Dopamine neuronal protection in the mouse Substantia nigra by GHSR is independent of electric activity

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ABSTRACT

Objective: Dopamine neurons in the Substantia nigra (SN) play crucial roles in control of voluntary movement. Extensive degeneration of this neuronal population is the cause of Parkinson’s disease (PD). Many factors have been linked to SN DA neuronal survival, including neuronal pacemaker activity (responsible for maintaining basal firing and DA tone) and mitochondrial function. Din-101, a naturally occurring splice variant of the human ghrelin gene, targets the ghrelin receptor (GHSR) present in the SN DA cells. Ghrelin activation of GHSR has been shown to protect SN DA neurons against 1-methyl-4-phenyl-1,2,5,6 tetrahydropyrimidine (MPTP) treatment. We decided to compare the actions of Din-101 with ghrelin and identify the mechanisms associated with neuronal survival.

Methods: Histological, biochemical, and behavioral parameters were used to evaluate neuroprotection. Inflammation and redox balance of SN DA cells were evaluated using histological and real-time PCR analysis. Designer Receptors Exclusively Activated by Designer Drugs (DREADD) technology was used to modulate SN DA neuron electrical activity and associated survival. Mitochondrial dynamics in SN DA cells was evaluated using electron microscopy data.

Results: Here, we report that the human isoform displays an equivalent neuroprotective factor. However, while exogenous administration of mouse ghrelin electrically activates SN DA neurons increasing dopamine output, as well as locomotion, the human isoform significantly suppressed dopamine output, with an associated decrease in animal motor behavior. Investigating the mechanisms by which GHSR mediates neuroprotection, we found that dopamine cell-selective control of electrical activity is neither sufficient nor necessary to promote SN DA neuron survival, including that associated with GHSR activation. We found that Din101 pre-treatment diminished MPTP-induced mitochondrial aberrations in SN DA neurons and that the effect of Din101 to protect dopamine cells was dependent on mitofusin 2, a protein involved in the process of mitochondrial fusion and tethering of the mitochondria to the endoplasmic reticulum.

Conclusions: Taken together, these observations unmasked a complex role of GHSR in dopamine neuronal protection independent on electric activity of these cells and revealed a crucial role for mitochondrial dynamics in some aspects of this process.

Keywords Parkinson’s; Mitochondrial dynamics; Dopamine neuron; Mitofusin 2; GHSR

1. INTRODUCTION

Parkinson’s Disease (PD) is a neurodegenerative disease affecting approximately 5–6 million individuals worldwide [1]. This number is expected to escalate to ~10 million by the year 2030 as the world’s population rapidly ages [2]. In the United States, more than 1 million individuals have PD, and around 60,000 new cases are diagnosed every year, causing a rapidly expanding social, medical, and financial burden. Clinical features of PD include resting tremor, muscle rigidity, bradykinesia, and postural instability, usually appearing when levels of dopamine (DA) in the dorsal striatum display a 70—80% reduction [3—5], making it very difficult for PD to be detected at early stages. Parkinson’s disease etiology is still elusive, and clinical interventions are focused on symptoms management rather than halt of disease progression. Appearing on the clinical scene in the late 60’s, L-Dopa administration was the first proposed treatment for PD and is still largely used today, as no major development has occurred regarding effective new treatments [6]. Therefore, a better understanding of how

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neurons with age and PD [35,36].

Increased accumulation of mutations in the mitochondrial DNA of encoding proteins important for mitochondrial dynamics [33,34], and mitochondrial dysfunction and fragmentation in postmortem studies, believed to be central to the pathophysiology of both familial and sporadic PD [37,38]. Anatomically, intoxication with MPTP induces the same selective loss of substantia nigra DA neurons that occurs in PD. In addition, some of the key biochemical changes elicited by MPTP treatment are the same as those identified in idiopathic and genetic forms of PD, such as deficiency in mitochondrial Complex I activity and induction of oxidative stress in DA neurons [39]. The rodent and nonhuman primate MPTP models have been used to develop both symptomatic and neuroprotective treatments for PD.

The aim of this study was to evaluate Dln101 as a potential neuroprotective compound, in comparison with ghrelin, and investigate its GHSR-mediated effects that increase DA cells is equivalent to that of ghrelin, its effect on electrical activity, dopamine release, locomotor behavior, and modulation of mitochondrial dynamics were very distinct despite the requirement of GHSR in mediating both molecules effects.

2. MATERIALS AND METHODS

2.1. Materials

Otherwise stated, all chemical were purchased from Sigma Aldrich (St. Louis, MO, USA). Dln101 and ghrelin were obtained from Peptides International (Louisville, KY, USA). CNO was obtained from Enzo Life Sciences and Salvinorin B from Cayman chemicals.

2.2. Animals

All animal procedures were performed in accordance to the National Institutes of Health Guide for the Care and Use of Laboratory Animals in research and were approved by the Institutional Animal Care and Use Committee of Yale University. All mice were handled once a day for three days prior to the start of experimental procedures for acclimation purposes. Mice harboring the dopamine-associated transporter-cre recombinase transgene (DAT-cre), the tamoxifen-inducible dopamine-associated transporter-cre (iCre-DAT), and the ribotag allele ribotag were all obtained from Jackson mice. DAT-cre-ribotag and iCre-DAT-ribotag animals permit, in a cre-dependent fashion (expressed in dopamine neurons), the immunoprecipitation of ribosome-associated mRNA. Mitofusin 2 (mfn2) floxed animals were available in our lab, as previously described [40]. Whole-body GHSR KO mice were also available in our laboratory [41].

Abbreviations

| Abbreviation | Definition |
|--------------|-----------|
| PD | Parkinson’s Disease |
| SN | Substantia nigra |
| MPTP | 1-methyl-4-phenyl-1,2,5,6 tetrahydropyridine |
| GH | ghrelin receptor |
| DREADD | Designer Receptors Exclusively Activated by Designer Drugs |
| Drp1 | dynamin-related protein 1 |
| Mfn | mitofusin |
| Mff | mitochondrial fission factor |
| OPA-1 | Dynamic-like 120 kDa protein mitochondrial |
| DAP | 2,3-diaminopropionic acid |
| TH | tyrosine hydroxylase |
| IBA1 | ionized calcium binding molecule 1 |
| CD | glycoprotein cluster of differentiation |
| CCL2 | C-C motif chemokine ligand 2 |
| TNFa | Tumor necrosis factor alpha |
| DHE | dihydroethidium |
| ROS | reactive oxygen species |
| SOD2 | superoxide dismutase 2 |
| UCP | uncoupling protein |
| DAT | dopamine-associated transporter |
| CN0 | clozapine-N-oxide |
| GOAT | ghrelin-0-acyl-transferase |
| RNS | reactive nitrogen species |
2.3. Genotyping

DNA was extracted from toe biopsy. Genomic DNA (gDNA) was extracted and standard PCR was performed using REDExtract-N-Amp™ Kit (Sigma). Cre recombinase, HA-RPL22, and Mfn2 gene presences were assessed according to PCR protocol from Jackson Laboratories. For Cre recombinase transgene, primers did not distinguish heterozygous from homozygous animals. Cre primers used were oMR1084 (5’-GGGTCTGGAGTAAAAACACT-3’) and oMR1085 (5’-GTGAAACAGCATTGCTGACTT-3’). For HA-RPL22, primers did not use 9508 (5’-GAGGAGCTGCTGGATAGT-3’) and 9509 (5’-TTTCAGACAGCAGCCTAGGA-3’). For Mfn2, primers were HC106 (5’-GAAGTAGGCGAGTCCATCG-3’) and HC 107 (5’-AACATCGCTCAGCTGACC-3’). For mCAT, primers were 18290 (5’-CCGTTGAGGACACACCTACA-3’) and 18291 (5’-TCAGGAATTCTCTCCTGGTCA-3’).

