Early ghrelin intervention protects against the progressive dopaminergic neuron loss in Parkinson's disease mice

Lingling Jiao
Qingdao University
Fengju Jia
Qingdao University
Xixun Du
Qingdao University
Pei Zhang
Qingdao University
Yong Li
Qingdao University
Dexiao Zhu
Qingdao University
Tingting Tang
Qingdao University
Qian Jiao
Qingdao University
Hong Jiang (hongjiang@qdu.edu.cn)
Qingdao University Medical College

Research

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Abstract

Background

Ghrelin has been identified as a multifunctional peptide that has many potential applications for the treatment of various diseases, including Parkinson's disease (PD). However, little is known about the pathophysiological function and mechanism of ghrelin in 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 PD model that overexpressing mutant human A53T α-syn mice exhibited a decreased levels of total and active ghrelin in plasma, fewer tyrosine hydroxylase (TH)-positive neurons in the substantia nigra (SN), lower DA content in the striatum (Str), and less weight. These changes were rescued by the subcutaneous administration of low-dose ghrelin. Interestingly, ghrelin had no effect on weight gain in wild-type mice but improved weight loss in A53T mice. In addition, ghrelin administration also attenuated the decreased Bcl-2/Bax ratio and superoxide dismutase1 (SOD1) protein levels and inhibited the upregulation pro-inflammatory cytokine interleukin-6 (IL-6) and tumour necrosis factor a (TNFa) and the downregulation of anti-inflammatory cytokine IL-10. In addition, ghrelin inhibited the increase in Iba1-positive cells in mice with PD.

Conclusions

Here we reported that ghrelin had a protective effect on dopaminergic neurons and against weight loss from PD via anti-oxidant, anti-inflammatory and anti-apoptotic mechanisms, which suggested that ghrelin could be an endogenous protective factor that prevents the onset or the progression of PD.

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. Dopamine-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. As of 2015, PD had affected 6.2 million people and led to 11,7400 deaths worldwide [1]. Both genetics and environmental factors contributing to the process of PD have complicated its diagnosis and management, and now, the Lewy body which contains dysfunctional aggregation of α-syn was thought to be the standard for diagnosis. However, because of the slow

progressive neurodegeneration caused by this disorder, clinical diagnosis were always made later than the early stage. The biomarkers of PD are rarely discovered at the early stage; however, our previous work reported that the plasma ghrelin concentration decreased before the onset of PD. Shortly after the discovery of a mutation in the gene encoding the protein α-syn in families with inherited PD (PARK1) [2], it was reported that the replacement of threonine at position 53 of the α-syn protein with alanine (A53T) causes the severe autosomal dominant trait of parkinsonism, which is characterized by early onset with a short disease course prior to death [3]. Of these, a mouse model overexpressing human α-syn with the A53T familial PD mutation was generated that exhibited the characteristic features of PD in terms of progressive neurodegeneration in the SN and age-dependent motor and non-motor deficits [4–7].

Ghrelin, a brain-gut peptide newly discovered in 1999, is the only endogenous ligand for the growth hormone secretagogue receptor (GHSR) [8]. The concentration of serum ghrelin is high during fasting and low after calorie intake [9]. Endogenous ghrelin is involved in food intake and energy metabolism [10]. Additionally, ghrelin could freely cross the blood-brain barrier [11], and it also has roles with the central nervous system, such as neuroprotection, anti-depression and anti-anxiety effects, and memory and cognition regulation, and ghrelin also protects neurons from apoptosis, oxidative stress and inflammation, which occur in many neurodegenerative disorders, including Alzheimer's disease, amyotrophic lateral sclerosis and PD [12]. Our previous study reported that ghrelin could regulate the electrical activity of dopaminergic neurons, affect the release of DA, and exert a protective effect on dopaminergic neurons through anti-oxidation and anti-apoptosis processes [13]. Furthermore, our recent study found that plasma total ghrelin and active ghrelin levels were significantly decreased in early PD patients [14]. There was no typical postprandial inhibitory or pre-meal secretion peak of plasma ghrelin in PD patient [15, 16]. Therefore, we first administered low and continuous doses of ghrelin to sustain its concentration before the onset of plasma ghrelin decreases and then detected whether recovered ghrelin could slow PD progression. However, the half-life of ghrelin is relatively short (27–31 min) [17], and regular injections to maintain the peptide at a constant effective level are difficult. Therefore, we adopted spontaneous *Alzet* mini-osmotic pumps for continuous administration.

