Early low-dose ghrelin intervention via mini-osmotic pumps could protect against the progressive dopaminergic neuron loss in Parkinson's disease mice

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Research

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Abstract

Background

Ghrelin has been identified as a multifunctional peptide that has a potential applications for the treatment Parkinson's disease (PD).

Method: ELISA was used for detecting plasma total and active ghrelin levels, dopamine (DA) content was measured by HPLC-ECD, immunofluorescence staining and Western blot were used to detect protein expressions, and cytokine was tested by Bio-PlexPro™ assay.

Results

Here, we reported a subcutaneous administration of low-dose ghrelin via mini-osmotic pumps to PD mice. The decreased levels of total and active ghrelin in plasma were rescued by ghrelin administration. Interestingly, ghrelin had no effect on weight gain in wild-type mice but improved weight loss in PD mice. We observed the attenuation of dopaminergic (DAergic) neuron loss in substantia nigra (SN) and low level of dopamine content in the striatum in PD mice with ghrelin treatment. Ghrelin administration could improve the environment of DAergic neuron by inhibiting microglia proliferation and pro-inflammatory cytokine expression, and could enhance cell survival by upregulating Bcl-2/Bax ratio and superoxide dismutase1 (SOD1) protein level in SN in PD mice.

Conclusions

Our results suggested that subcutaneous administration of low-dose ghrelin could prevent the onset or the progression of PD, and also provided a possible method for ghrelin application to cure PD.

1 Background

Parkinson's disease (PD) is characterized by the progressive degeneration of dopaminergic neurons projecting from the substantia nigra (SN) to the striatum (Str). Subsequently, the reduced release of dopamine in the Str leads to debilitating motor dysfunctions, such as resting tremor, muscle rigidity and bradykinesia. As of 2015, PD had affected 6.2 million people and led to 11,7400 deaths worldwide [1]. Dopamine (DA)-related therapies have not been effective measures to treat motor symptoms, as there are complications of long-term drug use. Treatments that halt disease progression are the greatest potential therapeutic strategies.

Ghrelin, a brain-gut peptide newly discovered in 1999, is the only endogenous ligand for the growth hormone secretagogue receptor (GHSR) [2]. The concentration of serum ghrelin is high during fasting and low after calorie intake to regulate food intake and energy metabolism [3]. Additionally, ghrelin also has roles with the central nervous system, such as neuroprotection, anti-depression and anti-anxiety effects, memory and cognition regulation, and neurogenesis regulation [4–6]. GI dysfunction may be a particularly early event in PD patients [12, 13]. Constipation, for example, is a well-described pre-motor

symptom reported by nearly 90% of all PD patients, with rates rising as the disease progresses. We found that plasma total ghrelin and active ghrelin levels were significantly decreased both in early PD patients, which indicated that plasma ghrelin levels might be irrelevant to PD progression [7]. For ghrelin's multifunction, it might be used as a biomarker and endogenous protective factor for PD diagnosis and therapy in future. Our previous studies also showed that the non-motor symptoms such as colon motility, anxiety-like and depressive-like behaviors, and motor coordination also changed from 3 months age, whereas no motor symptoms exhibited in homozygous mice which expressed human mutant A53T alpha synuclein (A53T mice) [8]. Thus, we wanted to know whether the progressive dopaminergic neuron loss were prevented if given protective administration in the early stage of PD.

In this work, we first tested the plasm ghrelin level in homozygous A53T mice. We showed that both total and active ghrelin in plasma were decreased in A53T mice at 3 month age. Because of the potential therapeutic application of ghrelin, we administered low and continuous doses of ghrelin to sustain its concentration using spontaneous *Alzet* mini-osmotic pumps before the onset of plasma ghrelin decrease, and then detected whether recovered ghrelin could prevent PD progression. The ghrelin treatment were divided into two types: one was administrating for 4 weeks, and another was administrating for 8 weeks. Finally, we observed the oxidant, apoptosis and inflammatory levels after ghrelin administration to evaluate ghrelin's neuroprotective effects. Our results revealed that subcutaneous administration of low-dose ghrelin could prevent the progression of PD and provided a possible method of ghrelin application for PD treatment.

2 Methods And Materials

2.1 Animals and treatments

All animal care and experimental protocols were performed in accordance with the guide from the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animal Ethics Committee of Qingdao University (20140522). A53T transgenic mice (B6; C3-Tg (Prnp-SNCA*A53T) 83Vle/J) were originally acquired from the Jackson Laboratory (004479) in breeding pairs to generate a stable breeding colony. Thirty homozygous A53T mice and thirty wild-type littermates aged 4 weeks were raised on a 12-hr light-dark cycle at room temperature (22 ± 2℃) with natural food and water. Genotyping was conducted according to the protocol used in our previous studies. Mice were randomly assigned to four groups for treatment: (1) P-WT group: wild-type mice with administered non-sense peptide (a misorder ghrelin peptide; amino acid sequences: G-L-S-F-E-H-Q-S-P-Q-Q-R-A-K-E-K-K-S-P-K-L-P-A-Q-P-R-K); (2) P-A53T group: homozygous A53T mice with administered non-sense peptide; (3) G-WT group: wild-type mice with administered ghrelin (Sigma, USA) (G-S-S-F-L-S-P-E-H-Q-K-A-Q-Q-R-K-E-S-K-K-P-P-A-K-L-Q-P-R), which contained acylated ghrelin as physiological level; (4) G-A53T group: homozygous A53T mice with administered ghrelin. Ghrelin and non-sense peptide were administered subcutaneously in saline via *Alzet* mini-osmotic pumps (Alzet, USA). There were three time points: (1) mice that were administered peptide starting at the age of 1 month, lasting for 4 weeks, and that were harvested at the age of 3 months (3 m (4 w); Fig. 1); (2) mice that were administered peptide starting at the age of 1 month, lasting

for 8 weeks, and that were harvested at the age of 3 months (3 m (8 w), Fig. 1); (3) mice that were administered peptide starting at the age of 1 month, lasting for 8 weeks, and that were harvested at the age of 6 months (6 m (8 w) Fig. 1).

All mice were fasted for approximately 8 hrs before treatment but had free access to water. At the end of treatment, mice were put under 45 mg/kg sodium pentobarbitone anaesthesia, and blood plasma was aliquoted into polypropylene tubes and stored at -80 °C for enzyme-linked immunosorbent assay (ELISA) without undergoing any additional freeze-thaw cycles. Brains were isolated from mice and divided into two hemispheres. The right hemisphere SN was used to assess some protein levels by western blot, and the Str was isolated for high-performance liquid chromatography electrochemical detection (HPLC-ECD); the remaining hemisphere was used for immunofluorescence

(IF) staining.

2.2 ELISA for plasma total and active ghrelin levels

The plasma sample was acidified with HCl (0.05 mol/L final concentration). Total and active ghrelin levels in plasma were measured by corresponding commercially available kits; mouse ghrelin (total) or mouse ghrelin (active) enzyme-linked immunosorbent assay kits (Millipore, USA) were applied according to the provided instructions.

2.3 HPLC-ECD for DA content

Str sample was homogenized in 300 μL liquid A (0.4 M perchloric acid). And the details for HPLC-ECD was described as our previous study [5, 9].

