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1 **Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie**

2 **Restriction in Parkinson's Disease**

3

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1 **Abbreviations**

2 **CR = Calorie Restriction, DA = Dopamine, GFAP = Glial Fibrillary Acidic Protein,**
3 **GHSR1a = Growth Hormone Secretagogue Receptor 1a, GOAT = Ghrelin-O-**
4 **Acyltransferase, IBA1 = Ionized Calcium Binding Adaptor Molecule 1, MPTP = 1-**
5 **methyl-4-phenyl-1,2,3,6-tetrahydropyridine, PD = Parkinson's Disease, PFA =**
6 **Paraformaldehyde, SN = Substantia Nigra, TH = Tyrosine Hydroxylase**

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9

1 **ABSTRACT**

2 Calorie restriction (CR) is neuroprotective in Parkinson's disease (PD) although the
3 mechanisms are unknown. In this study we hypothesized that elevated ghrelin, a gut
4 hormone with neuroprotective properties, during CR prevents neurodegeneration in
5 an MPTP model of PD. CR attenuated the MPTP-induced loss of substantia nigra
6 (SN) dopamine neurons and striatal dopamine turnover in Ghrelin WT but not KO
7 mice, demonstrating that ghrelin mediates CR's neuroprotective effect. CR elevated
8 phosphorylated AMPK and ACC levels in the SN of WT but not KO mice suggesting
9 that AMPK is a target for ghrelin-induced neuroprotection. Indeed, exogenous ghrelin
10 significantly increased pAMPK in the SN. Genetic deletion of AMPK β 1 and 2
11 subunits only in dopamine neurons prevented ghrelin-induced AMPK
12 phosphorylation and neuroprotection. Hence, ghrelin signaling through AMPK in SN
13 dopamine neurons mediates CR's neuroprotective effects. We consider targeting
14 AMPK in dopamine neurons may recapitulate neuroprotective effects of CR without
15 requiring dietary intervention.

16

17

18

1 **INTRODUCTION**

2 Parkinson's Disease (PD) is the second most common neurodegenerative disease
3 affecting approximately 160 per 100,000 people with an estimated incidence number
4 of new cases each year of 16-19 per 100,00 according to the World Health
5 Organization, creating a substantial medical, social and financial burden. The motor
6 symptoms of PD include rigidity and tremor of the extremities, postural instability and
7 bradykinesia.

8

9 The BMI of an individual affects PD progression, as obesity causes dopamine
10 neuronal cell loss in the substantia nigra (SN) in a mouse model of PD (Choi et al.,
11 2005) and midlife obesity and type-2 diabetes is associated with a greater incidence
12 of PD in humans (Chen et al., 2014b). In contrast to obesity, calorie restriction (CR)
13 attenuates MPTP-induced neurotoxicity in both mice (Duan and Mattson, 1999) and
14 non-human primates (Maswood et al., 2004). Indeed, CR in monkeys may delay the
15 aging process (Colman et al., 2009) and CR in humans has the potential to slow PD
16 disease progression (Chan et al., 1997), yet the beneficial effects of CR are
17 dependent on the adherence to strict dietary constraints that are not always practical
18 and achievable in society. Therefore, it is paramount to identify the key molecular
19 mechanisms linking CR and neuroprotection to circumvent the need to adhere to
20 CR.

21

22 Ghrelin is synthesized in the stomach where pro-ghrelin is acylated in the
23 endoplasmic reticulum by the enzyme Ghrelin O-acyltransferase (GOAT). Acyl
24 ghrelin is then released into the bloodstream where it crosses the blood-brain barrier
25 and binds to the ghrelin receptor (Growth Hormone Secretagogue Receptor 1a;

1 GHSR1a) in the brain. In addition to its well-known metabolic effects, ghrelin is
2 neuroprotective in PD as Ghrelin and GHSR KO mice exhibited significantly greater
3 loss of SN dopaminergic neurons compared to WT controls in an MPTP model of PD
4 (Andrews et al., 2009). The neuroprotective mechanisms include reducing apoptosis
5 and suppressing microglial activation and local inflammatory responses in the SN
6 (Dong et al., 2009; Moon et al., 2009). Moreover, postprandial ghrelin plasma
7 ghrelin concentrations are lower in human PD patients (Unger et al., 2011),
8 suggesting clinical relevance.