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); (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 [13, 18].

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 recovers decreased 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 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 restricts 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. 4A-B). 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. 4E). 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. 4A-B), and TH protein levels in the SN and DA content in the Str of P-A53T mice both decreased (Fig. 4C-E). 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 inhibits 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. 5A-B) and the protein level of IL-6 (Fig. 5C-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. 5A-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. 5C-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. 5E-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 boosts 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. 6A-B). In addition, the SOD1 protein levels of A53T homozygous mice decreased at the age of 6 months (Fig. 6A-B). Nevertheless, there was no significant change in WT mice (Fig. 6A-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. 6C-D). A53T homozygous mice showed a significant decrease in the Bcl-2/Bax ratio at the age of 6 months (Fig. 6C-D). Furthermore, no significant difference was observed in WT mice (Fig. 6C-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 ghrelin had neuroprotective effects in a mouse model of PD, with less dopaminergic neuron loss in the SN and increased DA levels in the Str. These protective effects may stem from three major functions of this peptide. First, ghrelin blocked microglial activation and inhibited the release of IL-6, both of which can mitigate neuro-inflammation and promote neuronal survivability. Second, ghrelin elevated the activity of SOD1, which acted as a homodimer to destroy naturally occurring, but harmful, reactive oxygen species (ROS) and, thus, prevented neurons from oxidative damage. Last,

ghrelin increased the Bcl-2/Bax ratio, which induced anti-apoptotic mechanisms and inhibited apoptosis during the course of the progressive degeneration of dopaminergic neurons.

The metabolic functions of ghrelin are well recognized and include the regulation of energy homeostasis, body weight and growth hormone release. The GHSR-1a, a G-protein-coupled receptor, is the only identified functional ghrelin receptor, and it was first cloned from the hypothalamus and pituitary gland and functions in growth hormone release [19]. Additionally, ghrelin is also thought to play pivotal roles in the central nervous system, in addition to its role in the hypothalamus [20, 21]. High expression of GHSR-1a has also been detected in the ventral tegmental area, subependymal region, SN and dorsal raphe nucleus [22, 23]. GHSR and TH co-expression in the SN has been recently discovered [24]. Collectively, GHSR-1a is also expressed outside the hypothalamus, indicating that ghrelin has vital neurological effects beyond its metabolic functions. Studies have shown that ghrelin plays vital roles in regulating reward [25], memory and mood [21, 26, 27]. Our previous study first reported that ghrelin can regulate the electrical activity of DA neurons, affect the release of DA, and exert a protective effect on DA neurons through anti-oxidative and anti-apoptotic processes. Ghrelin stimulated neural progenitors differentiating into TH-positive neurons in subventricular zone (SVZ) of adult mice [28]. Furthermore, our recent study found that plasma total ghrelin and active ghrelin were significantly decreased in PD patients. The present study found that homozygous A53T mice exhibited decreased plasma total ghrelin and active ghrelin from 3 to 6 months of age. After treatment with ghrelin for 4 weeks, plasma total ghrelin was not recovered; however, the increase in plasma active ghrelin was completely unexpected. Interestingly, a previous study showed that acylated ghrelin, but not des-acyl ghrelin, exerted a neuroprotective effect in a 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) mouse model of PD [29]. However, one study found that unacylated ghrelin prevented mitochondrial dysfunction in a mouse model of ischemia/reperfusion [30]. Another report indicated that both acylated and unacylated ghrelin inhibit apoptosis in myoblasts co-cultured with colon carcinoma cell lines [30, 31]. After treatment with ghrelin for 8 weeks, the plasma total level was recovered at the age of 3 months, and that at 6 months was lower than that at 3 months; however, the level was still much higher than that of homozygous A53T mice. The active ghrelin was recovered at 3 months and 6 months of age. These results indicate that early intervention with ghrelin administration recovered decreased plasma ghrelin in a PD mouse model.

PD patients exhibit non-motor symptoms, including hyposmia, sleep disturbances and gastrointestinal dysfunction, 10–20 years before they experience motor impairment [32]. Indeed, approximately 98.6% of PD patients suffered at least one non-motor symptom [33]. PD patients have a lower body weight than normal patients [34]. Correspondingly, homozygous A53T mice exhibited lower body weight in this study at 3–6 months old. Body weight is regulated by the balance between calorie intake and the amount of energy expended over a period of time. Ghrelin, the "hunger hormone", functions as a neuropeptide in the central nervous system [35]. In addition to regulating appetite, ghrelin increases body weight and fat mass by triggering receptors in the arcuate nucleus [9, 36, 37]. 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 [38]. However, 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. This study 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 [38], 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 [39]. This study also showed that ghrelin did not lead to obesity in normal mice.