2.4 IF staining for TH or Iba1-positive cells

The fresh left SN was postfixed in 4% paraformaldehyde (PFA) over 2 days at 4 °C, followed by dehydration in 20%, and then 30%, sucrose at 4 °C for approximately 2 days. The tissue was sectioned into 20-μm-thick slices. The sections were pre-treated with 0.1% Triton X-100 and blocked with 10% goat serum for 1 hr, and were incubated with rabbit monoclonal anti-TH (Millipore, USA) and anti-Iba1 (Wako, JPN) overnight at 4 °C. Secondary antibodies of Alexa Fluro®-555 conjugate H + L (Invitrogen, USA) were incubated for 1 hr, and images were taken with microscopy (Zeiss, Germany).

2.5 Western blot for protein levels

Total protein was extracted from the right SN with lysis buffer (RIPA (strong), Beyotime, CN) and a protease inhibitor cocktail (Roche, USA). Subsequently, protein was subjected to 10% SDS-PAGE gels and transferred onto PVDF membranes (Invitrogen, USA). After blocking with 10% skimmed milk for 1 hr at room temperature, the PVDF membranes were incubated with primary antibodies, including rabbit monoclonal anti-β-actin (Santa Cruz, USA), anti-TH (Millipore, USA), anti-Bcl-2, anti-Bax, anti-SOD1 and anti-interleukin-6 (IL-6) (all from CST, USA), respectively, overnight at 4 °C. The membranes were further probed with HRP-conjugated secondary antibodies (Santa Cruz, USA). Last, the membranes were exposed to ECL (Thermo Fisher Scientific, USA) for 1 min and visualized using a Tanon image system.

2.6 Bio-PlexPro™ mouse cytokine assays

Total protein was extracted from the SN with lysis buffer (RIPA (weak and without SDS), Beyotime, CN). Concentrations of TNFα, IL-6, and IL-10 were measured by Bio-Plex Pro™ mouse cytokine assays (Bio-Rad, USA) according to the provided instructions.

2.7 Statistical analysis

All data are shown as the mean ± SEM. Two-way analysis of variance (ANOVA) followed by the Student-Newman-Keuls test was used to detect differences between two groups using SPSS 19.0 (SPSS Inc, Chicago, IL, USA); $P < 0.05$ was considered statistically significant.

3 Results

3.1 Ghrelin recovered the decrease of plasma ghrelin levels and weight loss in homozygous A53T mice

A53T homozygous mice exhibited decreased plasma total and active ghrelin levels in the P-A53T group both at 3 months and 6 months of age (Fig. 2A-B). After 4 weeks of ghrelin administration, there was no significant difference in plasma total ghrelin levels in 3 month-old G-A53T mice (4.82 ± 1.49 ng/mL) (Fig. 2A). When the duration was doubled, the plasma total ghrelin levels of the G-A53T group at 3 months of age increased by 20% (Fig. 2A); however, at 6 months of age, no significant increase in ghrelin levels was found in the G-A53T group. Unexpectedly, the plasma active ghrelin levels of the G-A53T group at 3 m (4 w), 3 m (8 w) and 6 m (8 w) showed increases of 26%, 33% and 14%, respectively, compared with levels in the control group (Fig. 2B). Conversely, plasma active ghrelin levels in the P-A53T group decreased by 38% (3 m (4 w)), 27% (3 m (8 w)) and 26% (6 m (8 w)) (Fig. 2B). No significant differences were detected in WT mice in either plasma total or active ghrelin levels at any of these time points (Fig. 2). These results indicate that ghrelin administration could recover decreased plasma ghrelin in A53T homozygous mice.

However, at the beginning of this study, we were afraid that obesity would follow the recovery of ghrelin levels because ghrelin is considered to play an important role in food intake and metabolism, but beyond our expectations, the body weight of the mice in the A53T homozygous group decreased at the 3-month and 6-month time points (Fig. 2C). Meanwhile, body weight in G-A53T group mice increased compared with the control group (Fig. 2C). Furthermore, WT mice did not show significant changes at either of these time points (Fig. 2C). Our results suggested that ghrelin administration for 8 weeks could reverse weight loss in homozygous A53T mice.

3.2 Ghrelin restricted both the progressive loss of dopaminergic neurons in the SN and the depletion of DA in the Str of homozygous A53T mice

After 4 weeks of ghrelin administration, no significant changes in the number of TH-positive neurons or TH protein levels in the SN were detected in the G-A53T group at the age of 3 months (Fig. 3A-C). Interestingly, DA content (11.71 ± 1.37 ng/mg) in the Str of G-A53T mice at the age of 3 months was significantly increased (Fig. 3E). The A53T homozygous mice showed a 12% decrease in the number of TH-positive neurons in the SN (Fig. 3A-B). Correspondingly, TH protein levels in the SN were decreased, and DA content in the Str of P-A53T group mice decreased from 11.56 ± 2.46 ng/mg to 5.57 ± 2.48 ng/mg, with a 51% decrease at the age of 3 months (Fig. 3C-E).

To verify whether this kind of ghrelin administration is neuroprotective in A53T homozygous mice, we doubled the duration of ghrelin administration. After treatment with ghrelin for 8 weeks, the number of TH-positive neurons in the SN of G-A53T group mice increased at 3 months and 6 months by 13% and 15%, respectively (Fig. 3F-G). Correspondingly, TH protein levels in the SN and DA content in the Str of G-A53T group mice at the age of 3 months and 6 months both increased (Fig. 3H). The number of TH-positive neurons in the SN of P-A53T group mice at the ages of 3 months and 6 months decreased by 14% and 16%, respectively (Fig. 3F-G), and TH protein levels in the SN and DA content in the Str of P-A53T mice both decreased (Fig. 3H-J). In contrast, WT mice did not show significant changes at any of these time points. These results indicate that the progressive loss of dopaminergic neurons occurred in A53T homozygous mice between the ages of 3 and 6 months. Additionally, 4 weeks of ghrelin administration did not recover the loss of neurons in the SN, but it rescued the DA content in the Str. When the duration of ghrelin treatment was doubled, a neuroprotective effect of ghrelin on dopaminergic neurons was observed. More specifically, ghrelin administration for 8 weeks could rescue both the progressive loss of dopaminergic neurons in the SN and the depletion of DA in the Str of homozygous A53T mice 3–6 months old.

3.3 Ghrelin inhibited inflammation in the SN of homozygous A53T mice

After treatment with ghrelin for 4 weeks, no significant differences were observed in the number of microglia (Fig. 4A-B) and the protein level of IL-6 (Fig. 4C-D) in the SN of G-A53T group mice at the age of 3 months. When the duration of ghrelin administration was doubled, the number of microglia in the SN of G-A53T group mice decreased by 22% and 45% at the ages of 3 months and 6 months, respectively (Fig. 4A-B). Correspondingly, IL-6 protein levels in the SN of G-A53T group mice were decreased at the age of 3 months and 6 months, as well (Fig. 4C-D). In addition, A53T homozygous mice displayed more activated microglia and increased release of IL-6 at all of these time points. Furthermore, WT mice did not show changes at any of these time points.

Furthermore, we used Bio-Plex Pro™ mouse cytokine assays for mice administered ghrelin for 8 weeks. As expected, the concentration of IL-6 significantly increased, whereas the concentration of IL-10 decreased (Fig. 4E-F). There was no change in the concentration of TNFα in the SN of homozygous A53T mice at the age of 3 months. In addition, no difference was found between A53T homozygous and WT mice at the age of 6 months. Similarly, ghrelin administration reversed the changes in IL-6 and IL-10 levels at the

age of 3 months. These findings demonstrated that ghrelin administration has potent anti-inflammatory properties.