9
10 Plasma ghrelin is elevated during periods of negative energy balance, including CR
11 and previous studies showed that the anxiolytic and anti-depressant effects of CR
12 require GHSR signaling (Lutter et al., 2008). Ghrelin also prevented an excessive
13 decline in blood glucose levels during CR (Zhao et al., 2010). These studies provide
14 biological precedents that the ghrelin system mediates some of the beneficial effects
15 of CR. Because ghrelin protects against SN dopaminergic cell loss (Jiang et al.,
16 2008; Andrews et al., 2009; Moon et al., 2009), we reasoned that elevated plasma
17 ghrelin during CR contributes to the neuroprotective effects of CR in PD. Indeed,
18 cells treated with serum from CR rats show greater survivability, increased
19 mitochondrial function and mitochondrial biogenesis (Lopez-Lluch et al., 2006),
20 arguing that a hormonal signal mediates the effects of CR on mitochondrial function
21 and cell survivability. These findings above led us to hypothesize that increased
22 plasma ghrelin during CR acts on SN dopamine neurons to restrict SN dopamine
23 neuronal degeneration in a mouse model of PD.

24

1 **METHODS**

2 **Animals**

3 All experiments herein were conducted in accordance with Monash University
4 Animal Ethics Committee guidelines. Mice were maintained under standard
5 laboratory conditions with free access to food and water at 21°C with a 12-hour
6 light/dark cycle unless otherwise stated.

7

8 **Experimental protocol**

9 For the first set of experiments, Ghrelin WT/KO mice were individually housed. Male
10 Ghrelin WT/KO mice (~ 8-10 weeks old) on a C57/Bl6J background were obtained
11 from Regeneron Pharmaceuticals (Tarrytown, NY) and bred in the Monash Animal
12 Services facilities. Mice in ad libitum (ad-lib) groups had free access to food,
13 whereas the remaining mice were calorie-restricted (CR) to 70% of their baseline
14 food intake. Baseline food intake was calculated by measuring average food intake
15 over one week prior to the initiation of the restriction period. CR mice had daily blood
16 glucose and body weight measurements taken and then given access to a previously
17 calculated and weighed food pellet approximately 1 hour before the initiation of the
18 dark cycle (1800h) in an attempt to maintain normal physiological feeding times for
19 the duration of the experiment (27 days).

20 In the second set of experiments to test the effect of ghrelin administration on
21 neuronal function in the midbrain, we used group housed male C57/Bl6J mice (8-10
22 weeks old; Monash Animal Services, Victoria, Australia) that had free access to food
23 and water. C57/Bl6J mice were randomly allocated to receive saline, a low dose of
24 ghrelin (5mg/kg) or a high dose of ghrelin (15mg/kg). The mice were injected ip and

1 the food removed from the cage, they were subsequently culled 45 minutes later via
2 decapitation after being deeply anaesthetized, then the brains were dissected and
3 snap frozen (-70°C) for HPLC and western blot analysis.

4 In order to generate mice with selective deletion of AMPK $\beta 1$ & $\beta 2$ only in DAT-
5 expressing dopamine neurons, we crossed *Dat-Cre* knock-in mice obtained from Jax
6 Lab [Stock number 006660; B6.SJL-Slc6a3^{tm1.1(cre)bkmn}/j] with *Ampk beta 1*
7 *subunit* ($\beta 1$) and *beta 2 subunit* ($\beta 2$) floxed mice (O'Neill et al., 2011). The resultant
8 offspring (*Dat-Cre;Ampk beta 1^{fl/fl};Ampk beta 2^{fl/fl}* designated AMPK KO or *Ampk*
9 *beta 1^{fl/fl};Ampk beta 2^{fl/fl}* designated AMPK WT) were used as experimental mice. To
10 validate this model, AMPK WT and KO mice were also bred with cre-dependent
11 loxSTO^{lox} tdTOMATO reporter mice Stock number 007908; B6;129S6-
12 Gt(ROSA)26Sor^{tm14(CAG-tdTomato)Hze}/J). The resultant offspring *Dat-Cre;tdTomato* or
13 *Dat-Cre;Ampk beta 1^{fl/fl};Ampk beta 2^{fl/fl};tdTomato* mice allow tdTomato visualization
14 of DAT-expressing neurons that have undergone cre recombination. These mice
15 were also used for Fluorescence Activated Cell Sorting (FACS). The *Dat-*
16 *Cre;tdTomato* were used as AMPK WT mice and *Dat-Cre;Ampk beta 1^{fl/fl};Ampk beta*
17 *2^{fl/fl};tdTomato* mice were used as AMPK KO mice. These mice were culled via
18 inhalation anesthetic and the substantia nigra was collected. The cells were
19 dissociated using papain (Worthington Kit, LK003150) following kit instructions. After
20 collection of approximately 5000 tdTomato cells via FACS sorting using the influx v7
21 Sorter, the RNA was extracted and PCR was run to determine the presence/absence
22 of AMPK $\beta 1$ and 2.