Report has found that ghrelin increased the content of DA in the SN through its receptor [40]. Correspondingly, our previous study showed that homozygous A53T mice exhibited decreased dopamine transporter levels and decreased motility between approximately 3 and 12 months [41]. After treatment with ghrelin for 4 weeks, ghrelin had no protective effect on dopaminergic neurons at the age of 3 months. However, after treatment with ghrelin for 8 weeks starting at the age of 1 month, ghrelin restricted both the progressive loss of dopaminergic neurons in the SN and the depletion of DA in the Str of homozygous A53T mice at the age of 3 months, and this neuroprotective effect could last to the age of 6 months, followed by the recovery of plasma ghrelin. These results indicated that ghrelin administration prevented dopaminergic neuron degeneration in this PD mouse model, and we speculated that this early intervention of ghrelin may delay the progression of PD or even prevent the onset of PD.

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 [42]. Elevated pro-inflammatory cytokines were observed in the brain, cerebrospinal fluid and serum of PD patients [43–46]. Lipopolysaccharides (LPS) or viral pathogens could also induce PD-like neuronal death in rodents [47–49]. 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* [50]. There was a significant decrease in macrophage antigen-1 levels after ghrelin treatment, which is a classical marker of microglial activation in a threohydroxyaspartate-induced microglial activation model [51]. 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 [52]. 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 [51, 53, 54]. 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 [55–57]. 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. Oxidative stress (OS) has been well illustrated in the pathogenesis of many neurodegenerative diseases, particularly in PD [58, 59]. Increased levels of oxidation and its products, including nucleic acids, proteins and lipids, were detected in post-mortem PD brains [60, 61], suggesting that OS plays a vital role in the loss of dopaminergic neurons. An increase in intracellular ROS, particularly superoxide, was observed in

the 1-methyl-4-phenylpyridinium (MPP^+)-induced PD cell model [62]. Therefore, 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 [63]. The upregulation of malonaldehyde and the downregulation of SOD1 and catalase have been found in MPTP/MPP^+-induced PD models [64, 65]. 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 OS by reversing the reduced SOD1 [65]. 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 OS by increasing the activity of uncoupling protein 2 (an important mitochondrial protein in control of ROS production that acts as a sensor for mitochondrial OS), resulting in decreased dopaminergic neuron loss in the MPTP-induced PD model [40].

Report has shown that overexpression of mutant human α-synA53T mice was also led to neuronal degeneration and motor deficit [66]. Therefore, the ratio of Bcl-2/Bax was considered a determining factor of cell fate in response to apoptotic stimuli [67]. 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 [68–70]. 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 [12]. 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 [42]. Moreover, exogenous ghrelin also inhibited the activation of caspase-3, c-Jun and p-38 in oxygen-glucose deprivation-induced apoptosis [42, 71]. 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 [13]. 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.

Conclusions

1. Endogenous brain-gut peptide “ghrelin” can prevent the onset of PD.
2. Ghrelin restricts the degeneration of dopaminergic neurons in the SNpc.
3. Anti-antioxidant, anti-inflammatory and anti-apoptosis makes ghrelin be a new possible peptide for preventing PD.

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
SN	substantia nigra
SOD1	superoxide dismutase1
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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Figures