3.4 Ghrelin boosted decreased SOD1 levels and the Bcl-2/Bax ratio in the SN of homozygous A53T mice

No significant differences were observed in the protein levels of SOD1 in the SN among the four groups at the ages of 3 m (4 w) and 3 m (8 w). After ghrelin administration for 8 weeks, the protein levels of SOD1 in the G-A53T group mice increased at the age of 6 months (Fig. 5A-B). In addition, the SOD1 protein levels of A53T homozygous mice decreased at the age of 6 months (Fig. 5A-B). Nevertheless, there was no significant change in WT mice (Fig. 5A-B). These results illustrated that ghrelin administration boosts decreased levels of SOD1 in the SN of homozygous A53T mice.

No significant differences were observed in the Bcl-2/Bax ratio in the SN among the four groups at the ages of 3 m (4 w) and 3 m (8 w). After treatment for 8 weeks, the Bcl-2/Bax ratio in G-A53T group mice increased at the age of 6 months (Fig. 5C-D). A53T homozygous mice showed a significant decrease in the Bcl-2/Bax ratio at the age of 6 months (Fig. 5C-D). Furthermore, no significant difference was observed in WT mice (Fig. 5C-D). These results illustrated that ghrelin administration promotes a decreased Bcl-2/Bax ratio in the SN of homozygous A53T mice.

4 Discussion

In this study, we found that subcutaneous administration of low-dose ghrelin for 8 weeks provided a neuroprotective effect in a mouse model of PD. These protective effects may stem from three major functions of this peptide. First, ghrelin enhanced energy intake that induced the increase of body weight. Second, ghrelin blocked microglial activation and inhibited the release of IL-6, both of which can mitigate neuro-inflammation and promote neuronal survivability. Last, ghrelin elevated the activity of SOD1 and increased the Bcl-2/Bax ratio, which prevented neurons from oxidative damage and inhibited apoptosis during the course of the progressive degeneration of dopaminergic neurons, respectively.

PD patients exhibit non-motor symptoms, including hyposmia, sleep disturbances and gastrointestinal dysfunction, 10–20 years before they experience motor impairment [10]. Indeed, approximately 98.6% of PD patients suffered at least one non-motor symptom [11]. Moreover, PD patients usually have a lower body weight [12]. Ghrelin, the "hunger hormone", functions as a neuropeptide in the central nervous system [13]. In addition to regulating appetite, ghrelin increases body weight and fat mass by triggering receptors in the arcuate nucleus [3, 14, 15]. In PD patients, plasma total and active plasma ghrelin levels were decreased, and postprandial ghrelin suppression and preprandial peak responses were both attenuated [7]. In this study, we also found homozygous A53T mice plasma ghrelin were decreased at 3 months age when non-motor symptom exhibited. Injections of ghrelin in both humans and rodents have been shown to increase plasma ghrelin level, and thus increase food intake and motivation to seek out food as well [16]. Here, we showed that the plasma total and active ghrelin levels were both recovered at the age of 3 months after treatment with ghrelin for 8 weeks. Ghrelin injections increased food intake in a dose-dependent manner, and ghrelin could only stimulate food intake when it was elevated much

higher than normal levels. In our study, we first reported that nearly normal ghrelin levels rescued weight loss in homozygous A53T mice 3–6 months old. However, the plasma ghrelin level was lower in obese individuals than leaner individuals [16], suggesting that ghrelin did not contribute to obesity. In line with this research, ghrelin did not increase the body weight of normal mice, and there was “ghrelin resistance” in mice with adiposis [17]. Therefore, it could explain why normal physiological ghrelin dose treatment had no effect on WT mice body weight. Report has found that ghrelin increased the content of DA in the SN through its receptor [18]. Even though all ghrelin administrations were began at the age of 1 month, the administration process with 8 weeks rather than 4weeks, could recover the decreased plasma ghrelin and restrict both the progressive loss of dopaminergic neurons in the SN and the depletion of DA in the Str. The administration time was refer to Therefore, our results indicated that early intervention of ghrelin may delay the progression of PD or even prevent the onset of PD, and also suggested that the lasting time of drug delivery was an important factor for ghrelin clinical application.

Accumulating evidence has suggested a strong link between neurodegeneration and chronic inflammation resulting in the activation of microglia and astrocytes and the increased release of pro-inflammatory cytokines [19]. Elevated pro-inflammatory cytokines were observed in the brain, cerebrospinal fluid and serum of PD patients [20–23]. Lipopolysaccharides (LPS) or viral pathogens could also induce PD-like neuronal death in rodents [24–26]. This study found increased activation of microglia and increased release of IL-6 in the SN of homozygous A53T mice. Ghrelin markedly inhibited oligodendrocyte cell death in oligodendrocytes and LPS-stimulated BV-2 cell co-culture systems, which imitated microglial activation *in vitro* [27]. Ghrelin was reported to reduce microglial activation in an MPTP-induced PD model, inhibiting the increased expression of TNFα and IL-1β mRNA and inducible nitric oxide synthase in the SN [28]. Nevertheless, the anti-inflammatory effects of ghrelin may originate from the attenuation of the release of inflammatory cytokines, such as IL-6, TNFα, IL-1β and cyclooxygenase-2 [29–31]. However, no significant difference was found in the concentration of TNFα between the homozygous A53T and WT mice. In addition, *in vitro* and *in vivo* studies have revealed the neuroprotective activity of IL-10 on dopaminergic neurons when exposed to LPS and in a 6-OHDA PD model [32–34]. This finding is in accordance with results of the inhibition of microglia, decreased pro-inflammatory cytokine IL-6 and increased anti-inflammatory cytokine IL-10 in the SN of G-A53T mice, suggesting anti-inflammatory and neuroprotective roles of ghrelin on dopaminergic neurons.

Increased levels of oxidation and its products, including nucleic acids, proteins and lipids, were detected in post-mortem PD brains [35, 36], suggesting that oxidative stress plays a vital role in the loss of dopaminergic neurons. Superoxide dismutase Cu-Zn, also known as SOD1, can bind copper and zinc ions and act as a homodimer to destroy naturally occurring, but harmful, superoxide radicals in the body [37]. The upregulation of malonaldehyde and the downregulation of SOD1 and catalase have been found in MPTP/MPP^+-induced PD models [38, 39]. Correspondingly, this study found that homozygous A53T mice exhibited lower SOD1 protein levels in the SN at the age of 6 months. Nevertheless, exogenous ghrelin could antagonize MPTP-induced oxidative stress by reversing the reduced SOD1 [39]. This study observed that ghrelin administration for 8 weeks boosts the decreased levels of SOD1 in the SN of

homozygous A53T mice, indicating that ghrelin may exert a neuroprotective effect on dopaminergic neurons against oxidative stress. Additionally, ghrelin pre-treatment could reduce ROS generation and oxidative stress by increasing the activity of uncoupling protein 2 (an important mitochondrial protein in control of ROS production that acts as a sensor for mitochondrial oxidative stress), resulting in decreased dopaminergic neuron loss in the MPTP-induced PD model [18].