23 In the third set of experiments to test the effects of ghrelin administration in mice
24 lacking AMPK activation, we group housed AMPK WT and KO mice (8-10 weeks
25 old) with free access to water. The mice were administered ghrelin (1mg/kg) or

1 Saline daily at the beginning of the light cycle for 14 consecutive days. After
2 injections the food was subsequently removed for 6 hours to prevent excess
3 consumption of calories, after this period all mice had free access to food. Previous
4 studies (Andrews et al., 2009) indicate that if calories are consumed after injection of
5 acyl-ghrelin there is no neuroprotective effect observed. On days 7 and 8 mice were
6 injected with Saline or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP;
7 30mg/kg). Mice were culled on day 14 and perfused for immunohistochemical
8 analysis or fresh tissue collection for Western Blot and HPLC analysis.

9

10 **MPTP administration**

11 Experimental mice were injected with MPTP (30mg/kg, i.p.) dissolved in saline as
12 described previously (Andrews et al., 2005) over two consecutive days. Control
13 animals received sterile saline using the same timeline. Animals were injected with
14 MPTP or Saline and perfused 7 days later for immunohistochemical analysis or fresh
15 tissue collection for HPLC and western blot analysis.

16

17 **Immunohistochemistry**

18 Free-floating sections were stained with both Tyrosine Hydroxylase (TH) and Ionized
19 calcium Binding Adaptor (IBA1) or Glial Fibrillary Acidic Protein (GFAP). All mice
20 were deeply anesthetized and perfused with 0.05% PBS followed by 4%
21 Paraformaldehyde (PFA) to fix the tissue. Brains were stored in PFA overnight then
22 transferred to a 30% sucrose solution. Coronal sections (30 µm thick) of the entire
23 SN were collected with systematic sampling of every fifth section.

24 The sections was washed thoroughly in 0.1M PB and then endogenous peroxidase
25 activity was blocked using 1% H₂O₂ in 0.1M PB for 15 minutes and washed again.

1 The tissue was then transferred to 4% normal horse serum and 0.3% Triton X-100 in
2 0.1M PB for one hour, followed by a secondary mouse blocking step using AffiniPure
3 Goat Anti-Mouse IgG (H+L) (1:200, Jackson ImmunoResearch) to prevent non-
4 specific binding of mouse antibodies in mouse tissue. The tissue was then incubated
5 with the primary antibodies, in this case either anti-TH (mouse, 1:5000, Millipore) and
6 anti-IBA1(rabbit, 1:1000, Wako) or anti-GFAP (rabbit, 1:1000, DAKO) for 24 hours at
7 4°C. Following the primary antibody incubation the tissue was washed thoroughly
8 and incubated in the secondary antibody goat anti-mouse IgG (H+L) Alexa Fluor 488
9 (1:400, Invitrogen) and goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:400, Invitrogen)
10 for fluorescent staining for 90 minutes at room temperature. The tissue was then
11 thoroughly washed and mounted directly onto slides and coverslipped with anti-fade
12 mounting media.

13

14 **Stereological investigation of cell number and volume.**

15 In order to quantify the number of TH neurons, microglia (IBA1 stain) and astrocytes
16 (GFAP stain) in the SN we used design-based stereology. Using the
17 StereoInvestigator software (MicroBrightField, Williston, VT, USA) we analysed both
18 cell number (using the optical fractionator probe) and cell volume (using the
19 nucleator probe). To visualise the cells we used a Zeiss microscope with a motorised
20 stage and a MicroFibre digital camera connected to a computer.