2.4. Dln101, ghrelin, and MPTP treatment

Unless otherwise indicated in text, animals were injected with ghrelin or Dln101 for a total of 10 consecutive days, subcutaneous (s.c.), 15 min before the onset of the dark cycle, with either saline (vehicle), ghrelin (15nmoles), or Dln101 (15nmoles) and had their food removed during the dark cycle. Ad libitum access to food was re-established in the next morning (8:30-9am). On the seventh day, seven animals were challenged with MPTP (60 mg/kg, divided in 4 doses, 3–4 h apart). Samples were processed on the morning of day 11.

2.5. Home cage monitoring

Mice were left to acclimate in TSE LabMaster home cages for at least 3 days before recording food intake, water intake, and locomotor activity.

2.6. Food intake and body weight measurement

All mice were individually caged 5 days before the start of feeding studies to allow the animals to acclimate to their new environment. Animals and food pellets were weighed daily.

2.7. Pharmacokinetic analysis

Pharmacokinetic parameters were determined by non-compartmental analysis using the software package, PK Solutions 2.0 (Summit Research Services). Area under the curve (AUC) values were calculated by the trapezoidal method. Maximal drug concentration in tissue (Cmax), time to reach maximal tissue levels (Tmax), apparent elimination half-life (2–6 h; sc; 2–4 h—ip), AUC and brain/plasma ratios based on AUC (0–6 h) were evaluated for plasma and brain samples at six different time points following intraperitoneal and subcutaneous administration of ghrelin or Dln101.

2.8. Immunostaining

Mice were perfused transcardially with ice-cold 0.9% NaCl saline followed by 4% paraformaldehyde, 15% picric acid (vol/vol) in 0.1M phosphate buffer (PB). Brains were removed and fixed in 4% paraformaldehyde for at least 24 h before being cut with the vibratome. Brains 50-μm thick coronal sections were cut. Before antibody incubation, all sections were blocked in 5% normal goat serum containing 1% BSA, 0.1% glycine and 0.1% L-lysine to avoid nonspecific antibody binding, then incubated with mouse monoclonal anti-TH (1:3500, Millipore MAb 318) or rabbit polyclonal anti-Iba-1 (1:3000, Wako 019–19741). After overnight incubation at 4 °C, the sections were incubated in respective biotinylated secondary antibodies, and Vectastain ABC kit (Vector Laboratories) or Avidin conjugated to Alexa 488. For visualization after ABC kit, DAB and glucose oxidase were used. All staining was visualized by Axio Scope 1 (Zeiss) and analyzed using ImageJ.

2.9. In situ detection of reactive oxygen species

Dihydroethidium (Molecular Probes, Eugene, OR) was used to investigate the local in situ production of reactive oxygen species (ROS), because it is specifically oxidized by superoxide to red fluorescent ethidium. An intravenous injection of 200 μl of dihydroethidium (DHE; stock solution, 1 mg/ml in 1% DMSO vehicle) was administered through the tail vein to lightly restrained mice. Three hours after DHE injection, mice were perfused and processed for immunostaining, as described.

2.10. Stereological analysis

The SN was delineated from −2.54 to −3.88 mm from Bregma according to Paxinos Atlas for the mouse brain [42]. The number of TH+ cells in SN was estimated counting every other section throughout the rostro-caudal extent of the SN.

2.11. Electron microscopy

Under deep anesthesia, mice were perfused transcardially with ice-cold 0.9% NaCl saline followed by 4% paraformaldehyde, 15% picric acid (vol/vol), and 0.1% glutaraldehyde (vol/vol) in 0.1M Pb, and processed and labeled for immunostaining, except for the addition of a freeze-thaw step to disrupt cellular membranes before the incubation with the primary antibody. Sections were subsequently osmicated and dehydrated, and ultrathin sections were cut on a Leica Ultra-Microtome, collected on Formvar-coated single-slot grids and analyzed with a Tecnai 12 Biotwin transmission electron microscope (TEM - FEI).

2.12. Morphological analysis of mitochondria within SN

Measurements were generated from five to ten randomly acquired TEM images. The morphology of each mitochondrion was analyzed using ImageJ software. Mitochondria were identified by the presence of identifiable cristae and distinct double membrane; mitochondria with unclear morphology were excluded from the analysis.

Mitochondrial morphology parameters and terms: “mitochondrial area” = area of every individual mitochondria. “Average mitochondrial area” = summation of the area of every mitochondria divided by the total number of mitochondria, i.e. arithmetic mean. “Cytoplasmic area” = cell area – nucleus area. “Mitochondrial cytoplasmic coverage” = summation of the area of every mitochondria divided by the cytoplasmic area, in percentage. Two types of analysis were made: using histogram curves to describe and compare the distribution of the mitochondria population between groups and comparing the averages between groups.

2.13. Rotarod

The AccuRotor Rota Rod (Accuscan Instruments, Columbus, OH) apparatus consists of a rotating bar located 20 cm above the floor. On days 1–6, all mice were trained on the rotarod (three trials per day, initial speed = 0 rpm, maximum speed = 25 rpm, time to reach maximum speed = 90 s). The cut-off time on the rotarod was 180 s. The latency to fall from the rotating bar was recorded. The average latency of both last two trials in each day was used for analyzes.

2.14. High performance liquid chromatography (HPLC) determination of neurotransmitters dopamine, serotonin, and their metabolites

Mice were euthanized, and brain tissue was dissected and snap-frozen using liquid nitrogen. Samples were sonicated in 200 μl of 0.1N perchloric acid and 0.1 ng of 3,4-dihydroxybenzylamine hydrobromide (DHBA, as internal standard); after centrifugation at 14,000 xG for...
15 min, the supernatant was filtered through a 0.22 μm syringe filter (PALL Corp. PN4454). Tissue level of neurotransmitters was measured by HPLC analysis coupled with electrochemical detection as described [43]. Fast isocratic separation was obtained using a reverse phase Agilent HC-C18 column (5 μm particle size; Agilent Technologies) with the following mobile phase: 30 mM sodium citrate, 13.7 mM sodium monobasic phosphate, 0.025 mM Na2EDTA, 0.75 mM sodium octanesulfonate; pH 3.15–3.2, containing 0.6% tetrahydrofuran, 0.1% diethylamine and 6.5% acetonitrile.

2.15. Acute brain slice preparation and electrophysiology

Coronal brain slices (250 μm) were prepared from 4- to 8-week-old animals for all genotypes tested. The slices were sectioned into ice-cold oxygenated (5% CO2, 95% O2) dissection buffer (in mM: 87 NaCl, 2.5 KCl, 1.6 NaH2PO4, 25 NaHCO3, 75 sucrose, 10 glucose, 1.3 ascorbic acid, 0.5 CaCl2, 7 MgCl2), recovered for 15 min at 37 °C in oxygenated artificial cerebrospinal fluid (aCSF) (in mM: 122 NaCl, 3 KCl, 1.2 NaH2PO4, 26 NaHCO3, 20 glucose, 2 CaCl2, 1 MgCl2, 305–310 mOsm, pH 7.3) and acclimated at room temperature for 10 min before performing electrophysiological recordings. Borosilicate glass electrodes (Sutter Instruments, Novato, CA) were used for whole cell patch clamp recordings. Electropores were pulled with tip resistance of 2 MΩ, and filled with internal solution (in mM, 120 K-glucuronate, 5 KCl, 2 MgCl2, 0.05 EGTA, 10 HEPES, 2 Mg-ATP, 0.4 Mg-GTP, 10 creatine phosphate, 290–300 mOsm, pH 7.3). During recordings, coronal brain slices were placed in a room temperature chamber mounted on an Olympus upright microscope (BX50WI) and perfused with oxygenated aCSF. Cells were visualized under differential interference contrast imaging. Data were obtained via a Multiclamp 700B amplifier, low-pass Bessel-filtered at 4 kHz, and digitized on computer disk (Clamplex, Axon Instruments).