The present study found that homozygous A53T mice exhibited a lower Bcl-2/Bax ratio. Emerging evidence indicates that ghrelin has anti-apoptotic effects in multi-pathophysiological conditions, including ischaemia, inflammation, and nutrient deprivation [40–42]. Recently, it has been reported that the anti-apoptotic effects of ghrelin in neurons occur via multiple signalling pathways, including the PI3K/Akt, ERK1/2, GSK-3β, JNK and p38 pathways [4]. Report have shown that ghrelin could increase Bcl-2/Bax ratio, prevent of cytochrome c release and inhibit activation of caspase-3, thus promoting the survival of cortical neuronal cells [19]. Moreover, exogenous ghrelin also inhibited the activation of caspase-3, c-Jun and p-38 in oxygen-glucose deprivation-induced apoptosis [19, 43]. Our previous study demonstrated that ghrelin could also attenuate Bax expression and caspase-3 activation to promote the survival of dopaminergic neurons in a MPTP-induced PD mouse model [5]. Additionally, ghrelin administration for 8 weeks promoted a decreased Bcl-2/Bax ratio in the SN of homozygous A53T mice. These findings suggest that ghrelin exerts its neuroprotective effect on dopaminergic neurons by anti-apoptosis processes.

In summary, with the progression of disease in homozygous A53T mice, the plasma ghrelin levels decreased, and continuous ghrelin administration could restore the decreased plasma ghrelin to normal levels. Continuous dose of ghrelin did not lead to obesity in WT mice. With the recovery of the plasma ghrelin, the degeneration of dopaminergic neurons in homozygous A53T mice was improved significantly in terms of increased numbers of TH-positive cells and TH protein levels in the SN. Treatment with ghrelin for 4 weeks had no neuroprotective effects on dopaminergic neurons at the age of 3 months; however, treatment with ghrelin for 8 weeks had a neuroprotective effect on dopaminergic neurons in the SN at the age of 3 months, and this effect lasted through 6 months of age. This early intervention with ghrelin may delay the progression of disease in homozygous A53T mice by inhibiting apoptosis decreasing oxidative stress and inflammation. Therefore, ghrelin can improve the progression of neurodegenerative diseases, which will have important potential clinical value for preventing the onset of PD, especially familial PD.

5 Conclusions

In the present study, we found endogenous brain-gut peptide “ghrelin” could recover the decrease of plasma ghrelin levels and weight loss of PD mice. Ghrelin administration could restrict the degeneration of dopaminergic neurons in the SNpc and prevent the onset of PD. Anti-antioxidant, anti-inflammatory and anti-apoptosis effects of ghrelin, which might make ghrelin as a new possible peptide for preventing PD.

List Of Abbreviations

DA	dopamine
ELISA	enzyme-linked immunosorbent assay
GHSR	growth hormone secretagogue receptor
HPLC-ECD	high-performance liquid chromatography electrochemical detection
IL	interleukin
LPS	lipopolysaccharides
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MPP^+	1-methyl-4-phenylpyridinium
PD	Parkinson's disease
PFA	paraformaldehyde
IF	immunofluorescence
ROS	reactive oxygen species
OS	oxidative stress
SDS	sodium dodecyl sulfate
Str	striatum
SVZ	subventricular zone
TH	tyrosine hydroxylase
TNFα	tumor necrosis factor α
WT	wild type

Declarations

- Ethics approval and consent to participate

All animal care and protocols were approved by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and Animal Ethics Committee of Qingdao University (20140522).

- Consent to publication

Informed consent for publication was obtained from all participants.

- Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

- Competing interests

There is no conflict of interest or commercial relationship and/or support from pharmaceutical or other companies.

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- Authors' contributions

Hong Jiang and Qiao Jiao were responsible for the overall conception of the project and provided supervision. Lingling Jiao, Fengju Jia, Xixun Du, and Yong Li conducted the experimental work, and/or analyzed the data, prepared the figures and wrote the manuscript. Pei Zhang, Tingting Tang and Dexiao Zhu assisted with sample processing, data discussion and conducted all immunohistochemistry. Qian Jiao and Xixun Du assisted with maintenance of availability of reagents, sample processing, data discussion and data analysis. All authors contributed to the editing of the paper.

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Not applicable

References

1. Mortality GBD, Causes of Death C: **Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980-2015: a systematic analysis for the Global Burden of Disease Study 2015.** *Lancet* 2016, **388:**1459-1544.
2. Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K: **Ghrelin is a growth-hormone-releasing acylated peptide from stomach.** *Nature* 1999, **402:**656-660.
3. Tschop M, Smiley DL, Heiman ML: **Ghrelin induces adiposity in rodents.** *Nature* 2000, **407:**908-913.
4. Jiao Q, Du X, Li Y, Gong B, Shi L, Tang T, Jiang H: **The neurological effects of ghrelin in brain diseases: Beyond metabolic functions.** *Neurosci Biobehav Rev* 2017, **73:**98-111.

5. Jiang H, Li LJ, Wang J, Xie JX: **Ghrelin antagonizes MPTP-induced neurotoxicity to the dopaminergic neurons in mouse substantia nigra.** *Exp Neurol* 2008, **212:**532-537.
6. Shi L, Bian X, Qu Z, Ma Z, Zhou Y, Wang K, Jiang H, Xie J: **Peptide hormone ghrelin enhances neuronal excitability by inhibition of Kv7/KCNQ channels.** *Nat Commun* 2013, **4:**1435.
7. Song N, Wang W, Jia F, Du X, Xie A, He Q, Shen X, Zhang J, Rogers JT, Xie J, Jiang H: **Assessments of plasma ghrelin levels in the early stages of parkinson's disease.** *Mov Disord* 2017, **32:**1487-1491.
8. Wang W, Song N, Jia F, Tang T, Bao W, Zuo C, Xie J, Jiang H: **Genomic DNA levels of mutant alpha-synuclein correlate with non-motor symptoms in an A53T Parkinson's disease mouse model.** *Neurochem Int* 2018, **114:**71-79.
9. Jiang H, Luan Z, Wang J, Xie J: **Neuroprotective effects of iron chelator Desferal on dopaminergic neurons in the substantia nigra of rats with iron-overload.** *Neurochem Int* 2006, **49:**605-609.
10. Lee HM, Koh SB: **Many Faces of Parkinson's Disease: Non-Motor Symptoms of Parkinson's Disease.** *J Mov Disord* 2015, **8:**92-97.
11. Gaig C, Tolosa E: **When does Parkinson's disease begin?** *Mov Disord* 2009, **24 Suppl 2:**S656-664.
12. Akbar U, He Y, Dai Y, Hack N, Malaty I, McFarland NR, Hess C, Schmidt P, Wu S, Okun MS: **Weight loss and impact on quality of life in Parkinson's disease.** *PLoS One* 2015, **10:**e0124541.
13. Dickson SL, Egecioglu E, Landgren S, Skibicka KP, Engel JA, Jerlhag E: **The role of the central ghrelin system in reward from food and chemical drugs.** *Mol Cell Endocrinol* 2011, **340:**80-87.
14. Chebani Y, Marion C, Zizzari P, Chettab K, Pastor M, Korostelev M, Geny D, Epelbaum J, Tolle V, Morisset-Lopez S, Pantel J: **Enhanced responsiveness of Ghsr Q343X rats to ghrelin results in enhanced adiposity without increased appetite.** *Sci Signal* 2016, **9:**ra39.
15. Hewson AK, Dickson SL: **Systemic administration of ghrelin induces Fos and Egr-1 proteins in the hypothalamic arcuate nucleus of fasted and fed rats.** *J Neuroendocrinol* 2000, **12:**1047-1049.
16. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S: **Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion.** *J Clin Endocrinol Metab* 2002, **87:**240-244.
17. Briggs DI, Lockie SH, Wu Q, Lemus MB, Stark R, Andrews ZB: **Calorie-restricted weight loss reverses high-fat diet-induced ghrelin resistance, which contributes to rebound weight gain in a ghrelin-dependent manner.** *Endocrinology* 2013, **154:**709-717.
18. Andrews ZB, Erion D, Beiler R, Liu ZW, Abizaid A, Zigman J, Elsworth JD, Savitt JM, DiMarchi R, Tschoep M, et al: **Ghrelin promotes and protects nigrostriatal dopamine function via a UCP2-dependent mitochondrial mechanism.** *J Neurosci* 2009, **29:**14057-14065.
19. Chung H, Seo S, Moon M, Park S: **Phosphatidylinositol-3-kinase/Akt/glycogen synthase kinase-3 beta and ERK1/2 pathways mediate protective effects of acylated and unacylated ghrelin against oxygen-glucose deprivation-induced apoptosis in primary rat cortical neuronal cells.** *J Endocrinol* 2008, **198:**511-521.