21

22 **Analysis of blood chemistry**

23 Trunk blood was collected via decapitation from deeply anaesthetised mice and
24 collected into EDTA tubes pre-treated with pefabloc (SC Roche Applied Science,

1 Mannheim, Germany) to achieve a concentration of 1mg/mL. The blood was then
2 briefly centrifuged and the plasma was collected and acidified with HCl (final
3 concentration 0.05N). Plasma ghrelin levels were determined using Active Ghrelin or
4 Des-acyl Ghrelin Enzyme-Linked Immunoassay Kits (Mitsubishi Chemical Medicine,
5 Tokyo, Japan). Active and des-acyl ghrelin were measured according to kit
6 instructions. Plasma Insulin concentration was determined through an in-house
7 ELISA assay.

8

9 **High Performance Liquid Chromatography (HPLC)**

10 We used HPLC to identify, separate and quantify **dopamine** (DA) and DOPAC
11 concentrations within samples of striatal tissue. Striatal (both sides) tissue was
12 rapidly dissected and snap frozen (approximately -70°C). The samples were then
13 sonicated in 0.4mL cold 0.1M perchloric acid containing internal standard. Following
14 centrifugation, DA DOPAC and internal standard in the supernatant were extracted
15 on alumina at pH 8.4, eluted in 0.1M perchloric acid, separated by reverse-phase
16 HPLC and detected using electrochemical detection. Both dopamine and DOPAC
17 concentrations in the striatum were calculated by reference to the internal standard
18 and external standards. The protein content of each sample was determined from
19 the centrifuged pellet by the Lowry method. The concentrations of DA and DOPAC
20 are expressed as ng/mg of protein present (mean ± SEM)

21

22 **Western Blot**

23 Whole tissue samples of the SN and Striatum or SN4741 cells were processed for
24 western blot analysis. Briefly, tissue was sonicated in RIPA buffer (50mM Tris.HCl,
25 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100) containing a

1 protease inhibitor (Sigma), then centrifuged (10,000 rpm, 10min, 4°C) to remove cell
2 debris and the supernatant was collected. For cell culture studies SN4741 cells were
3 maintained at 37°C in a 5% CO₂ humidified environment in Dulbecco's modified
4 Eagle medium (DMEM, 41965, life technologies) supplemented with 10% fetal
5 bovine serum (FBS), 2mM glutamine, 100U/ml penicillin and 0.1mg/ml streptomycin,
6 0.6% glucose. Once cells had reached approximately 90-100% confluency, cells
7 were sub-cultured.

8 SN4741 cells were treated with vehicle (compound diluent), 1µM acyl-ghrelin
9 (Tocris), 6nM JMV2894 (ghrelin receptor agonist, Aeterna Zentaris) or 0.5µM
10 oligomycin (Sigma) for 5 minutes. Cells were washed 3 times with ice cold PBS and
11 lysed in ice cold RIPA lysis buffer (50mM Tris.HCl, pH 7.5 containing 1% NP40,
12 0.1% SDS, 0.5% sodium deoxycholate and 150mM NaCl) with 1% mammalian
13 protease (Sigma P8340) and phosphatase inhibitors (Sigma P0044)(Ho et al., 2013).
14 Cell lysates were incubated at 4°C for 15 min and then centrifuged at 22,000 x g for
15 10min at 4°C. The supernatant was collected and 1 volume of 2x SDS- PAGE
16 sample loading buffer (Sigma S3401) was added and left at room temperature for
17 1h.

18
19 An aliquot was then used to identify the amount of protein present in each sample
20 using a BCA kit (Pierce, Rockford, IL) according to kit instructions. The samples
21 concentrations were then standardised and the supernatants were mixed with
22 Laemmli's buffer and boiled for 5 minutes. Samples (20µl) were loaded onto 10%
23 acrylamide gels and separated by SDS polyacrylamide gel electrophoresis. The
24 separated proteins were then transferred from the gel to the PVDF membrane
25 (Biorad). The blots were then blocked for 1 hour in Tris-Buffered Saline Solution