2.16. Real-time PCR

Gene expression was investigated using real-time qPCR, performed in 96 or 384-well PCR plates using the Roche 480 LightCycler Thermal Cycler. The real-time PCR reaction mixture contained iTaq SYBR green master mix (BioRad), 0.3 mM primer pairs, and diluted cDNA in a total volume of 10 μL. The mixture was heated initially to 95 °C for 3 min to activate hot-start iTaq DNA polymerase and then followed by 50 cycles with denaturation at 95 °C for 10 s, annealing and extension at 60 °C for 60 s. Samples and standards were run in triplicate. Primers were carefully designed to span exon-junction in the mRNA and intron-spanning in the qDNA, when possible. Briefly, NCBI Primer-Blast was used, and gene and mRNA sequences were obtained from NCBI website. Primers were tested for efficiency, specificity, and primer dimer formation using 10-fold dilution curve of cDNA concentration. Additionally, a melt curve protocol designed for increment temperatures of 0.5 °C with a starting temperature of 57 °C and ending at 92 °C was performed at the end of all PCR-reactions. RNA was extracted from target tissues using RNasy Micro Kit (QIAGEN #74004) and cDNA was synthesized using Quantitect Reverse Transcription Kit (QIAGEN #205311). Real-time PCR analysis was made using the comparative threshold cycle method (ΔΔCt) with actin as reference gene. Potential reference genes, including actin, cyclophilin A, beta-2 microglobulin, glyceraldehyde-3-phosphate dehydrogenase (gapdh), and nicotinamide were tested and actin was found to show the least variation among samples. Selected human RNA samples (Kidney, Pancreas, Stomach, Duodenum, Placenta, and non commercial A172 glioblastoma cell line - Brain) were obtained from Stratagene. Primer sets were: Actin F(+)AGTGTGACGGTGGATGACAGT; R(–)GCAACTGATACATCGATGACC, TNFa F(+)(+)-CTTCTGTCTAGTCCAGTTCCAGTA, R(–)CTTCATCATCAGTTCTGTTTATAC; IFNγ F(+)GAGCTGCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; CCL5 F(+)GAGCTGCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; CD68 F(+)GAGCTGCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; ACTCCGGAAATAAGGCTGCT, IL1β F(+)(+)-AAGACTCAGGAGGGAGGCTCCCT; ThFNα F(+)(+)-ATGAGTCCAGAGGAGGCTCCCT; IFNγ F(+)GCCTGTCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; CCL5 F(+)GAGCTGCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; CD68 F(+)GAGCTGCTGACCTGTGTTTATAC; R(–)CTTCATCATCAGTTCTGTTTATAC; IL6 F(+)AGAAGAGGAGGGAGGCTCCCT; R(–)ATGAGTCCAGAGGAGGCTCCCT; 2.17. Viral vectors and stereotaxic injections

DAT-cre ribogate mice were anesthetized with a ketamine/xylazine cocktail and injected into the SN (coordinates from Bregma — AP: 3.6; ML: ±1.4; DV: 4.0 and AP: 3.65; ML: ±1.2; DV: 4.2) with 500 nL of excitatory DREADD virus (rAAV9-hSyn-DIO-mCherry; 5 × 1012 vg/ml), 500 nL of inhibitory DREADD virus (rAAV9-hSyn-DIO-KORD-ires-mCitrine; 5.3 × 1012 vg/ml), or the control virus lacking the DREADD construct rAAV5-hSyn-DIO-mCherry; 5.3 × 1012 vg/ml), or 0.9% saline vehicle control; or Salvinorin B (inhibitory DREADD agonist 0.3 mg/kg, Cayman Chemical) or DMSO vehicle control, for seven consecutive days, permitting chemosignaling of the viral injection, this procedure allows the expression of DREADDs exclusively in SN DA neurons. Mice were allowed to recover for approximately ten days and then injected daily subcutaneously 15 min before the onset of the dark phase, with either clozapine-N-oxide (CNO, excitatory DREADD agonist 0.3 mg/kg, ENZO Life Sciences) or 0.9% saline vehicle control; or Salvinorin B (inhibitory DREADD agonist 0.3 mg/kg, Cayman Chemical) or DMSO vehicle control, for seven consecutive days, permitting chemo- genetic excitation of SN DA cells with CNO or inhibition of SN DA cells with salvinorin B. In addition, for chronic and continuous release of either CNO or Salvinorin B, mice were implanted with subcutaneous osmotic mini-pumps (Alzet). Viral vector placement was assessed via florescent microscopy based on mouse brain atlas landmarks.

2.18. Osmotic mini pump implantation

Before implantation, the Alzet osmotic minipumps (Model 1007D; pumping rate, 0.5 μL/h for seven days) were filled with the appropriate solution and incubated at 37 °C for 24 h for priming and flow stabilization. At the time of the surgery, mice were given intraperitoneal injection of ketamine/xylazine (0.3 mL), and had the pump subcutaneously implanted in the space between the scapula.

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2.19. Statistics
Data are expressed as mean ± SEM unless otherwise indicated. GraphPad Prism 7 was used for all statistical analysis, and we considered p < 0.05 as statistically significant; labeled as: * = p < 0.05, ** = p < 0.01, *** = p < 0.001. When ANOVA was performed, post-hoc test was used to compare the mean of each column to the mean of the control column and correcting for multiple comparisons using the Tukey test. When comparing the mean of each column to the mean of every other column, letters (a,b,c) were used to indicate differences; unless otherwise indicated in the figure legend.

3. RESULTS

3.1. Dln101 is a human splice-variant of the ghrelin gene with different expression pattern but similar effects over food intake and body weight gain
Structurally similar to ghrelin, Dln101 is an acylated 24-amino acid peptide that is derived from a naturally occurring splice variant of human ghrelin. To increase the stability of Dln101 peptide, the ester bond between the serine at position 3 (Ser3) and the n-octanoyl group was modified to an amide bond, resulting in replacement of Ser3 by 2,3-diaminopropionic acid (DAP) (Figure 1A). Dln101 mRNA expression pattern also differs from the one observed for ghrelin. Ghrelin is mainly expressed in gastro-intestinal (GI) organs and, to a lower extent, in kidney and neural tissue. Dln101 had low to no expression in the GI tract but displayed elevated expression in kidney and neural tissue (Figure 1C). When administered to mice for 7 consecutive days, Dln101 displayed an effect similar to ghrelin, promoting increases in body-weight. We also observed a positive trend (at day 6, p = 0.09; at day 7 p = 0.056) in cumulative food intake (Figure 1B).
Next, we decided to compare acylated Dln101 (henceforth Dln101) and acylated ghrelin (henceforth ghrelin) plasma pharmacokinetic concentrations, following subcutaneous and intraperitoneal administrations as shown in Table 1. Absorption was rapid across all doses with TMAX at 5 min. Intraperitoneal (i.p.) injections of 1.2 and 7.2 mg/kg of Dln101 raised plasma concentration to peak (CMAX) at 36.3 and 588 ng/mL. Ghrelin i.p. injections reached CMAX at 7.5 and 171.4 ng/mL for 1.2 and 7.2 mg/kg doses, respectively. Subcutaneous (s.c.) injections of Dln101 displayed similar PK parameters when compared to i.p. injections. Increases in both AUC and CMAX were proportional to administered dose and route, with s.c. administrations displaying higher CMAX and AUC for 7.2 mg/kg dosage. Mean half-life of Dln101 ranged between 11.9 and 20.9 min compared to 14.8 for ghrelin.