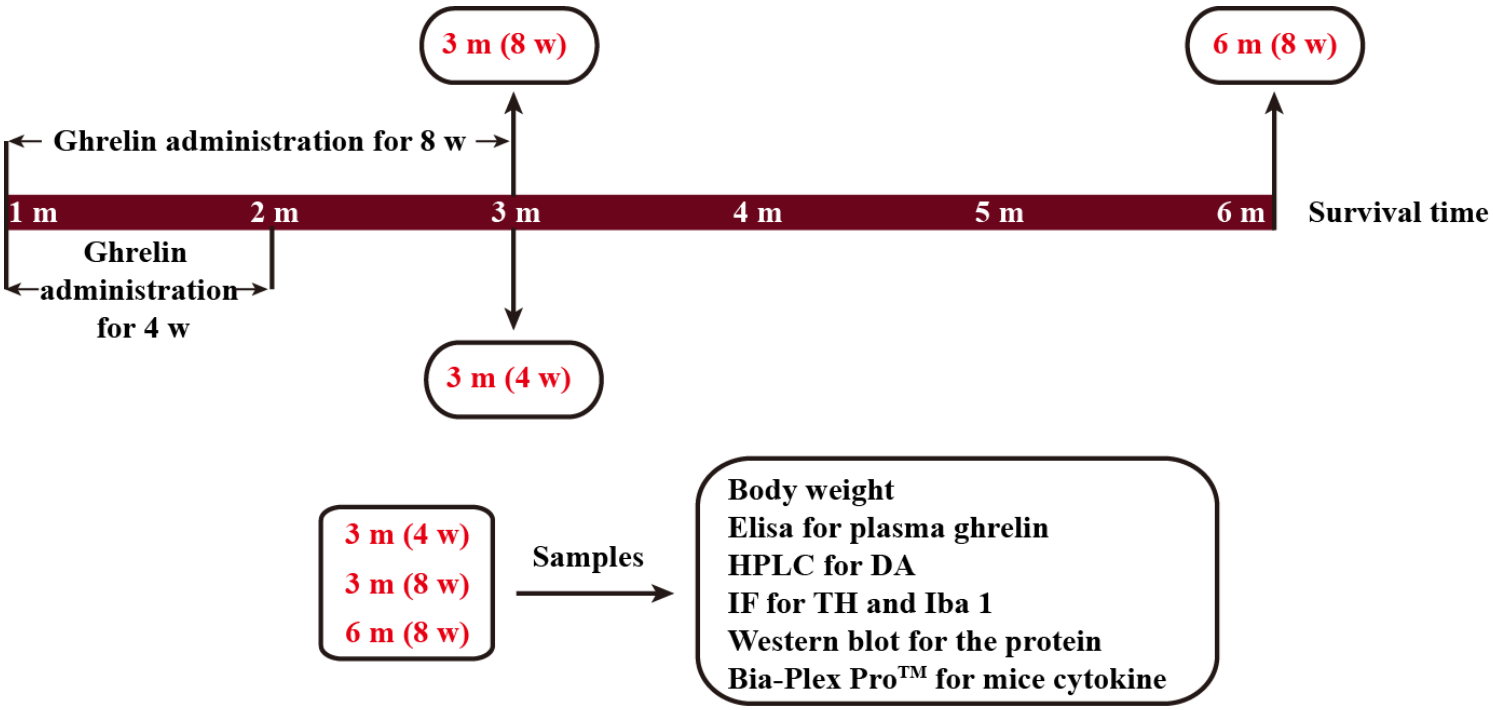

Figure 1

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.