20. Dobbs RJ, Charlett A, Purkiss AG, Dobbs SM, Weller C, Peterson DW: **Association of circulating TNF-alpha and IL-6 with ageing and parkinsonism.** *Acta Neurol Scand* 1999, **100:**34-41.
21. Scalzo P, Kummer A, Cardoso F, Teixeira AL: **Serum levels of interleukin-6 are elevated in patients with Parkinson's disease and correlate with physical performance.** *Neurosci Lett* 2010, **468:**56-58.
22. Orr CF, Rowe DB, Halliday GM: **An inflammatory review of Parkinson's disease.** *Prog Neurobiol* 2002, **68:**325-340.
23. Chen WW, Zhang X, Huang WJ: **Role of neuroinflammation in neurodegenerative diseases (Review).** *Mol Med Rep* 2016, **13:**3391-3396.
24. Ogata A, Tashiro K, Nukuzuma S, Nagashima K, Hall WW: **A rat model of Parkinson's disease induced by Japanese encephalitis virus.** *J Neurovirol* 1997, **3:**141-147.
25. Qin L, Wu X, Block ML, Liu Y, Breese GR, Hong JS, Knapp DJ, Crews FT: **Systemic LPS causes chronic neuroinflammation and progressive neurodegeneration.** *Glia* 2007, **55:**453-462.
26. Jang H, Boltz D, Sturm-Ramirez K, Shepherd KR, Jiao Y, Webster R, Smeyne RJ: **Highly pathogenic H5N1 influenza virus can enter the central nervous system and induce neuroinflammation and neurodegeneration.** *Proc Natl Acad Sci U S A* 2009, **106:**14063-14068.
27. Lee JY, Yune TY: **Ghrelin inhibits oligodendrocyte cell death by attenuating microglial activation.** *Endocrinol Metab (Seoul)* 2014, **29:**371-378.
28. Moon M, Kim HG, Hwang L, Seo JH, Kim S, Hwang S, Kim S, Lee D, Chung H, Oh MS, et al: **Neuroprotective effect of ghrelin in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson's disease by blocking microglial activation.** *Neurotox Res* 2009, **15:**332-347.
29. Beynon AL, Brown MR, Wright R, Rees MI, Sheldon IM, Davies JS: **Ghrelin inhibits LPS-induced release of IL-6 from mouse dopaminergic neurones.** *J Neuroinflammation* 2013, **10:**40.
30. Lee ES, Yoon YS, Park CY, Kim HS, Um TH, Baik HW, Jang EJ, Lee S, Park HS, Oh SW: **Eradication of Helicobacter pylori increases ghrelin mRNA expression in the gastric mucosa.** *J Korean Med Sci* 2010, **25:**265-271.
31. Lee S, Kim Y, Li E, Park S: **Ghrelin protects spinal cord motoneurons against chronic glutamate excitotoxicity by inhibiting microglial activation.** *Korean J Physiol Pharmacol* 2012, **16:**43-48.
32. Qian L, Hong JS, Flood PM: **Role of microglia in inflammation-mediated degeneration of dopaminergic neurons: neuroprotective effect of interleukin 10.** *J Neural Transm Suppl* 2006**:**367-371.
33. Johnston LC, Su X, Maguire-Zeiss K, Horovitz K, Ankoudinova I, Guschin D, Hadaczek P, Federoff HJ, Bankiewicz K, Forsayeth J: **Human interleukin-10 gene transfer is protective in a rat model of Parkinson's disease.** *Mol Ther* 2008, **16:**1392-1399.
34. Arimoto T, Choi DY, Lu X, Liu M, Nguyen XV, Zheng N, Stewart CA, Kim HC, Bing G: **Interleukin-10 protects against inflammation-mediated degeneration of dopaminergic neurons in substantia nigra.** *Neurobiol Aging* 2007, **28:**894-906.

35. Alam ZI, Jenner A, Daniel SE, Lees AJ, Cairns N, Marsden CD, Jenner P, Halliwell B: **Oxidative DNA damage in the parkinsonian brain: an apparent selective increase in 8-hydroxyguanine levels in substantia nigra.** *J Neurochem* 1997, **69:**1196-1203.
36. Rodriguez-Rocha H, Garcia-Garcia A, Pickett C, Li S, Jones J, Chen H, Webb B, Choi J, Zhou Y, Zimmerman MC, Franco R: **Compartmentalized oxidative stress in dopaminergic cell death induced by pesticides and complex I inhibitors: distinct roles of superoxide anion and superoxide dismutases.** *Free Radic Biol Med* 2013, **61:**370-383.
37. Sea K, Sohn SH, Durazo A, Sheng Y, Shaw BF, Cao X, Taylor AB, Whitson LJ, Holloway SP, Hart PJ, et al: **Insights into the role of the unusual disulfide bond in copper-zinc superoxide dismutase.** *J Biol Chem* 2015, **290:**2405-2418.
38. Barkats M, Horellou P, Colin P, Millecamps S, Faucon-Biguet N, Mallet J: **1-methyl-4-phenylpyridinium neurotoxicity is attenuated by adenoviral gene transfer of human Cu/Zn superoxide dismutase.** *J Neurosci Res* 2006, **83:**233-242.
39. Dong J, Song N, Xie J, Jiang H: **Ghrelin antagonized 1-methyl-4-phenylpyridinium (MPP(+))-induced apoptosis in MES23.5 cells.** *J Mol Neurosci* 2009, **37:**182-189.
40. Granata R, Settanni F, Trovato L, Destefanis S, Gallo D, Martinetti M, Ghigo E, Muccioli G: **Unacylated as well as acylated ghrelin promotes cell survival and inhibit apoptosis in HIT-T15 pancreatic beta-cells.** *J Endocrinol Invest* 2006, **29:**Rc19-22.
41. Miao Y, Xia Q, Hou Z, Zheng Y, Pan H, Zhu S: **Ghrelin protects cortical neuron against focal ischemia/reperfusion in rats.** *Biochem Biophys Res Commun* 2007, **359:**795-800.
42. Li B, Zeng M, He W, Huang X, Luo L, Zhang H, Deng DY: **Ghrelin protects alveolar macrophages against lipopolysaccharide-induced apoptosis through growth hormone secretagogue receptor 1a-dependent c-Jun N-terminal kinase and Wnt/beta-catenin signaling and suppresses lung inflammation.** *Endocrinology* 2015, **156:**203-217.
43. Chung H, Kim E, Lee DH, Seo S, Ju S, Lee D, Kim H, Park S: **Ghrelin inhibits apoptosis in hypothalamic neuronal cells during oxygen-glucose deprivation.** *Endocrinology* 2007, **148:**148-159.