1 containing 0.1% Tween-20 (TBST) and 5% bovine Serum albumin (BSA). The
2 membranes were subsequently incubated overnight at 4°C in TBST with 5% BSA
3 with either of the following antibodies: TH (1:1000, Milipore), Parkin (1:1000, Santa
4 Cruz), PINK (1:1000, Santa Cruz), LC3B (1:1000, Cell Signaling), pACC (1:1000,
5 Cell Signaling) or pAMPK (1:1000, Cell Signaling), where AMPK α (1:1000, Cell
6 Signaling) antibodies, ACC (1:1000, Cell Signaling) and anti- β actin (1:1000, Abcam)
7 were used as controls. Blots were visualised using the chemiluminescence method
8 (ECL, Amersham) and levels were detected using ImageLab Software, version 4.1,
9 Biorad.

10

11 **RNA extraction and PCR**

12 After FAC's sorting cells were stored in Qiazol for RNA extraction. Briefly, chloroform
13 was added, samples were centrifuged (12,000g, 15min, 4°C) and supernatant was
14 collected. Isopropanol and glycogen was added and the samples centrifuged
15 (12,000g, 10 minutes, 4°C). The pellet formed was washed with ethanol (75%) and
16 vortexed. cDNA was synthesized using the iScript cDNA synthesis kit (number 170-
17 8890; Biorad Laboratories). The cDNA collected was combined with Mastermix and
18 primers (either AMPK β 1, AMPK β 2 or GHSR) and exposed to a heat block in the
19 Mastercycler. We used TaqMan Gene Expression Mastermix (Applied Biosystems)
20 and GHSR primers (GHSR forward: GCTGCTCACCGTGATGGTAT and reverse:
21 GCTGCTCACCGTGATGGTAT) as our control. A PCR reaction was required to
22 amplify the AMPK β 1 and AMPK β 2 transcripts from the cDNA. We used nested PCR
23 to enhance accuracy using two PCR reactions involving outer and inner primers
24 (AMPK β 1 outer forward: CCACTCCGAAGAGATCAAGG and reverse:
25 GTGCTGGGTCACAAGAGATG, AMPK β 1 inner forward:

1 CACGACCTGGAAGCGAAT and reverse: CATGTAAGGCTCCTGGTGGT and
2 AMPK β 2 outer forward: GTTATCCGCTGGTCTGAAGG and reverse:
3 CAGCAGCGTGGTGACATACT and AMPK β 2 inner forward:
4 GAGCACCAAGATCCCTCTGA and reverse: GGAAGTAAGGCTGGGTCACA). This
5 process was repeated with inner primers and then visualized in a gel mounting
6 media (agarose gel) and exposed to electrical current (120V) for 25 minutes. The
7 results were viewed using gene snap technology. The specificity of the primers was
8 confirmed using a blast search. Positive control was hypothalamic tissue from
9 C57BL/6 mice and negative control contained no cDNA.

10

11 **Rotarod**

12 Mice were trained prior to testing by being placed on a rotating rod (Ugo Basile Rota-
13 Rod 47600), spinning at 4 rotations per minute (RPM) for 5 minutes. Lane width =
14 5cm. On training day mice were subjected to incrementally increasing speed over
15 300 seconds going from 4-40 RPM. Each animal underwent 4 trials. The length of
16 time that the mice remained on the rod was recorded and analysed.

17

18 **Statistical Analysis**

19 All data is represented as Mean \pm Standard Error of the Mean (SEM). Two-Way
20 ANOVA with a Bonferroni post hoc test was used to determine statistical significance
21 between treatment and genotype and One-Way ANOVA with a Tukey post hoc test
22 was used to determine statistical significance between injection groups. Cell lysate
23 analysis used a two-tailed Student's *t*-test. $p < 0.05$ was considered statistically
24 significant.

25

1 RESULTS

2 Effect of Calorie restriction on metabolic parameters in Ghrelin WT/KO mice

3 **Calorie Restriction** (CR) significantly elevated acylated (Figure 1A) and des-acyl
4 plasma ghrelin in WT, with no detectable levels in KO mice (Data not shown),
5 confirming reports that CR increases plasma ghrelin. There were no genotypic
6 differences in plasma insulin from ad-lib or CR mice, although CR significantly
7 reduced plasma insulin levels compared to ad-lib mice (significant main effect ad-lib
8 vs. CR) (Figure 1B&C). Both body weight and blood glucose measurements
9 exhibited a significant overall reduction in response to CR (Data not shown).