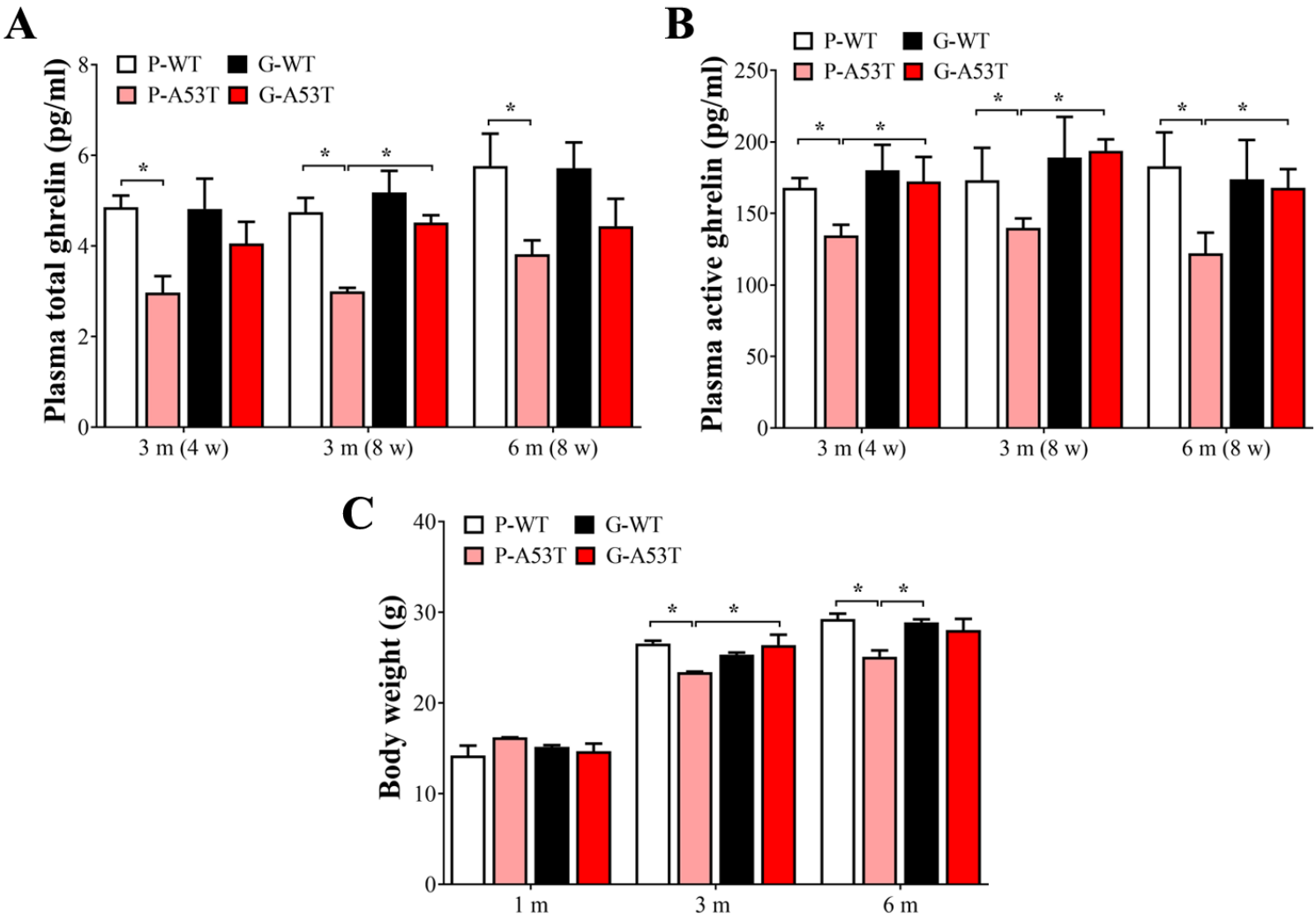

Figure 3

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.