Figures

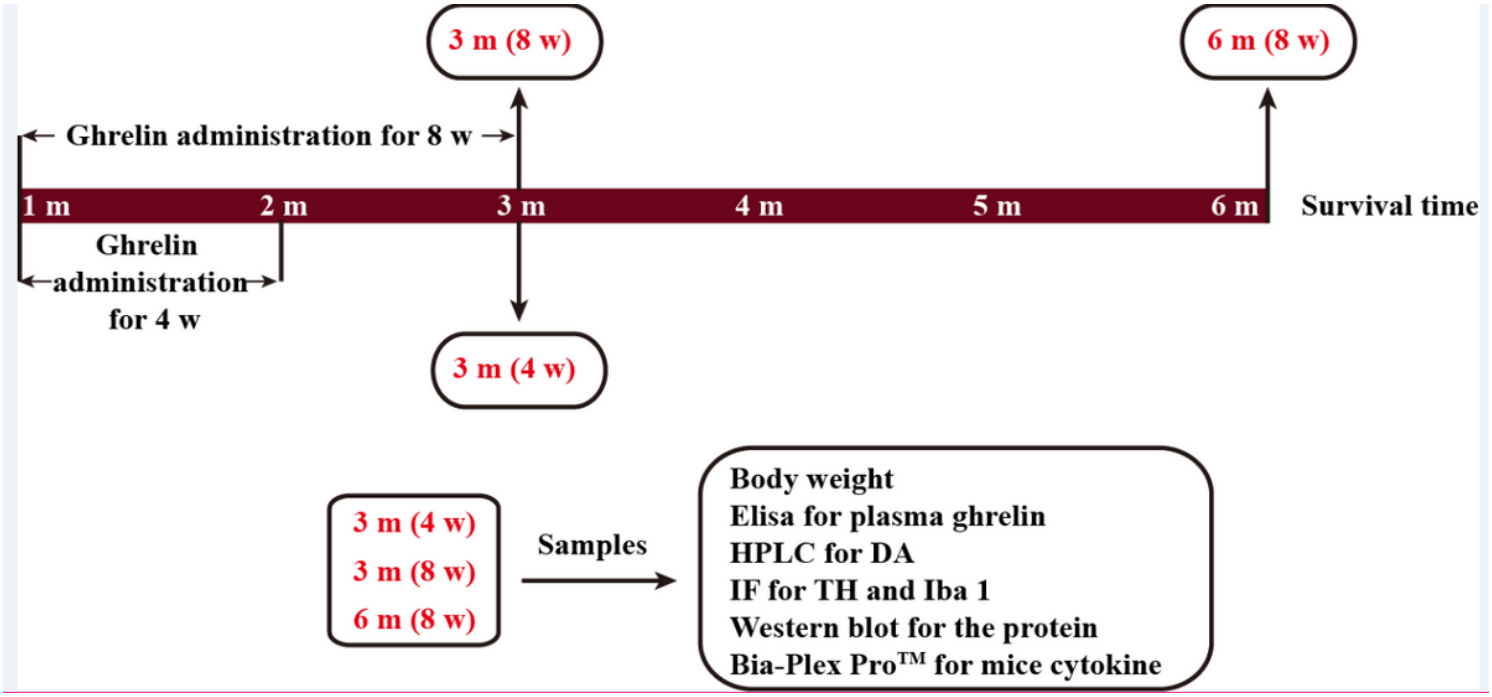

Figure 1

Experimental design. The chart illustrates the experimental design, including drug administration, body weight, enzyme-linked immunosorbent assay (ELISA), immunofluorescence (IF), high-performance liquid chromatography electrochemical detection (HPLC-ECD), western blot and Bio-Plex ProTM mouse cytokine assay.

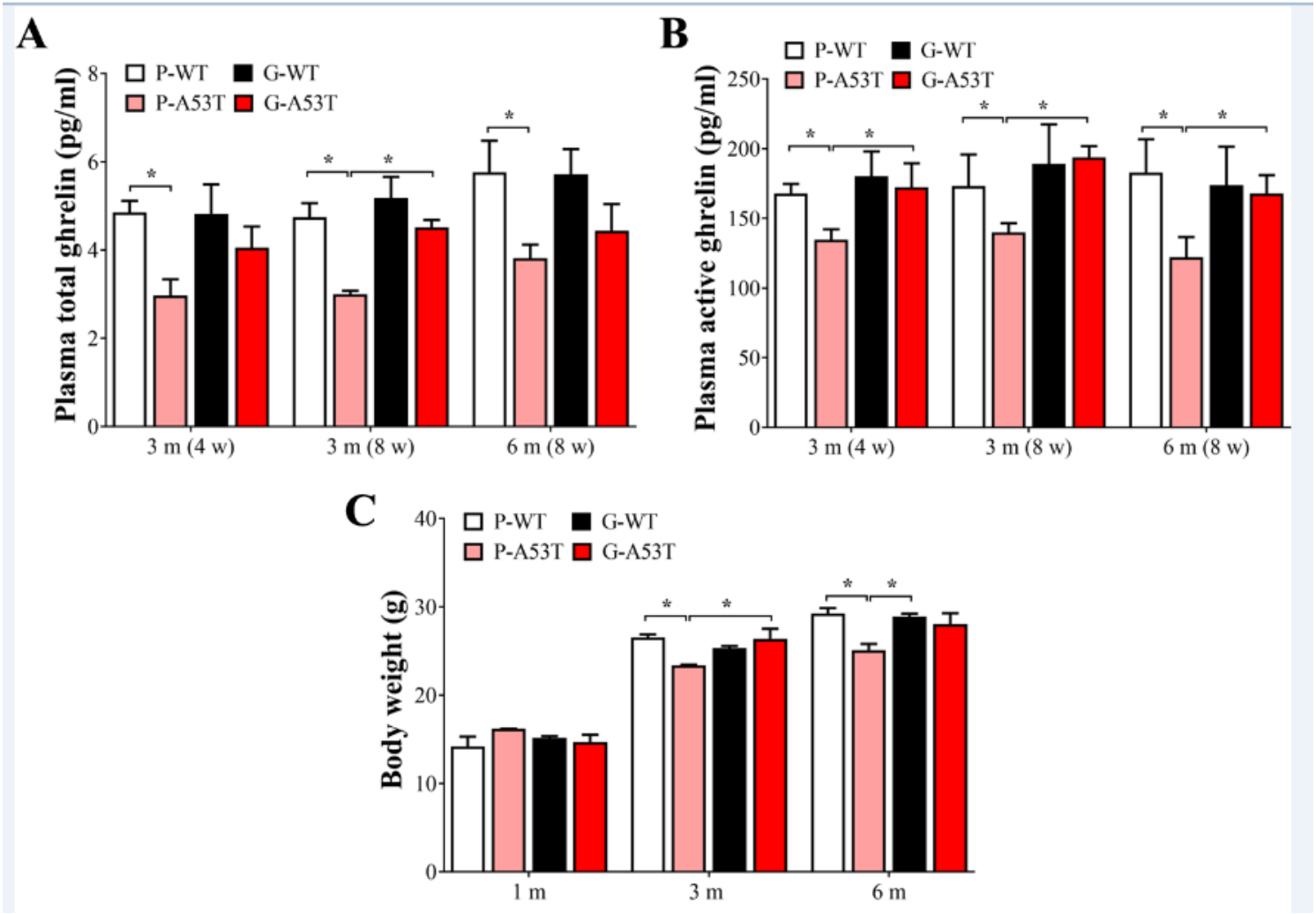

Figure 2

The effect of early ghrelin intervention on plasma ghrelin and body weight in A53T+/+ mice. Quantification of plasma total ghrelin (A) and active ghrelin (B) in WT and A53T mice at 3 m (4 w), 3 m (8 w) and 6 m (8 w) by ELISA. C: The body weight of WT and A53T mice at 1 m (N=10), 3 m (N=10) and 6 m of age. Data are the mean±SEM, *P<0.05, N=5.