10

11 Ghrelin restricts MPTP-induced nigrostriatal damage during CR

12 Tyrosine Hydroxylase (TH) neurons

13 We used the stereological optical fractionator probe to estimate total TH-positive (i.e.
14 dopamine) neurons in the **substantia nigra** (SN). MPTP administration significantly
15 reduced the number of SN TH neurons in ad-lib and CR Ghrelin WT and KO mice
16 **(Figure 1D)**. CR partially attenuated SN TH neuronal loss in Ghrelin WT (Figure 1E),
17 however this protective effect was lost in Ghrelin KO mice (Figure 1F).

18

19 **Gliosis**

20 MPTP treatment exhibited a significant elevation in microglia (IBA1+ cells) present in
21 the SN of both genotypes, although CR did not prevent the MPTP-induced increase
22 of **ionized calcium binding adaptor 1** (IBA1) cell number in either Ghrelin WT or
23 Ghrelin KO mice (Figure 1G & H). Astrocytes, as represented by **Glial Fibrillary**
24 **Acidic Protein** (GFAP) staining, are the most abundant cell type found throughout the

1 **central nervous system** and play a critical role during cellular damage to minimize
2 overall cell loss (Hailer et al., 2001). Elevated GFAP+ cells in any specified region
3 indicates greater cellular damage in that area. MPTP treatment initiated a significant
4 increase in GFAP cells in both Ghrelin WT and KO ad-lib mice compared to saline
5 controls (Figure 1I & J). CR reduced GFAP expression in the SN of both **MPTP-**
6 **treated** ghrelin WT and KO mice relative to MPTP ad-lib mice (Figure 1I & J), This
7 result indicates that CR restricts GFAP cell expression in the SN, **although this does**
8 **not appear to be directly mediated by ghrelin.**

9

10 TH cell volume

11 To accurately measure cell volume we used the nucleator stereological probe.
12 There was a significant main effect for MPTP to reduce average cell volume in
13 Ghrelin WT but not Ghrelin KO (Figure 2A & B). We performed a cell volume
14 distribution analysis in order to determine if diet or treatment preferentially affected
15 neuronal number within a certain volume range. MPTP treatment to CR Ghrelin WT
16 mice prevented the loss of TH neurons with volumes between 1000-2000 μm^3
17 compared to ad-lib MPTP-treated Ghrelin WT mice (Figure 2C). Remarkably, no
18 beneficial effects of CR on TH neuronal cell volume between were observed in
19 Ghrelin KO mice (Figure 2D). Thus, ghrelin influences both TH cell number and cell
20 volume distribution during CR.

21

22 HPLC analysis

23 HPLC analysis of dopamine (DA) in the striatum revealed a significant overall
24 ($p < 0.05$) reduction with MPTP administration and CR significantly attenuated the loss
25 of dopamine in WT but not KO mice (Figure 2E & H). MPTP also significantly

1 reduced DOPAC in the striatum however there was no effect of diet on DOPAC
2 levels regardless of genotype (Figure 2F & I). CR also prevented in the increase in
3 the DOPAC:DA ratio observed after MPTP in ghrelin WT ad-lib but not ghrelin KO
4 ad-lib mice (Figure 2G & J).

5

6 TH protein expression

7 Reduced dopamine levels in the striatum indicate impaired dopamine synthesis,
8 which is controlled by the rate-limiting enzyme TH. In the SN of Ghrelin WT and KO
9 mice, MPTP administration significantly reduced TH protein expression in both ad-lib
10 fed and CR mice with no protective effect of CR in either genotype (Figures 3A,
11 C&D). In the striatum however, CR significantly attenuated the lower TH protein
12 levels in MPTP-treated ghrelin WT but not ghrelin KO mice (Figure 3B, E&F). These
13 results highlight that CR has site-specific effects acting to increase TH in the striatum
14 but not the SN. Together, these results corroborate with the TH neuronal counts, cell
15 volume analysis and HPLC DA content results indicating that CR has a protective
16 effect only in Ghrelin WT mice. Overall these results imply that ghrelin is responsible
17 for these protective effects in a mouse model of PD.