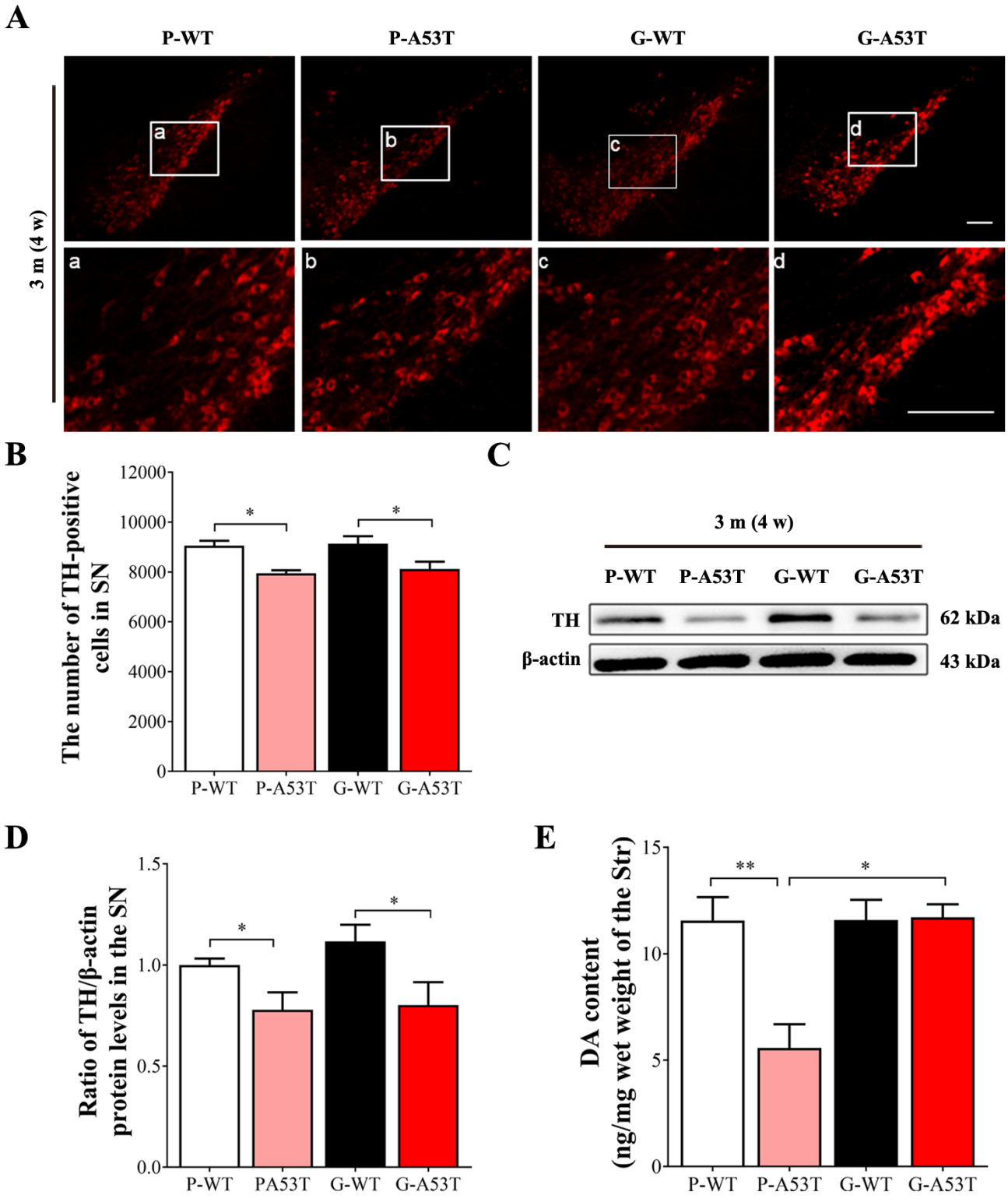

Figure 5

The effect of 4 w 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). 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.