3.2. Dln101 protects SN DA neurons against MPTP-induced degeneration, inflammation, and increased ROS production
Initially, we evaluated neuroprotection of SN DA neurons by ghrelin and Dln101 by counting SN tyrosine hydroxylase-positive (TH⁺) neurons in brain sections from mice injected, s.c., for 10 consecutive days, 15 min before the onset of the dark cycle, with either saline (first and second bar on the graph), ghrelin (15nmoles), or Dln101 (15nmoles). On day 7, animals were challenged with MPTP (60 mg/kg, i.p.), or not (control group). MPTP injection promoted vast nigral degeneration in vehicle-treated animals, with 55.9% reduction in TH⁺ cells, equally mitigated by either ghrelin or Dln101 pre-treatment, with 19.8% and 16% reduction, respectively (Figure 2AB). Preservation of motor

Table 1 — Pharmacokinetic profile of Dln101 and ghrelin. TMAX = time of maximal plasma concentration, CMAX = maximal plasma concentration, AUC0−∞ = area under the plasma concentration vs time curve from time 0 to infinity, T1/2 = half-life.

| PEPTIDE | DOSE (mg/Kg) | ROUTE | TMAX (min) | CMAX (ng/mL) | AUC0−∞ (ng.min/ml) | T1/2 (min) |
|---------|--------------|-------|------------|--------------|---------------------|------------|
| Dln101  | 1.2          | i.p.  | 5          | 36.3         | 622                 | 20.9       |
|         | 7.2          |       | 588        | 9160         | 16106               | 11.9       |
|         | 7.2          | s.c.  | 828        |             | 16106               | 12.6       |
| Ghrelin | 1.2          | i.p.  | 7.5        | NA           | NA                  |            |
|         | 7.2          |       | 171.4      | 40788        | 14.8                |            |

Figure 1: Dln101 is a human splice-variant of the ghrelin gene with different expression pattern but similar effects over food intake and body weight gain. (A) Dln101 and ghrelin peptide sequences. Mature peptides display homology of 57% and same acylation site in the third residue. (B) Chronic peripheral (s.c.) injection of Dln101 or ghrelin (7.2 mg/kg) had similar effects on Body Weight Gain and Food Intake in mice. Two-way ANOVA followed by multiple comparisons test; n = 8 per group. (C) Human ghrelin gene mRNA splicing and their respective expression pattern. Two splicing patterns of ghrelin mRNA. The boxes represent exons, short boxes represent untranslated sequences, and long boxes represent coding sequences. Top pattern yields mature ghrelin 117 amino acids preproghrelin, while bottom pattern yields Dln. Qualitative RT-PCR was conducted on 6 selected human samples (Stratagene: Kidney, Pancreas, Stomach, Duodenum, Placenta, and non-commercial A172 glioblastoma cell line — neural tissue). Water was used as negative control. Dln and ghrelin bands are indicated with arrows.
behavior was evaluated using the accelerating Rotarod test, with vehicle-treated animals used as controls. A shorter latency to fall from the apparatus is indicative of motor impairment, a hallmark of MPTP-treated animals, which displayed a reduction of 42.7% in latency to fall time when compared to controls. Animals that received either ghrelin or Dln101 before MPTP challenge, displayed comparable levels of motor performance, with 16.3 and 16% reduction in latency to fall time respectively, supporting their role as being neuroprotective of the nigrostriatal dopamine system (Figure 2C). In line with these observations, dopamine levels in dorsal striatum of animals exposed to MPTP displayed a 90% reduction (Figure 2D). Animals pre-treated with ghrelin or Dln101 showed significantly attenuated dopamine depletion of 60% and 56%, respectively. Moreover, analysis of dopamine turnover products DOPAC and HVA suggests protection of dopamine release in animals pre-treated with ghrelin and Dln101 exposed to MPTP (Figure 2D).

Next, we decided to analyze Dln101 effects over SNDA neurons that bring about protection. Based on current literature of PD, we hypothesized that mitigation of MPTP-induced nigral degeneration by Dln101 could be achieved by dampening inflammation, regulating ROS production/detoxification, augmenting midbrain dopamine neuron electrical activity, changes in mitochondria, or any combination of...
aforementioned effects. Following MPTP exposure, we observed microglial activation using ionized calcium binding adaptor molecule 1 (IBA1) immunostaining (Figure 2E) and increased levels of gene expression of activation markers, such as the glycoprotein cluster of differentiation (CD) 68 gene, the C-C motif chemokine ligand 2 (CCL-2) gene, and the inflammasome protein Nlrp3 gene. Pro-inflammatory cytokine TNFα gene also displayed increased levels after MPTP exposure. Interestingly, animals pre-treated with Din101 displayed microglial morphology similar to vehicle-treated animals (Figure 2E) and no increase in levels of TNFα, CD68, and CCL-2 genes was observed (Figure 2F). As microglia cells are activated during cellular damage, these results suggest less microglial activation in the SN and, therefore, decreased overall dopaminergic cell loss in animals pre-treated with Din101.

Accumulating evidence indicates that oxidative damage contributes to the cascade of events leading to degeneration of SN DA neurons. Evaluation of ROS levels using dihydroethidium (DHE) demonstrated a 7.46 fold increase in ROS (red) production in SN DA neurons (TH⁺ - green) following MPTP exposure (Figure 2G). Animals pre-treated with Din101 displayed reduced levels of ROS (5.25 fold increase). In further investigating mRNA levels of enzymes involved in ROS production and detoxification in SN DA neurons using the DAT-ribotag animals, we found upregulation of superoxide dismutase 2 (SOD2) and catalase (Figure 2H), involved in detoxification of superoxide and peroxide species, respectively. No changes were observed in mRNA levels for glutathione peroxidase. Surprisingly, opposite to what has been described for ghrelin treatment, we found no changes in UCP2 mRNA levels following Din101 treatment, an increase in mRNA levels of UCP5, and a tendency towards increase in UCP4 (Figure 2H), both also involved in uncoupling mechanisms in neuronal mitochondria and potential reduction of ROS formation.

3.3. Din101 and ghrelin have opposite electrophysiological effects over SN DA neurons

During the course of characterizing GHSR-mediated effects on SN DA neuron physiology, we discovered that the GHSR agonist, ghrelin, and Din101 had opposite effects on neuronal firing, while both signals act on GHSR receptors. In electrophysiological recordings, ghrelin induced an increase in the firing frequency of SN DA neurons, while Din101 decreased it (Figure 3A). Neither molecule had an effect on firing activity in SN DA cells of GHSR KO animals (Figure 3B), suggesting the requirement of GHSR for the effect on modulation of neuronal activity. To further investigate whether those effects on electrical activity of SN DA neurons in slice preparations for patch clamp analysis would indeed reflect in changes in vivo of dopamine release into the dorsal striatum and/or changes in the animals’ locomotor behavior, we evaluated the effect of ghrelin and Din101 on dopamine release in the dorsal striatum, the synaptic target of SN DA neurons. In line with the electrophysiology experiments, 30–45 min after subcutaneous injection, ghrelin induced an increase in dopamine release (Figure 3C-top) whereas Din101 induced a decrease when compared to littersmates injected with 0.9% NaCl (saline — CTR) (Figure 3C-bottom). Again, no change was observed in the dorsal striatum of GHSR KO mice (Figure 3D). To show specificity of modulation of the dopaminergic system, serotonin release in the dorsal striatum of WT animals was measured under the same conditions and neither ghrelin nor Din101 induced a change (Figure 3E).

The nigrostratal dopaminergic pathway modulates both the direct and indirect pathways of motor control by basal ganglia, and dopamine release in the dorsal striatum leads to increased motor activity. To fully characterize the role of ghrelin and Din101 in the activation of the nigrostral pathway and their effect over locomotor activity, WT mice were monitored using infrared monitors after ghrelin (15 nmoles) or Din101 (15 nmoles) injection 15 min before the onset of the dark phase. As expected for scenarios of increased electrical activity of SN DA neurons and increased dopamine release in the dorsal striatum, animals injected with ghrelin displayed a trend towards increased locomotor activity in comparison to saline-injected mice. The opposite was found in Din101-injected animals (Figure 3F).

