2AR.For	ACAGGAACTGCTGTGTGAGGAT
b2AR.Rev	ACGCTAAGGCTAGGCACAGT

hydrogen water (see Methods), which resulted in H_2 concentration of 0.04–0.8 mM, and we observed that the effects of hydrogen water on ghrelin induction and protection of dopamine neurons were dose-independent over this range. Thus, small amounts of oral H_2 are sufficient for gastric induction of ghrelin and subsequent neuroprotection, in the absence of detectable H_2 in the brain. Interestingly, gut microbes can produce molecular hydrogen constitutively and lactulose, a synthetic disaccharide, is an effective substrate to enhance bacterial H_2 production in the colon[31]. However, ingestion of lactulose had no significant effect on dopaminergic neuron survival in 6-OHDA-induced PD model rats (although alveolar H_2 concentrations were elevated)[19]. These results emphasize the importance of gastric ghrelin induction in the neuroprotective action of H_2. Oral H_2-water is being explored as a therapeutic for PD as well as a variety of other human pathophysiological conditions[17,32,33] under the generally held assumption that the mechanism of action of supplemental H_2 is likely to reflect an antioxidative role. Our findings that oral H_2 water exerts a neuroprotective effect through activation of an endogenous, gastric ghrelin system that is tightly coupled to β-adrenergic receptor signaling suggests the possibility of novel applications of H_2 therapy for various diseases.

Methods

Animals. Procedures in animal experiments were approved by the university review board for animal care in Chiba University and Kyushu University, and performed in accordance with the guidelines established by the Science Council of Japan. All mice (C57BL/6J) were maintained in the animal facility under controlled temperature and lighting (12-hour light, 12-hour dark), and received a standard mouse chow diet and filter-sterilized tap water *ad libitum*.

Preparation and administration of H_2- water. H_2 water was prepared by one of three methods. The first method utilized the spontaneous ionization reaction of magnesium in water[14]. A magnesium stick (AZ31, Nakagawa Metal, Japan, composed of 96% magnesium, 3% aluminum, and 1% zinc) was polished to remove the oxidized surface and wiped clean with 1 N hydrogen chloride before dipping into drinking water for 15–20 min at 25°C. The second method was based on the electrolysis of water with platinum electrodes at 150 V DC for 20 min (open-air water electrolysis method). The anode was placed inside a drinking straw to vent generated oxygen. In the third method (employed in the experiments with MPTP-induced PD model mice), H_2 gas was produced by solid polymer electrolyte water electrolysis method at 5 V DC in which the cathode and anode were separated by a solid polymer electrolyte membrane (Nafion® E.I., du Pont de Nemours & Co., Inc.) and collected through a polyethylene tube. Saturated H_2 water was then generated by bubbling water in a drinking bottle with H_2 gas such that H_2 gas filled the headspace. H_2 water made with this method and kept in sealed drinking bottles remained saturated with H_2 for at least 24 hr.

In most experiments, each mouse received 0.8 ml of H_2 water (administered within 30 min of preparation) or control water (obtained by boiling H_2 water to degas) once every morning via feeding needle according to the experimental schedule. In experiments with MPTP-induced PD model mice, fresh H_2 water was supplied every 24 hr and water intake was *ad libitum* for the 7 days prior to MPTP administration.

Measurements of ghrelin expression. Male and female mice aged between 41 and 48 weeks were chosen for the initial screening for the altered expression of gastric enzymes (n = 4 for each group), as they are relatively stable in dietary intake, which may influence mostly on the digestive enzyme expressions. Since the previous study was done with male mice[14], the following experiments chose male mice. For the time-course analysis of ghrelin induction by hydrogen water, male mice (16–21 weeks,

average 17.9 weeks, n = 5–10 in each group) were selected. Adrenoceptor mediated changes of ghrelin secretion was analyzed in male mice (age between 11–13 weeks, n = 4–5 for each group). Of note, there was no body weight change more than one gram during the period of experiment (4 days). These results are in line with the previous report with a recombinant ghrelin injection to mice, where the body weight change was below one gram in three days[34].

Mice were administrated H_2 water or control water for 4 days according to the experimental schedule. At the end of the experiment, mice were sacrificed by cervical dislocation and blood was collected by cardiopuncture with EDTA as anti-coagulant. Blood was centrifuged at 2000 g for 5 min at 4°C and the plasma was collected and mixed with 1 N hydrogen chloride (10% of plasma volume) to avoid inactivating deacylation of ghrelin[20]. Samples were stored at −80°C until analysis. Following blood collection, the stomach was removed intact and snap-frozen in liquid nitrogen for further analysis. For consistency, mice were killed 4–5 hours after the final administration of H_2 water and at the same time of day. At the time of blood sampling the stomach was almost full of chow, which suggests that the water administration at a time (0.8 ml/time) through the feeding needle did not cause the sustained reduction on appetite.