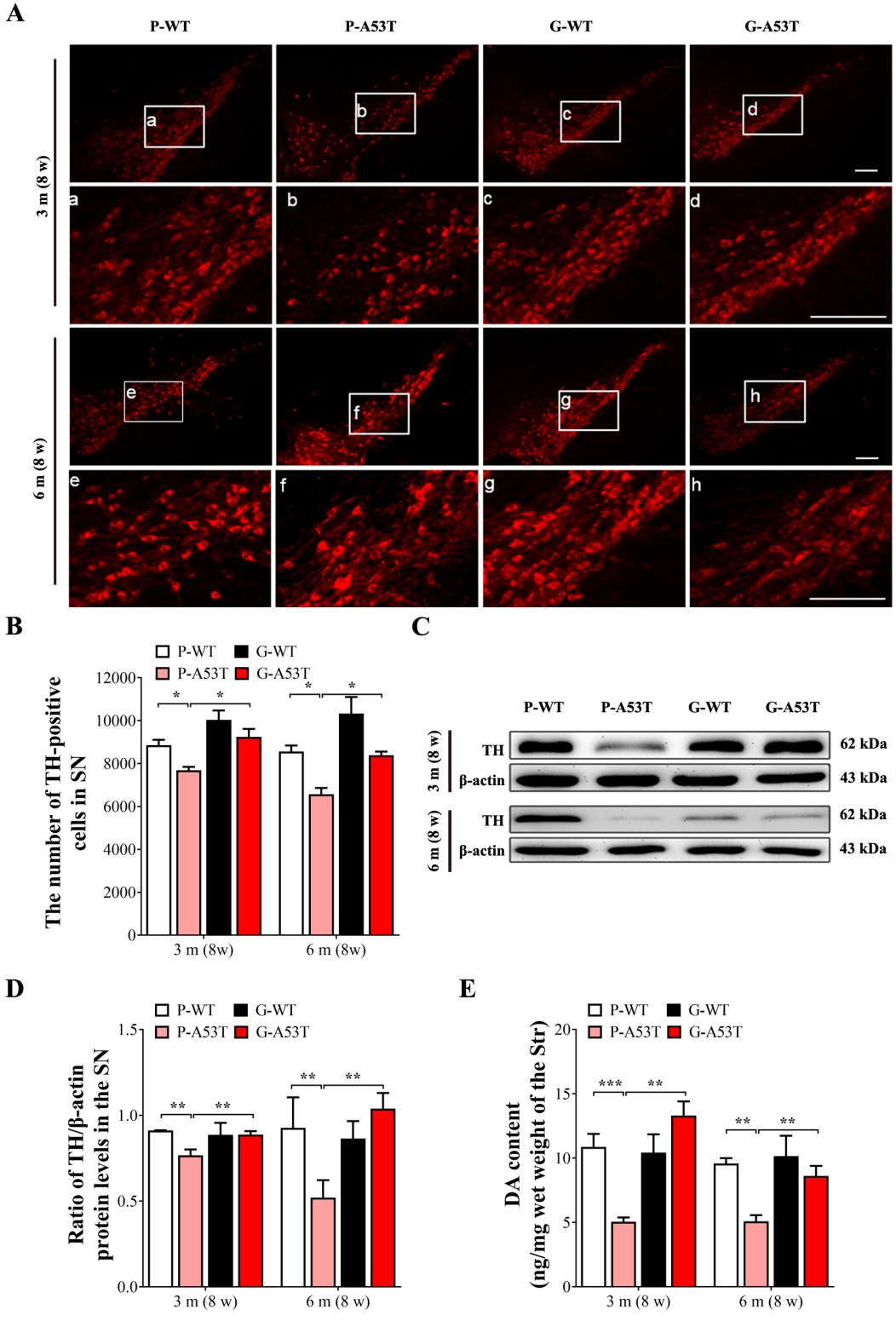

Figure 7

The effect of 8 w of early ghrelin intervention on dopaminergic neurons in the SN of α-SynA53T+/+ mice. A: 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. B: Quantification of TH staining in the SN. C: Representative immunoblots of TH and

β-actin from 3 m (8 w) and 6 m (8 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 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.

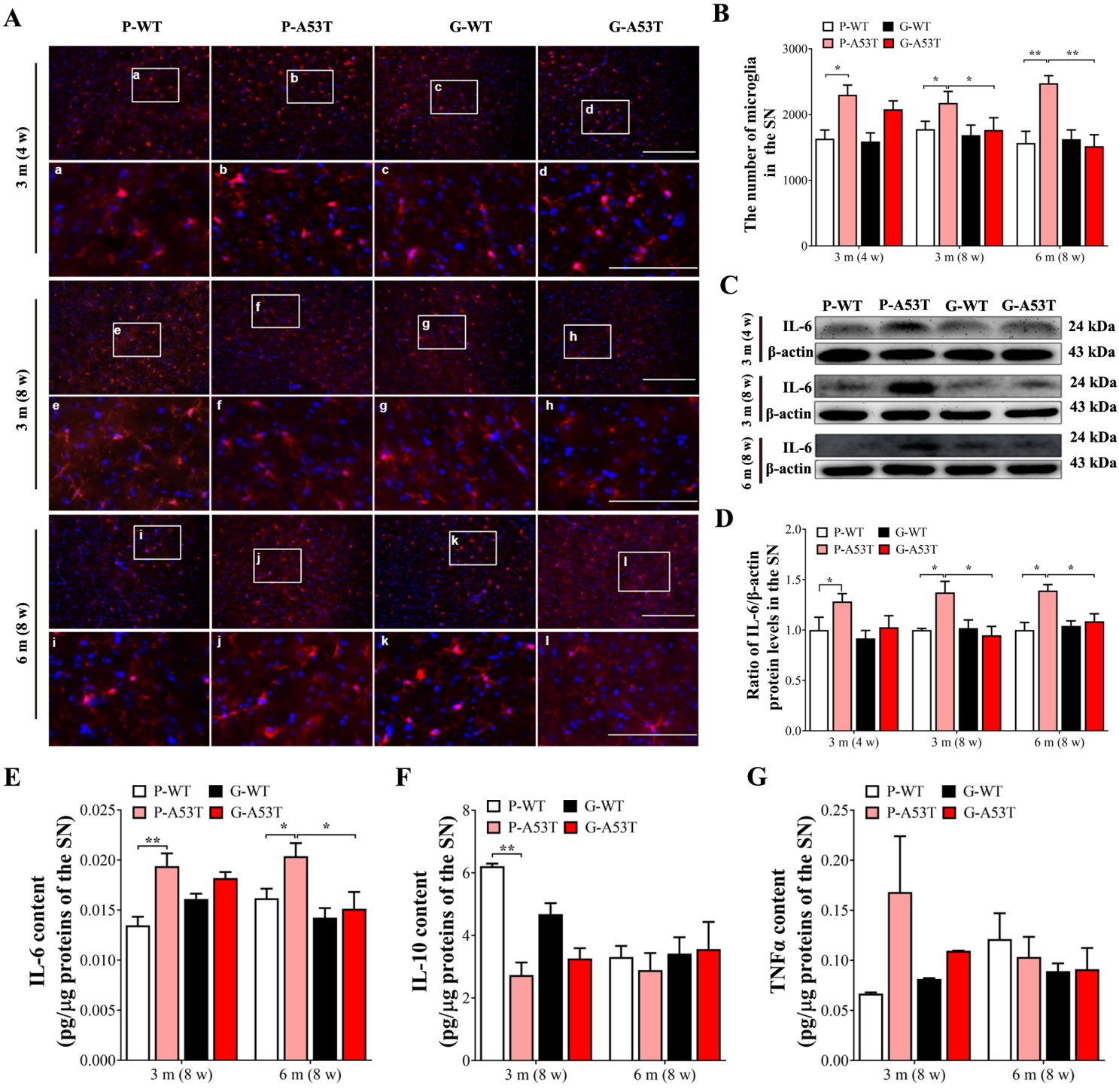

Figure 9

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.