3.4. Modulation of SN DA cell electrical activity does not interfere with MPTP-induced degeneration

The presence of GHSR was protective of SN dopamine neurons in the MPTP model regardless of whether the ligand electrically activated (ghrelin) or inhibited (Din101) the dopamine neurons. We sought to verify whether dopamine neuronal activation or inactivation per se would impact SN DA neuronal survival upon MPTP-induced cellular stress. To address that, we used mice harboring the dopamine-associated transporter-cre recombinase transgene (DAT-cre), which permits control over neuronal electrical activity, in a cre-dependent fashion (expressed in dopamine neurons). DAT-cre mice were injected bilaterally into the SN with excitatory DREADD virus (rAAV5-hSyn-DIO-Hm3Dq-mCherry; DAT-SN<sup>MM3</sup>) or inhibitory DREADD virus (rAAV9-hSyn-DIO-KORD-ires-mCitrine; DAT-SN<sup>KORD</sup>) (UNC Vector Core). DREADD receptors are only expressed in cre recombinase-positive cells, and, due to spatial localization of the viral injection, this procedure allows the expression of DREADDs exclusively in SN DA neurons (Figure 4A). Mice were then injected subcutaneously with either clozapine-N-oxide (CNO, excitatory DREADD agonist) or 0.9% saline vehicle control; or salvinorin B (inhibitory DREADD agonist) or DMSO vehicle control, permitting chemigenetic excitation or inhibition of SN DA cells with CNO or salvinorin B, respectively. To confirm proper control of activity of SN DA neuronal population, we evaluated dopamine release in the dorsal striatum and locomotor activity of DAT-SN<sup>MM3</sup> (Figure 4B-top) and DAT-SN<sup>KORD</sup> (Figure 4B-bottom). To check neuroprotection of SN DA cells after MPTP (60 mg/kg) insult, DAT-SN<sup>MM3</sup> animals were administered subcutaneously daily for 7 consecutive days, 15 min before the onset of the dark cycle, with either vehicle (first and third bar), CNO (second and fourth bar), or had an osmotic minipump filled with CNO solution implanted (fifth bar) and then challenged with MPTP (Figure 4C). After MPTP, animals were maintained in injection protocol for additional 3 days (10 days total). Similarly, DAT-SN<sup>KORD</sup> animals received either vehicle (first and third bars), Salvinorin B (second and fourth bars), or had an osmotic minipump filled with Salvinorin B solution implanted (fifth bar) (Figure 4D).

The exact same procedure was performed in GHSR-KO animals injected with DREADD virus (Figure 4E,F). Control animals underwent the same injection routine but did not receive MPTP at the end of the 7 consecutive days. Quantification of SN DA cells (TH⁺ cells) showed that neither increase nor decrease of SN DA cell electrical activity had any neuroprotective effect and both WT and GHSR-KO animals displayed the exact same level of MPTP-induced degeneration as observed in control animals. Additionally, we decided to use the DREADD approach to neutralize the effect of ghrelin and Din101 over the electrical activity of SN DA cells and verify the impact on the neuroprotection observed with these two molecules (Figure 4A). DAT-SN<sup>KORD</sup> animals treated with ghrelin and DAT-SN<sup>MM3</sup> animals treated with Din101 had their electrical activity effect neutralized with Salvinorin B or CNO, respectively. Analysis of dopamine release in the dorsal striatum confirmed neutralization (Figure 4G). However, neutralization of the electrical activity did not interfere with the neuroprotective effect of both ghrelin and Din101 (Figure 4H). Altogether,
our data suggest that modulation of electrical activity of SN DA cells is completely irrelevant for acyl-ghrelin or Dln101-mediated neuroprotection in the MPTP-induced neurodegeneration model.

3.5. Dln101 and ghrelin have opposite effects over SN DA neuron mitochondria

Mitochondrial dynamics has a direct impact over mitochondrial functions and thus is of central importance for the pathophysiology of neurodegenerative processes. However, the in vivo significance of changes in mitochondrial dynamics in the context of nigrostriatal dopaminergic cells has remained undetermined. We decided to evaluate mitochondrial morphology following ghrelin and Dln101 treatment and its impact on neuroprotection of SN DA cells using electron microscopy. Initially, we compared animals treated with either Dln101 or ghrelin for 10 consecutive days under the same protocol used to achieve neuroprotection. Analysis of the distribution of mitochondrial area demonstrated a higher percentage of bigger mitochondria in SN DA cells from Dln101-treated animals, compared to controls, shifting the histogram curve to the right and indicative of increased fusion (Figure 5A,B). Average mitochondrial area was also increased following Dln101 treatment (Figure 5C). No change in area was observed in the ghrelin-treated group (Figure 5A).

Figure 3: Effects of ghrelin and Dln101 over SN DA neuron function of GHSR KO mice and their WT littermates. (A) Using patch-clamp slice electrophysiology analysis, the firing frequency (generation of action potentials) was recorded in SN DA neurons for 15 min followed by acute ghrelin (1 μM) or Dln101 (1 μM) administration. In WT neurons, ghrelin increased (120.3% relative to baseline) neuronal activity whereas Dln101 decreased it (55.5%). (B) No effect was observed in GHSR KO animals. Paired t test; n = 4–13 per group. (C) Dopamine turnover (DOPAC/DA ratio) was evaluated by HPLC-ED 30 min after s.c. administration of saline (CTR), ghrelin 15 nM, or Dln101 15 nM. In WT mice, ghrelin promoted increased dorsal striatum dopamine release (CTR = 0.040 ± 0.002, Ghrelin = 0.066 ± 0.009, top graph). After Dln101 administration, dopamine release was reduced in dorsal striatum (CTR = 0.038 ± 0.002, Dln101 = 0.005 ± 0.001, bottom graph). Unpaired t tests, n = 4–5. (D) Dopamine release in dorsal striatum of GHSR KO animals was unaffected by ghrelin or Dln101. Unpaired t tests, n = 3–4. (E) For control purposes, serotonin turnover (5HIAA/5HT) was quantified in dorsal striatum following ghrelin or Dln101 injection and no change was observed. Unpaired t tests, n = 5–7. (F) Average locomotor activity was evaluated by infrared monitoring up to 3 h after subcutaneous administration of saline, ghrelin 15 nM, or Dln101 15 nM. Total number of beam breaks on the day of ghrelin/Dln101 administration was subtracted from total number of beam breaks from the day before, when animals received saline (baseline). Ghrelin promoted a tendency to increase average locomotor activity (p = 0.10) whereas Dln101-injected animals had the opposite effect, promoting a substantial decrease in locomotor activity. Paired t test; n = 4 per group. All other data were analyzed using unpaired t tests.
Figure 4: Modulation of electrical activity of SN DA neurons and its effects on survival, dopamine release in dorsal striatum, and locomotor activity in DAT-cre-ribotag mice. (A) GHSR-mediated protection of substantia nigra dopamine neurons by ghrelin or Dln101 occurs despite their opposite effects on neuronal electrical activity. To address whether modulation of electrical activity is sufficient or necessary for neuroprotection, we used DREADDs to evaluate neuroprotection in scenarios of controlled neuronal firing. (B) Dopamine turnover (DOPAC/DA ratio) and locomotor activity in DAT-cre-ribotag mice was evaluated 30–45 min after subcutaneous administration of vehicle (CTR), CNO (0.3 mg/kg) or Salvinorin B (5 mg/kg). Excitatory DREADD activation with CNO promoted elevated dopamine release in dorsal striatum in comparison to saline (CTR = 1.00 ± 0.13, CNO = 1.77 ± 0.08), n = 3. Inhibitory DREADD activation with Salvinorin B produced a tendency towards decreased dopamine release (CTR = 1.00 ± 0.47, Salvinorin B = 0.55 ± 0.16), n = 5. Animals had their locomotor activity recorded for 10 min. CNO promoted a tendency towards increased locomotor activity, while Salvinorin B decreased it (CTR = 10.87 ± 2.01, CNO = 13.62 ± 0.90) and (CTR = 13.37 ± 1.61, Salvinorin B = 6.67 ± 1.17), Unpaired t test; n = 3–10 per group. (C) Total number of TH⁺ cells in SN was evaluated in WT animals with expression of excitatory DREADD. CNO (0.3 mg/kg) was injected daily using the same routine used for ghrelin and Dln101 administration, or chronically released using osmotic minipump placed in the interscapular space. MPTP administration promoted vast nigral degeneration that was not prevented by the excitatory DREADD agonist, in any of the delivery formats. (D) Total number of TH⁺ cells in SN was evaluated in WT animals with expression of inhibitory DREADD. Salvinorin B (5 mg/kg) was injected or chronic released using osmotic minipump. (E) Total number of TH⁺ cells in SN was evaluated in GHSR KO animals with expression of excitatory DREADD. (F) Total number of TH⁺ cells in SN was evaluated in GHSR KO animals with expression of inhibitory DREADD. (G) Ghrelin-induced increase in dopamine release in dorsal striatum was prevented with injection of Salvinorin B in animals with expression of inhibitory DREADD. Likewise, Dln101-induced decrease in dopamine release in dorsal striatum was prevented with injection of CNO in animals with expression of excitatory DREADD. (H) Dln101 and ghrelin displayed their neuroprotective effect even with neutralization of their effects on neuronal firing. Data from C to H were analyzed using One-way ANOVA test followed by multiple comparisons test, n = 6–8 per group.
Mitochondrial cytoplasmic coverage was not changed in the Dln101 group but was increased in the ghrelin group, suggesting increased mitochondrial biogenesis promoted by ghrelin (Figure 5D), as previously described [12]. Next, we decided to evaluate changes in mitochondrial morphology following MPTP exposure (15 mg/kg) and how pre-treatment with Dln101 would impact mitochondrial changes. Animals were euthanized at 2 different time points: 4 h, and 1 day after MPTP exposure. At the 4-hours’ time point, MPTP-treated animals displayed a shift to the left in the mitochondrial histogram curve and a decrease in average mitochondrial area, all indicative of increased fission (Figure 5E—G, red). No change in mitochondrial cytoplasmic coverage was observed (Figure 5H). Surprisingly, 1 day after MPTP exposure, we actually observed a shift to the right in the mitochondrial histogram curve and an increase in average mitochondrial area (Figure 5E—G, orange). However, a marked decrease in mitochondrial cytoplasmic coverage was observed (Figure 5H), indicative of degeneration of small mitochondria and preservation of bigger mitochondria, shifting the population towards bigger mitochondria but in lower number. Interestingly, animals pre-treated with Dln101 before MPTP displayed a more pronounced shifting to the right on the mitochondrial histogram curve at 1-day time point, with associated increase in the average area of remaining mitochondria (Figure 5E—G, green). Additionally, no changes in cytoplasmic coverage were observed. Taken together, our working hypothesis was that MPTP treatment initially leads to mitochondrial fragmentation, with following degeneration of small and preservation of bigger mitochondria. Dln101 treatment induces fusion of mitochondria and therefore protects mitochondria against MPTP-induced fragmentation and degeneration.