The active form of ghrelin was measured in plasma by ELISA (Active Ghrelin ELISA kit, Sceti, Japan) according to the manufacturer's instructions. For semi-quantitative PCR, stomach tissue frozen in liquid nitrogen was crushed into fine granules and total RNA was extracted with RNAiso (Takara, Japan) according to the manufacturer's instructions. Two μg of total RNA was then used to synthesize first strand cDNA with a SuperScript VILO cDNA Synthesis Kit (Invitrogen, USA). mRNA expression levels were quantified by real-time PCR with SYBR green dye (Thunderbird Sybr qPCR Mix, Toyobo, Japan) with the specific primer sets shown in Table 1, and normalized to ribosomal protein L4 (RPL4) mRNA[35–37].

Administration of ghrelin receptor antagonist, β₁-adrenoceptor blocker, and MPTP. The ghrelin receptor antagonist, D-Lys³ GHRP-6 (Sigma-Aldrich, USA; 100 nmol/day), β₁-adrenoceptor blocker, atenolol (ICI, USA; 10 mg/kg), or saline was administered by i.p. injection daily for 7 days in conjunction with supplying fresh control or H_2 water. On day 7, MPTP-HCl (Sigma-Aldrich, USA; 15 mg/kg in 0.9% NaCl per injection) or saline as control was administrated by i.p. injection four times at 2 hr intervals. Mice were supplied with untreated tap water for the next 7 days before removal of brains under deep anesthesia (pentobarbital, 50 mg/kg i.p.).

Stereological and immunological analyses of nigral dopaminergic neurons. Stereological analysis was carried out as described[14,38–40]. In brief, coronal sections (30 μm thickness) were obtained through SNpc (−2.70 mm to −3.80 mm relative to bregma)[41] with a MICROM cryostat. Free-floating sections were incubated with Block Ace (Dainippon Pharmaceutics, Japan) for 30 min followed by incubation with anti-tyrosine hydroxylase (TH) antibody (Chemicon, USA, 1 : 3000 in 10% Block Ace) for 2 days at 4°C. After rinsing, sections were immersed in a solution of 3% H_2O_2 in methanol/PBS (1 : 1) for 10 min at room temperature, followed by incubation for 2 hr in biotinylated goat anti-rabbit IgG (1 : 400, Vector, USA) and processing with a Vectastain ABC kit (Vector, USA) using 3′3′-diaminobenzidinetetrahydrochloride (DAB, Vector, USA) as peroxidase chromogen.

Unbiased stereological counts of TH-immunoreactive cell bodies in the SNpc were obtained using an optical fractionator method[42] and Stereo Investigator software (Stereo Investigator 8, MicroBrightField Inc., USA). The boundary of SNpc was delineated under 100 × magnification and immunopositive neurons were counted in every third section (eight sections per brain) at 400 × magnification on a Nikon ECLIPSE 80 i microscope using a grid of 70 × 70 μm on a counting grid (75 × 100 × 12 μm) with 2 μm upper and lower guard zones. Gundersen's coefficient of error in all samples was < 0.07.

Immunological detection and quantification of TH expression in the substantia nigra tissue were performed by western blotting with actin as control. Animals were prepared identically to those of the stereological analysis. The substantia nigra was removed and stored at −80°C until use. Samples were lysed on ice in hypotonic buffer (20 mM Hepes pH 7.6, 10 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 0.1% Triton X-100) with protease inhibitor cocktail (Roche, USA), and cleared by a centrifugation at 20 k G for 10 min at 4°C. Following quantification of protein concentration using the BCA method with BSA as control, five μg of tissue lysate reduced in sample buffer was resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, and probed with anti-TH antibody (1 : 2000) overnight at 4°C. Proteins were visualized using anti-rabbit secondary antibody conjugated to HRP and a chemiluminescence detection system (Immobilon Western Chemiluminescent HRP Substrate, Millipore, USA). Chemiluminescence image was quantified by Gauge application (Fuji Film, Japan) and the value was normalized to the level of actin band on the same sample specified by anti-Actin monoclonal antibody (Clone C4, Millipore, USA).

Statistical analysis and data managing. The statistical significance of data was assessed by unpaired Student t-test (two-tailed) unless otherwise mentioned and results were considered significant at p ≤ 0.05.

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Author contributions

A.M., Y.N., and M.N. conception and design of research; A.M., M.Y., T.T. performed experiments; A.M., M.Y., and M.N. analyzed data; A.M., M.Y., Y.N., and M.N. interpreted results of experiments; A.M. and M.Y. prepared figures; A.M., M.Y., Y.N., M.N., and H.N. wrote and revised manuscript; A.M., M.Y., T.T., Y.N., M.N., and H.N. approved final version of manuscript.

Additional information