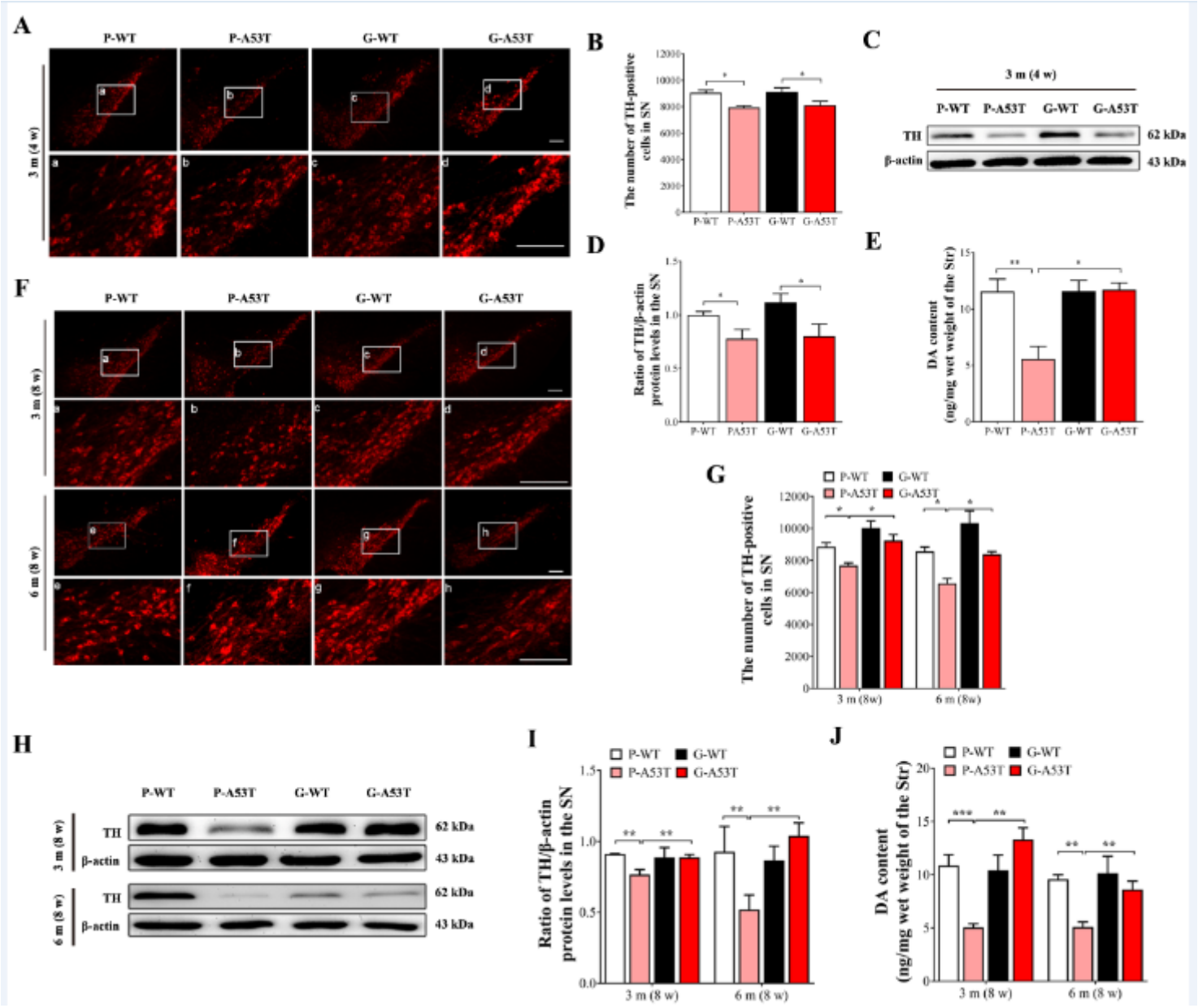

Figure 3

The effect of early ghrelin intervention on dopaminergic neurons in the SN of α-SynA53T+/+ mice. A: Labelling for TH (red) in 20-μm-thick sections of the SN from 3 m (4 w) WT and A53T mice. B: Quantification of TH staining in the SN. C: Representative immunoblots of TH and β-actin from 3 m (4 w) WT and A53T mice. D: Data analysis of the TH/β-actin ratio E: Quantification of the DA contents in the Str of mice at the age of 3 m (4 w). F: Labelling for TH (red)-positive neurons in 20-μm-thick sections of the SN from 3 m (8 w) and 6 m (8 w) WT and A53T mice. G: Quantification of TH staining in the SN. H: Representative immunoblots of TH and β-actin from 3 m (8 w) and 6 m (8 w) WT and A53T mice. I: Data analysis of the TH/β-actin ratio. J: Quantification of the DA contents in the Str of mice at the ages of 3 m (8 w) and 6 m (8 w). SN neurons were confirmed by TH IF in a serial section. Data are the mean±SEM, *P<0.05, **P<0.01, ***P<0.001, N=5, Scale bar=100 μm.