To support the idea of Dln101-induced mitochondrial fusion, we decided to evaluate the expression of several genes related to the control of mitochondrial dynamics. Using DAT-cre-ribotag animals, we compared the gene expression between controls, Dln101, ghrelin, and MPTP-treated (all after 4h) animals. For genes involved with mitochondrial fission (Fis-1, Mff and Drp-1), no change was observed with Dln101 treatment. Ghrelin treatment led to an increase in Mff and MPTP treatment increased expression of Drp-1, the main regulator of mitochondrial fission. For genes related to mitochondrial fusion (Mitofusin 1 and 2, Opa-1), Dln101 treatment led to an increase in mfn 2 expression, but not in mfn 1. No change in these two genes was observed with ghrelin treatment. Inversely, MPTP treatment led to a markedly decrease in both mitofusins expression. Taken together, real-time pcr data indeed support the notion of increased mitochondrial fission following MPTP treatment and increased mitochondrial fusion following Dln101 treatment (Figure 5I). Moreover, data suggests that mfn2 might have an important role in this process.

3.6 Knockout of mitochondrial fusion protein 2 (MFN2) blocks Dln101-induced mitochondrial fusion in SN DA neurons

To determine if mfn2 is indeed a key factor controlling mitochondrial fusion and Dln101-mediated neuroprotection in SN DA cells, we generated mice with tamoxifen-induced deletion of mfn 2 gene in dopamine cells (iCre-DATMFN2 KO). Mice were allowed normal development and had their series of tamoxifen injections between 4 and 6 weeks of age. Deletion of mfn2 gene per se in dopamine cells did not alter dorsal striatum dopamine levels, number of SN DA cells, locomotor activity (Figure 6A—C), or generated any noticeable behavioral alteration on these animals, checked until 4 months after tamoxifen exposure. Electron microscopy analysis of mitochondria of iCre-DATMFN2 KO SN DA cells revealed no changes in mitochondrial morphology, area, or coverage (Figure 6D,E). However, when iCre-DATMFN2 KO animals were treated with Dln101, no increased rate of fusion was observed (Figure 6F—H).

3.7 Knockout of MFN 2 prevents Dln101 neuroprotective effect and, in combination with MPTP, induces an aberrant mitochondria phenotype SN DA cells

Lack of increased fusion in the Dln101-treated iCre-DATMFN2 KO animals was correlated with absence of neuroprotection against MPTP, as evaluated by total TH+ cell number in SN of those animals (Figure 7A), and motor performance in the rotarod test (Figure 7B). In fact, MPTP-treated iCre-DATMFN2 KO animals displayed loss of small mitochondria, leading to an increase in average mitochondrial area, but to a decrease in both total number of mitochondria per cell and mitochondrial cytoplasmic coverage (Figure 7D). This was similar to what was observed in WT animals (Figure 5E), after 1 day of MPTP-exposure. Noteworthy, in MPTP-treated iCre-DATMFN2 KO animals, these changes in mitochondrial content happened already 4 h after MPTP challenge. Finally, swollen mitochondria, with undefined cristae, typical of dysfunctional, mitophagy-prone mitochondria was predominant in iCre-DATMFN2 KO animals after 3 days of MPTP challenge (Figure 7C), leading to a further increase in both average mitochondrial area and coverage (Figure 7D). Supporting the notion of severe loss of mitochondrial content, we observed a continuous drop in the average number of mitochondrial units per cell following MPTP exposure (Figure 7D).