18

19 **Ghrelin influences AMPK activation in the striatum**

20 Ghrelin enhances AMPK activity in the hypothalamus (Andrews et al., 2008) and
21 AMPK also increases mitochondrial biogenesis and function in the periphery
22 (Bergeron et al., 2001; Horvath et al., 2011). Thus, we reasoned that the
23 neuroprotective actions of CR induce a ghrelin-dependent increase in AMPK function
24 in SN TH neurons. We found that both metabolic (CR) and chemical (MPTP) stress
25 increased AMPK phosphorylation (pAMPK/AMPK ratio) and subsequent Acetyl CoA

1 Carboxylase (ACC) in the striatum, but not the SN as seen for TH expression, in
2 Ghrelin WT but not Ghrelin KO mice (Figure 3G-P). CR in Ghrelin KO MPTP treated
3 mice significantly reduced AMPK and ACC phosphorylation in the SN compared to
4 ghrelin KO MPTP ad-lib mice (Figure 3J&N). The maintenance of autophagy is one
5 downstream effect of AMPK activation (Mihaylova and Shaw, 2011), therefore we
6 examined LC3 II, the membrane-bound form of autophagosomes (Kimura et al.,
7 2007). We observed significantly reduced LC3 II in the SN of CR Ghrelin WT mice
8 compared to ad-lib controls (Figure 3S), with a significant overall elevation in
9 response to MPTP in striatum (Figure 3U). No effect was observed in Ghrelin KO in
10 the SN (Figure 3T) or Striatum (Figure 3V). The LC3 II results in the SN are inversely
11 related to SN TH cell counts suggesting there is less autophagosome formation
12 required in cells with less MPTP-induced degeneration.

13 PINK1 and Parkin regulate mitophagy and mutations in PINK and Parkin cause early
14 onset PD, therefore, we also measured the expression of these two proteins in the
15 SN and striatum from CR and ad-lib Ghrelin WT and KO mice. PINK1 and Parkin
16 expression showed a significant reduction in protein expression post MPTP
17 administration in the striatum with no significant effect of CR or genotype (Data not
18 shown). There was no change in protein levels in response to metabolic state, MPTP
19 or genotype in the SN (Data not shown).

20

21 **Exogenous ghrelin influences the phosphorylation of AMPK and ACC**

22 In order to support the notion that increased endogenous ghrelin is the critical to CR-
23 induced neuroprotection, we examined the effects of exogenous acyl-ghrelin on
24 AMPK and ACC phosphorylation both *in vivo* and *in vitro*. The addition of either acyl-

1 ghrelin or the ghrelin agonist JMV2894 increased AMPK activation in cultured
2 dopaminergic cell line SN4741 (Figure 4A-D).

3 For *in vivo* studies, we injected acyl-ghrelin ip at 2 different doses (low: 5mg/kg and
4 high: 15mg/kg). The high dose of acyl-ghrelin significantly increased AMPK and ACC
5 phosphorylation in the SN (Figure 4E,G&H). However, there was no significant
6 difference in the striatum in response to either a low or high dose of acyl-ghrelin
7 (Figure 4F,I&J). This is in contrast to the effect of CR on AMPK activation, as we
8 observed a significant difference in the striatum but not the SN.

9

10 Injection of ip acyl-ghrelin at a high dose significantly increased TH expression in
11 both the SN and Striatum (Figure 4K-M). Moreover, ip acyl-ghrelin increased LC3 II
12 in the SN but not the striatum (Figure 4N-P). There was no change in PINK1
13 expression in either the SN or the striatum (Data not shown). Parkin expression
14 remained unchanged in the SN, however, in the striatum there was a significant
15 increase with a high dose of acyl-ghrelin (Data not shown). These results indicate
16 that peripheral acyl-ghrelin injection affects AMPK and ACC phosphorylation, as well
17 as TH, Parkin and LC3 II protein expression in the nigrostriatal system.