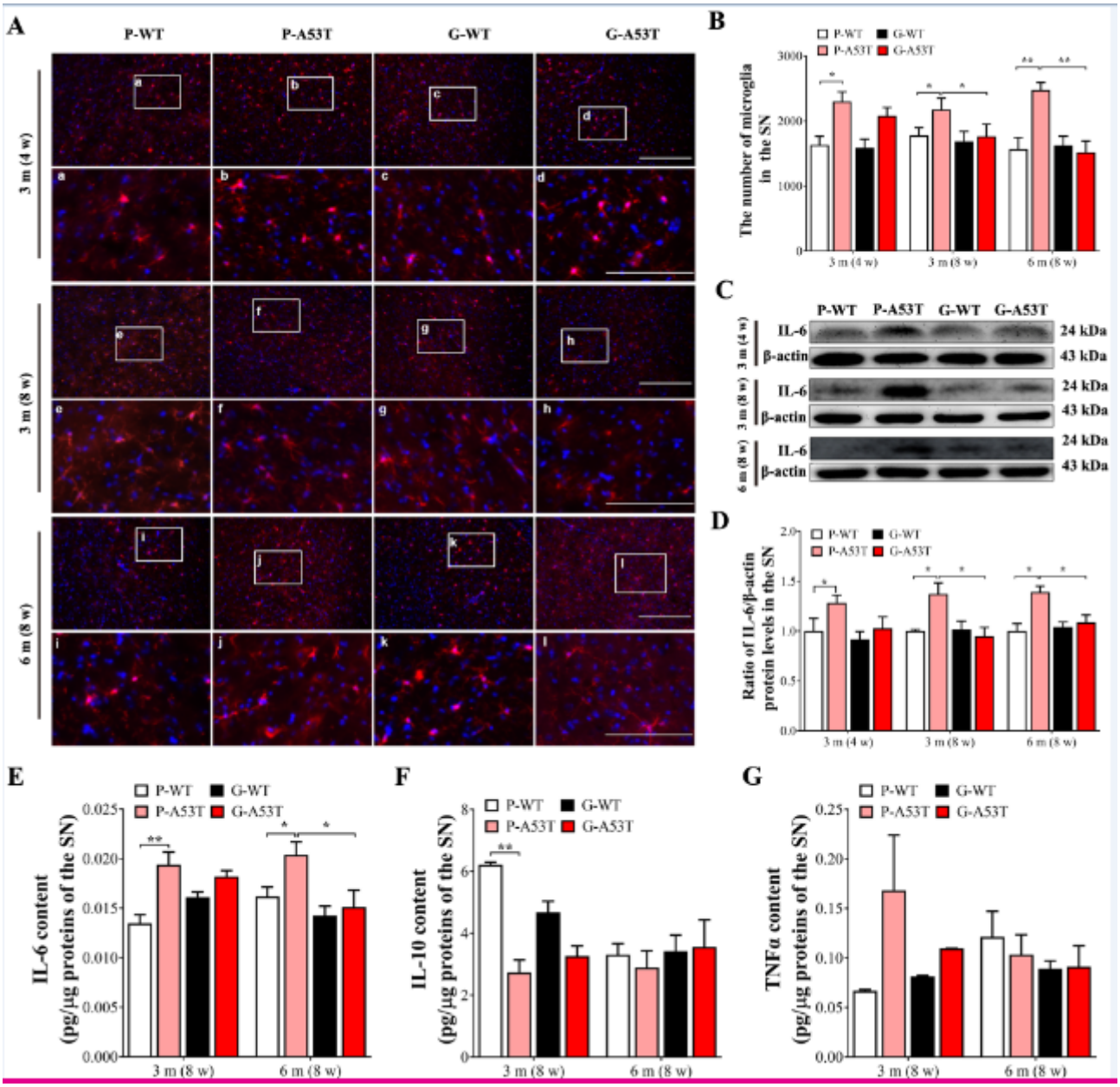

Figure 4

The effect of early ghrelin intervention on the number of microglia and inflammatory cytokines in the SN of α-SynA53T+/ + mice. A: Double labelling for Iba1 (red)-positive cells and Hoechst 33258 (blue)-staining nucleus in 20-μm-thick sections of the SN from 3 m (4 w), 3 m (8 w) and 6 m (8 w) WT and A53T mice. B: Quantification of Iba1 staining in the SN. C: Representative immunoblots of IL-6 and β-actin from 3 m (4 w), 3 m (8 w) and 6 m (8 w) WT and A53T mice. D: Data analysis of the IL-6/β-actin ratio. E-G: Bio-Plex ProTM assay for mouse cytokine levels: IL-6 (E), IL-10 (F) and TNFα (G) at the age of 3 m (8 w) and 6 m (8 w) in the SN of mice. Microglia was confirmed by Iba1 IF in a serial section. Data are the mean±SEM, *P<0.05, **P<0.01, N=5, Scale bar=100 μm.

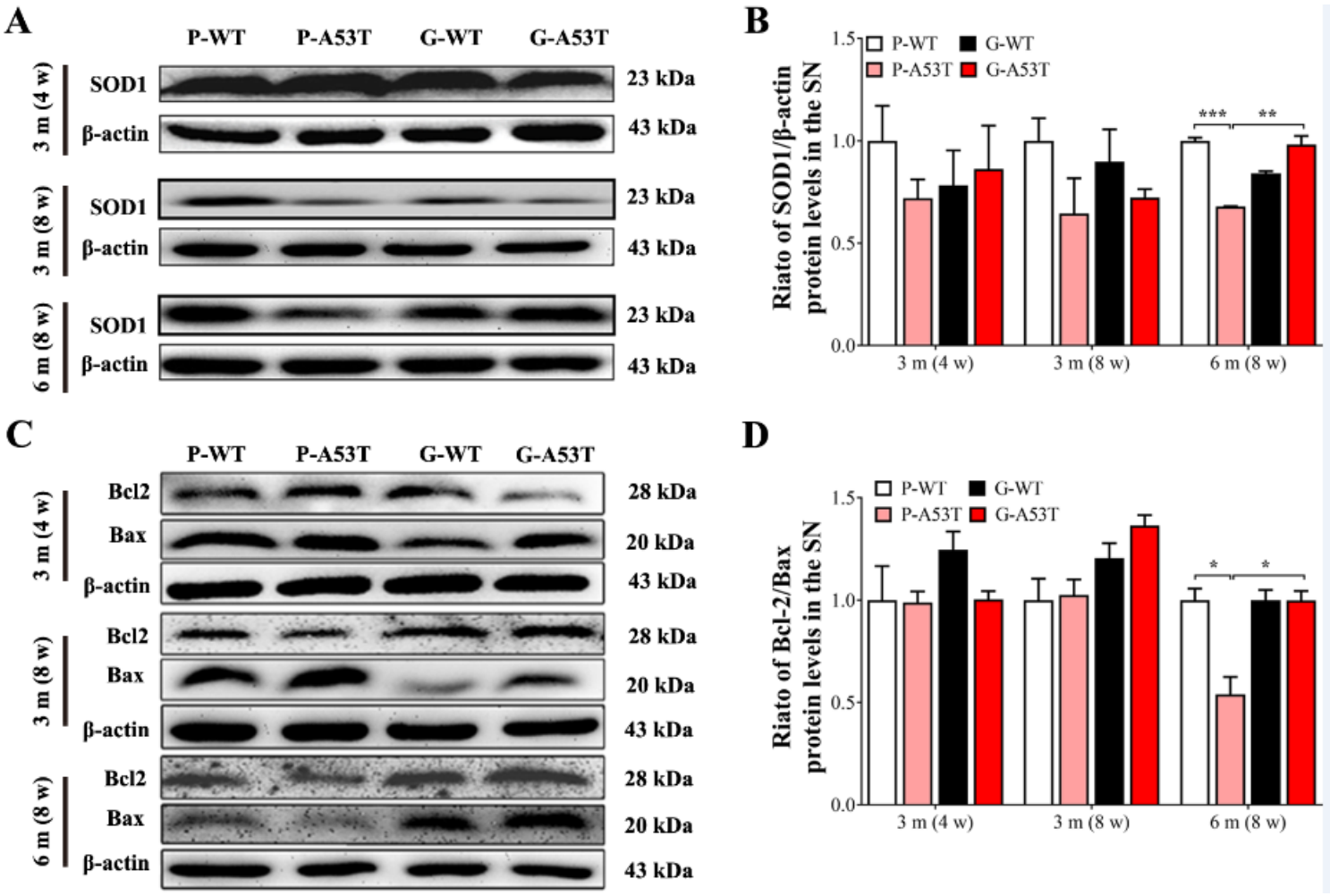

Figure 5

The effect of early ghrelin intervention on the SOD1 protein level and the Bcl-2/Bax ratio in the SN of α-SynA53T+/+ mice. A: Representative immunoblots of SOD1 and β-actin in WT and A53T mice at ages of 3 m (8 w) and 6 m (8 w). B: Data analysis of the SOD1/β-actin ratio. C: Representative immunoblots of Bcl-2, Bax and β-actin in WT and A53T mice at ages of 3 m (8 w) and 6 m (8 w). D: Data analysis of the Bcl-2/Bax ratio. Data are the mean±SEM, **P<0.01, ***P<0.001, N=5.