4. DISCUSSION

Ghrelin is mainly produced in the X/A-like gland cells of the gastric fundus mucosa [44] and, to a lesser degree, in the intestines, pancreas, gall bladder, liver, gonads, and breasts [45]. Ghrelin peptide is formed after post-translational processing of the 117-amino-acid peptide proghrelin. Then, native ghrelin peptide is subject to a unique modification consisting of acylation (addition of an octanoyl group) of the third serine residue (Ser3), a process well conserved among mammalian species [46]. The enzyme responsible for ghrelin acylation, before its secretion, is known as ghrelin-O-acyl-transferase (GOAT), and is predominantly expressed in gastrointestinal organs [47,48]. According to its acylation status, the peptide can be termed des-acyl or acyl ghrelin. De-acylation process is performed by esterases, including acyl-protein thioesterase-1/lysophospholipase-1 (APT-1), thought to be mainly responsible for ghrelin de-acylation in vivo [49]. Physiological action of acyl ghrelin gene products specifically in the midbrain has been matter of debate as ghrelin does not seem to be expressed in the central nervous system [50,51] despite immunolabelling in arcuate nucleus neurons [52,53], and some cells around the third ventricle [54]. On one hand, although only acyl ghrelin is able to activate GHSR, 66—94% of circulating ghrelin is actually found in the des-acyl form [55—57], due to the activity of esterases and APT-1 [49]. In addition, ghrelin has a limited capacity to cross the blood-brain barrier [58]. On the other hand, Dln101 emerges as a relevant candidate to maintain GHSR activation due to its increased stability in the acylated residue. Des-acyl ghrelin is the predominant form of ghrelin, but the lack of acylation precludes it from binding to GHSR and subsequent receptor activation. Despite this, there are several reports describing des-acyl ghrelin’s biological effects, including neuroprotection [59] and attenuation of proinflammatory cytokines release [60]. Despite the biological importance of des-acyl ghrelin has started to be elucidated, chronic administration of des-acyl ghrelin was shown inefficient in mitigating MPTP-induced SN DA neuronal degeneration in ghrelin KO animals.
Figure 5: Effect of ghrelin and Dln101 on mitochondrial dynamics of WT SN DA neurons. (A) Representative EM pictures of WT SN DA cells under various treatments, with drawing of overall mitochondrial distribution in the representative cell. Scale bar = 1 μm. (B) Cumulative probability distribution for mitochondrial area (vehicle = black, Dln101 = purple, ghrelin = red). Kolmogorov–Smirnov test, n = 315–555 per group. (C) Mitochondrial average area (vehicle = 0.143 ± 0.004 μm², Dln101 = 0.181 ± 0.006 μm², ghrelin = 0.138 ± 0.004 μm²). (D) Mitochondrial cytoplasmic coverage (vehicle = 9.94% ± 1.1%, Dln101 = 11.2% ± 0.75%, ghrelin = 13.05% ± 0.79%, as percentage of total cytoplasm area). Area and coverage were analyzed using One-way ANOVA followed by multiple comparisons test; n = 315–555 per group. (E) MPTP-induced mitochondrial fragmentation and degeneration in SN DA neurons. Representative EM pictures of SN DA cells. (F) Cumulative probability distribution for mitochondrial area (vehicle = blue, MPTP 4h = red, MPTP 1day = orange, Dln101 + MPTP 1day = green). (G) Mitochondrial average area (vehicle = 0.143 ± 0.004 μm², MPTP 4h = 0.112 ± 0.006 μm², MPTP 1day = 0.176 ± 0.006 μm², Dln101 + MPTP 1day = 0.234 ± 0.009 μm²). (H) Mitochondrial cytoplasmic coverage (vehicle = 9.94% ± 1.1%, MPTP 4h = 8.66% ± 0.68%, MPTP 1day = 6.144% ± 0.51%, Dln101 + MPTP 1day = 8.14% ± 0.55%). Kolmogorov–Smirnov test on cumulative distribution, One-way ANOVA on G and H; n = 102–473 per group. (I) Real-time PCR data of genes coding mitochondrial fission (FIS-1, MFF, DRP-1) and fusion proteins (MFN 1, MFN 2, OPA-1) at 4h time point. One-way ANOVA test followed by multiple comparisons test; n = 4–7 per group.
Figure 6: Mitofusin 2 deletion in DA neurons. (A) HPLC measurement of dorsal striatum dopamine levels (iCre-DATWT = 184.9 ± 9.1, iCre-DATMFN2KO = 139.4 ± 30.5, in ng/mg of tissue protein). Unpaired t-test, n = 4–5. (B) SN/DA neuron number did not differ between control groups and iCre-DATMFN2KO animals (Controls = 7532 ± 62, 7482 ± 198, 7464 ± 86; iCre-DATMFN2KO = 7912 ± 383). One-way ANOVA, n = 3–7. (C) Open field locomotor behavior analysis (B = before, A = after tamoxifen injections; iCre-DATWT grey bars and iCre-DATMFN2KO black bars; Total distance travelled (iCre-DATWT, B = 8.0 ± 1.7, A = 8.2 ± 1.9; iCre-DATMFN2KO, B = 10.0 ± 1.0, A = 7.4 ± 0.91; in meters). Average speed (iCre-DATWT, B = 0.026 ± 0.005, A = 0.027 ± 0.005; iCre-DATMFN2KO, B = 0.033 ± 0.003, A = 0.025 ± 0.003; in meters/second). Total time mobile (iCre-DATWT, B = 134 ± 19, A = 136 ± 20; iCre-DATMFN2KO, B = 138 ± 14, A = 132 ± 13, in seconds), total behavior test time was 300 s. Two-way ANOVA analysis retrieved no statistically significant differences, n = 11–14. (D) Representative EM pictures of SN/DA cells of control (WT, either cre negative or iCre-DATMFN2 fl/fl animals that did not receive tamoxifen) and iCre-DATMFN2KO animals, with drawing of overall mitochondrial distribution in the representative cell. (E) Cumulative probability distribution for mitochondrial area (cre negative = grey, no tamoxifen = black, iCre-DATMFN2KO = red). No change was observed between groups. Kolmogorov–Smirnov test, n = 421–723 per group. (F) Din101 does not induce mitochondrial fusion in iCre-DATMFN2KO animals. Representative EM pictures of SN/DA cells. (G) Differently of what was observed in WT animals, Din101 treatment did not induce a shift to the right in the cumulative distribution curve, suggesting no increased rate of mitochondrial fusion observed in iCre-DATMFN2KO animals. (H) No change in mitochondrial average area, or mitochondrial cytoplasmic coverage was observed with Din101 treatment in iCre-DATMFN2KO animals. One-way ANOVA test followed by multiple comparisons test, n = 421–723 per group.
proinflammatory cytokine production, microglia activation, mitochondrial dysfunction, and oxidative stress, reactive gliosis, and T-cell infiltration [61], suggesting that the neuroprotective effect of ghrelin over SN DA cells indeed requires GHSR activation. Another possible explanation for midbrain action of ghrelin gene products is based on the local activity of GOAT [62], which mRNA has been shown to have a broad expression among tissues [63], including the central nervous system [64,65]. This fact supports the idea that ghrelin gene products could potentially be acylated in situ, permitting local activation of GHSR [66]. The potential therapeutic actions of GHSR in the central nervous system has been in the limelight lately. Peripheral administration of GHSR agonists have been shown to inhibit oxidative stress, apoptosis, and mitochondrial dysfunction, and excitotoxicity both in vivo and in vitro [16,67–71], as well as exert neuroprotective effects against hippocampal and cortical neuronal death [68,72–74]. Through these actions, GHSR has thus been linked to neuroprotection in several CNS diseases such as stroke [75], Alzheimer disease [72,76], Parkinson disease [11–13,69,77], multiple sclerosis [78,79], epilepsy [80,81], and spinal cord injury [82]. Nevertheless, GHSRs have been shown to display higher constitutive basal activity in the absence of ligand [83], suggesting that (1) GHSR-mediated pathways do not need acyl ghrelin gene products to be activated but are modulated by them; (2) the expression of other endogenous ligand(s) able to regulate GHSR activity in the midbrain and other brain areas. In different cell types and tissues, acyl-ghrelin binds GHSR to activate several intracellular cascades, including ERK1/2, PI3K/Akt/mTOR, and AMPK signaling pathways, thus mediating different physiological functions. The first described pathway associated with GHSR activation was the inositol 1,4,5-trisphosphate/diacylglycerol (IP3/DAG) pathway, leading to GH release in the anterior pituitary. In other non-neuronal tissues and cell types, the binding of acyl-ghrelin to GHSR activates either the phospholipase C/inositol trisphosphate or the adenylate cyclase/protein kinase A pathways, resulting in an increase in intracellular calcium levels. These activation of these different signaling pathways could be due to different endogenous ligands, differential concentration of acyl ghrelin [84], and/or differential GHSR dimerization to other receptors (as described for dopamine, serotonin, melanocortin receptors, among others) [85–88]. In SN DA cells, mitochondrial function is regulated to some extent by 5’-AMP-activated protein kinase (AMPK), a ubiquitous enzyme that is considered a master sensor of intracellular energy stress that plays a crucial role in adaptive responses to falling energy levels (e.g., from low nutrient availability or cellular stress). AMPK signaling is activated by GHSR and declines in PD patients [89]. Ghrelin-mediated activation of AMPK is associated with neuroprotection of SN DA cells [13,14]. Thus, activating AMPK may be an effective neuroprotective strategy to help restore energy balance and redox homeostasis in SN DA neurons, but other signaling pathways might also be involved in GHSR-mediated neuroprotection and must be considered. GHSR-mediated activation of AMPK is also observed in other brain regions, including the arcuate nucleus of the hypothalamus [90]. Further characterization of GHSR-mediated signaling pathways is required to support the development of clinical therapeutics targeting GHSR in SN DA cells to promote survival. Oxidative stress, reactive gliosis, and T-cell infiltration are major pathological features in human PD and are recapitulated in the mouse MPTP model [91]. Oxidative stress defines an imbalance between the levels of reactive oxygen species (ROS) and reactive nitrogen species.