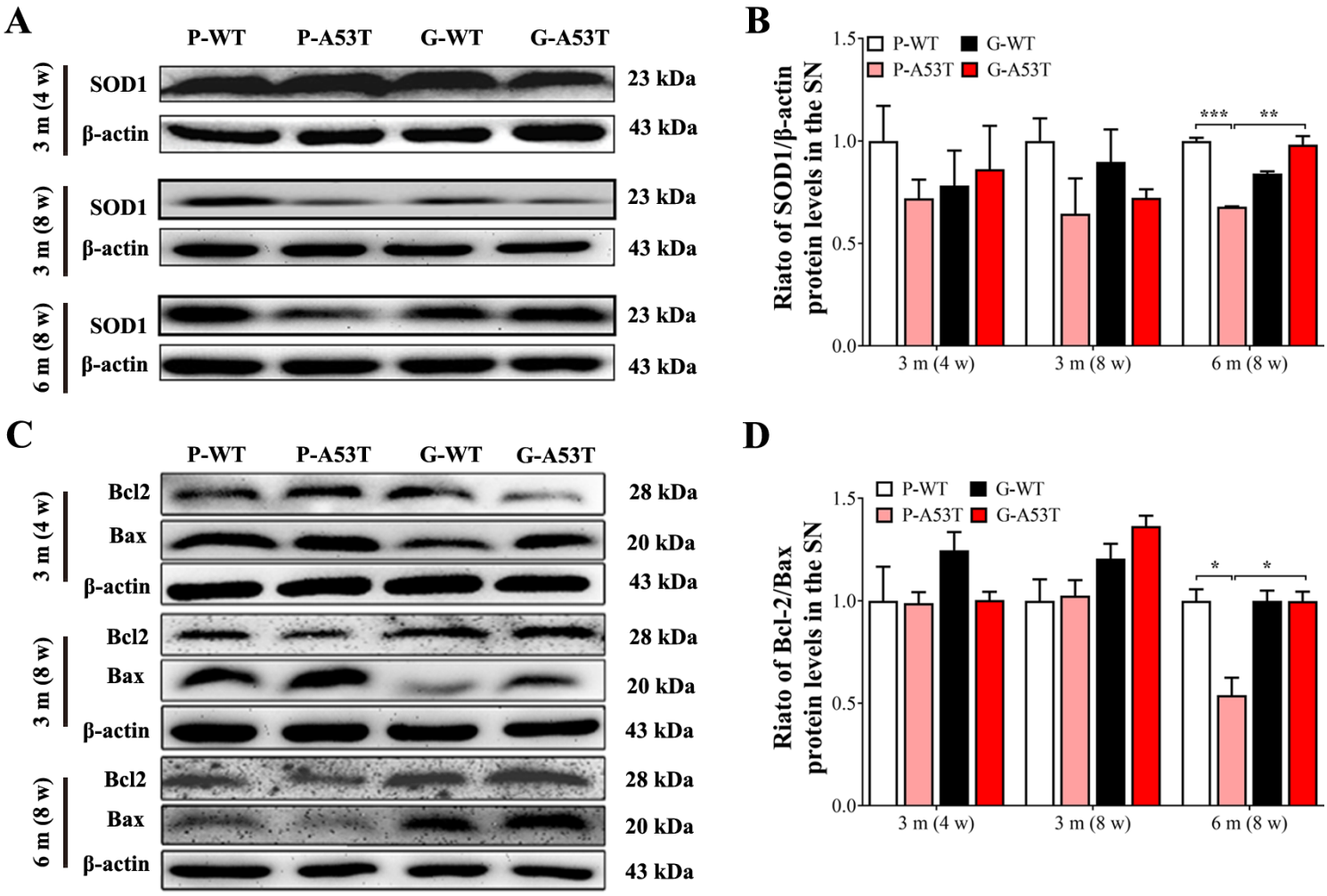

Figure 11

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.