features: microglia activation and reactivation of astroglia. Speci-
postmortem brain tissue analyses (reviewed in [91]). Overall, activated
in the MPTP model [93].
In PD, the roles of oxidative stress and reactive gliosis are supported by
also generate lipid peroxidation [92] and DNA fragmentation, ultimately
leading to cell death. In addition, impairment of dopamine synthesis
has been observed as a result of oxidation of tyrosine hydroxylase (TH)
in the MPTP model [93].
In PD, the roles of oxidative stress and reactive gliosis are supported by
postmortem brain tissue analyses (reviewed in [91]). Overall, activated
glial cells contribute to increase local oxidative stress [94] and increase
proinflammatory cytokine production [91]. Reactive gliosis happens as a
consequence of cellular stress and can be divided in two major features: microglia activation and reactivation of astroglia. Specifically
in the MPTP model, microglial activation precedes the loss of DA
neurons and upregulation of proinflammatory cytokines [95]. In-
terventions that halt microglial activation have a direct impact on SN
DA neuroprotection [96]. Reactive astrogliosis and T-cell infiltration in
the SN are also present in the MPTP model [97,98], and neuro-
protection of SN DA cell is achieved with strategies that tackle either
one of those features [99–101]. Here, we demonstrated that Dln101
treatment was correlated with decreases in TH⁺ cell oxidative stress,
and microglia activation, as measured by decreased CD68 expression
in SN region, similar to observed in interventions in monkeys [102]. We
also reported prevention of upregulation of proinflammatory genes
such as TNFα and the inflammasome component Nlrp3, both of which
have been associated with MPTP-induced loss of SN DA neurons
[103,104]. Moreover, Dln101 ability to activate GHSR might have a
deeper relevance to neuroprotection beyond regulation of mitochon-
drial dynamics, once GHSR signaling has been linked to negative
control of neuroinflammation [104,105], which is believed to sustain
and exacerbate the loss of the DA neurons in PD. Taken together, our
data suggest that Dln101 can act in different components of the
pathophysiology of SN DA neurodegeneration, perhaps providing a
better outcome in clinical therapies for PD.
Another key component of proper DA cell physiology is the basal firing
activity of SN DA neurons, which maintains extracellular levels of
dopamine in the dorsal striatum that are essential for voluntary
movement [106]. Changes in SW DA neuron activity directly impacts the
amount of dopamine released in target areas and are largely observed in
PD subjects [107] as well as in PD animal models and may occur in
the absence of changes in other indirect markers of DA neuron function
(e.g. neuron number, DA content) [108,109].
The proper control of SW DA cell electrical activity is of crucial
importance because, on one hand and despite the yet elusive mech-
anism underlying such effect and identification of all cell types involved
[110], augmentation of electrical activity of SW DA neurons is thought to
be neuroprotective, as increased survival of DA cells has been re-
ported with the use of depolarizing agents such as veratridine [19] and
K⁺ [111]. Also in line with these observations, prospective cohort and
case-control studies have identified tobacco smoking as the most
significant protective factor against the risk of developing PD, decreasing it by 60% in active cigarette smokers in comparison to
those who never smoked [112,113]. The mechanisms involved in-
cludes reinforcing cholinergic stimulation of SW DA neurons with
nicotine, which also showed to be protective in mouse and monkey
MPTP models [114–116]. Consistently, degeneration of DA neurons
has been observed in situations of diminished electrical activity
[117,118], including the use of MPTP, which is linked to the activation
of KAATP channels and consequently silencing of SW DA neurons [119].
Interestingly, KAATP channels-mediated hyperpolarization of SW DA
neurons induced by mitochondrial dysfunction was mediated by
mitochondrial reactive oxygen species (ROS) [120]. These data suggest
that ROS could possibly participate in the demise of SW DA neurons by
rendering them electrically inactive [121].
One another hand, SW DA neuron activity and dopamine release relies
on a controlled modulation of cytosolic calcium levels [122]. Over-
activation of SW DA cells leads to excitotoxicity, causing mitochondrial
dysfunction and ultimately neuronal degeneration. Therefore, the fine
balance between excitation/inhibition is mandatory for proper SW DA
cell function. Based on that, we decided to investigate effects of
electrical activity per se on SN DA neuroprotection but also on GHSR-
mediated neuroprotection. Our data suggest that, at least for the acute
mouse MPTP model, modulation of electrical activity is not associated
with neuroprotection and, in fact, is not required for GHSR-mediated
ghrelin and Dln101 neuroprotection.
Mitochondrial dynamics are tightly coordinated in association with the
cell cycle and state, with complex structural and functional interactions
leading to fusion and fission of mitochondria to alter the balance of
oxidative phosphorylation, rescue damaged mitochondria and regulate
reactive oxygen species production. Mitochondrial dynamics are also
central to the pathogenesis of idiopathic and toxin-induced cases of PD,
as well as several monogenic familial PD forms. Mitochondrial length
is the result of competitive balance between GTPase dynamin-like proteins
mediators of mitochondrial fusion and fission. Mitofusin 1 (MFN1) and 2
(MFN2), located in the outer mitochondrial membrane, and optic atrophy
protein 1 (OPA1), in the inner membrane, regulate mitochondrial fusion
whereas dynamin-related protein 1 (DRP1) regulates mitochondrial
fission; among other supporting proteins involved [27,28]. Disruption
of mitochondrial fission leads to an extensively interconnected and
collapsed mitochondrial network, while defects in mitochondrial fusion
lead to mitochondrial fragmentation and loss of mitochondrial DNA. As
a promoter of mitochondrial fusion, Dln101 is thought to facilitate the
exchange of mitochondrial DNA, protein components, and metabolites
required for mitochondrial function, conferring increased resilience
against MPTP-induced cellular stress. Efficient mitochondrial fusion is
important for cell viability, because cells defective in fusion exhibit
reduced growth, decreased mitochondrial membrane potential (∆Ψm),
and defective respiration, resulting in impaired cellular energy meta-
bolism [123,124], exactly as observed in patients with PD [125], in both
sporadic and genetic forms of the disease [126].
In fact, changes in mitochondrial dynamics directly affects many things
including cellular metabolism [127], mitochondrial mass and turnover
[128], mitochondrial transport, and calcium buffering [129]. Nigro-
striatal DA neurons seem to have a preferential susceptibility to loss of
Mfn2 when compared to VTA DA neurons [130]. Although a specific
mitochondrial fusion or fission protein has not been implicated in classic
PD, recent data suggest that impaired mitochondrial fusion due to
mutations in Opas1 cause parkinsonism in the absence of clinically
significant optic atrophy [131]. Furthermore, it is well known that α-
synuclein and MPTP cause fragmentation of mitochondria [132]. Taken
together, these studies support the notion that interventions to increase
 mitochondrial fusion are crucial to re-establish proper SW DA neuronal
function and avoid neurodegeneration, as demonstrated in [133,134].
Moreover, Rappold and colleagues [134] suggest that increased mito-
chondrial fusion not only can be a strategy to prevent neuro-
degeneration but also can be used to restore proper function of
remaining cells, as impairment in mitochondrial dynamics also disrupts
synaptic release of dopamine. Spared TH⁺ cells in PD patients and
animal models of PD after establishment of Parkinsonism could benefit
from mitochondrial fusion inducing agents to re-establish proper syn-
aptic dopamine release and thus ameliorate the symptoms of PD.
Finally, disruption of cellular metabolism is a key feature of PD, with both α-synuclein and PD-related neurotoxins such as MPTP acting as disruptors [135,136]. Bioenergetic failure produced by these and other factors may also contribute to the degeneration of vulnerable neurons, such as SN DA cells. Dopamine neurons of the SN are particularly vulnerable to energy failure because of their substantial energy demands. Neurons in general consume a huge amount of energy in order to maintain the ionic gradient across their plasma membrane. SN DA neurons have long unmyelinated axons and extensive arborization making their energy demands especially high [137,138]. Furthermore, they exhibit pacemaking activity that maintains basal DA tone across an expansive region, increasing their energy demands even further [139]. Neurons do not store glycogen so they are especially sensitive to fluctuations in energy demand and reliant on neighboring astrocytes to provide nutrients [140]. Unfortunately, SN DA neurons have relatively few surrounding astrocytes to provide supplementary energy [140]. Thus, the nigrostriatal DA system is probably the most vulnerable neuronal population to compromised bioenergetic status [141], becoming very sensitive to changes in mitochondrial dynamics that impact oxidative capacity and cellular bioenergetics.

**AUTHOR CONTRIBUTION**

BS, MN, ZWL, XBG, LM, and TLH designed the experiments. BS, CN, MN, DC, ZWL, XBG, JDE, and LM performed experiments. BS, LM, and TLH designed the project and wrote the paper.

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**CONFLICT OF INTEREST**

The authors declare no conflict of interest regarding studies of this paper.

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