18

19 **Exogenous ghrelin requires AMPK in dopamine neurons to elicit** 20 **neuroprotection**

21 To prove that ghrelin-induced neuroprotection requires AMPK activation in SN
22 dopamine neurons, we generated a novel mouse line in which AMPK activation was
23 disabled in dopaminergic neurons. These mice were generated by cross breeding
24 *Dat-Cre* mice with *Ampk beta 1^{fl/fl}*; *Ampk beta 2^{fl/fl}* mice in order to generate AMPK

1 WT and AMPK KO mice. AMPK $\beta 1$ & $\beta 2$ are regulatory subunits required for AMPK
2 activity (O'Neill et al., 2011). To determine the specificity of the knockout, we bred
3 AMPK WT and KO with Rosa26loxSTOPlox tdTomato reporter mice to generate *Dat-*
4 *Cre;tdTomato* and *Dat-Cre;Ampk beta 1^{fl/fl};Ampk beta 2^{fl/fl};tdTomato*. TH and
5 tdTomato co-expression in the SN was >90% (Figure 5A-C), indicating cre
6 recombination had occurred in >90% of SN TH neurons. Deletion of AMPK $\beta 1$ and
7 AMPK $\beta 2$ in dopamine neurons was confirmed by FACS of tdTomato-labelled
8 neurons from midbrain dissections from *Dat-Cre;tdTomato* and *Dat-Cre;Ampk beta*
9 *1^{fl/fl};Ampk beta 2^{fl/fl};tdTomato* cells and nested PCR for AMPK $\beta 1$ and AMPK $\beta 2$
10 (Figure 5D). Positive bands for both AMPK $\beta 1$ and AMPK $\beta 2$ were observed in AMPK
11 WT but not AMPK KO mice (Figure 5D). As a positive control, nested PCR for GHSR
12 was performed to confirm the presence of the ghrelin receptor in both AMPK WT and
13 KO mice (Figure 5D).

14 In order to show that ghrelin elicits neuroprotection in a mouse model of PD, we
15 chronically administered acyl-ghrelin to DAT AMPK WT & KO mice. In AMPK WT
16 mice, acyl-ghrelin administration significantly attenuated TH cell loss in MPTP-
17 treated mice (Figure 5E & F). This effect was abolished in the AMPK KO mice
18 (Figure 5G). Acyl-ghrelin reduced IBA1+ cell number in AMPK WT MPTP treated
19 (5H), however no significant effect was observed in AMPK KO mice (Figure 5I). A
20 similar pattern was observed with GFAP cells, in which acyl-ghrelin reduced GFAP
21 cell number in AMPK WT but not AMPK KO mice (Figure 5J & K).

22 Despite the attenuated TH cell loss in the acyl ghrelin-treated AMPK WT mice there
23 was no overall change in cell volume or distribution (Figure 6A-D). HPLC analysis of
24 dopamine and DOPAC in the striatum revealed that acyl-ghrelin attenuated the

1 MPTP-induced loss of dopamine and prevented the MPTP-induced rise in the
2 DOPAC/dopamine ratio in AMPK WT but not AMPK KO mice (Figure 6E-J).
3 Changes in motor behaviour were determined using an accelerating Rotarod by
4 measuring latency to fall. There was no overall change between Saline or Ghrelin
5 treated AMPK WT and KO mice without MPTP treatment (Figure 6K & L). When
6 pretreated with saline and given MPTP there was no effect of genotype (Figure 6M)
7 however, there was a protective effect of ghrelin administration prior to MPTP in
8 AMPK WT but not KO mice (Figure 6N). Collectively, these experiments highlight
9 that ghrelin activates AMPK in SN dopamine neurons, restricts dopaminergic cell
10 loss, maintains striatal dopamine concentrations and promotes locomotor behaviour
11 after MPTP treatment to provide a neuroprotective effect.

12

13 We previously showed that ip ghrelin can elicit an increase in the pAMPK/AMPK and
14 pACC/ACC ratio in SN (Figure 4G&H) and that chronic ghrelin treatment to AMPK
15 WT, but not AMPK KO is neuroprotective in a mouse model of PD. In further support
16 of this neuroprotection, ghrelin treatment attenuated the MPTP-induced loss of TH in
17 both the SN and striatum of AMPK WT but not AMPK KO mice (Figure 7A-F). To
18 determine if chronic ghrelin differentially affected AMPK and subsequent ACC
19 phosphorylation, we measured the pAMPK/AMPK and pACC/ACC ratio in AMPK WT
20 and KO mice in response chronic daily ghrelin injections. There was no effect of
21 chronic ghrelin treatment on the pAMPK/AMPK (Figure 7G, I&J) or the pACC/ACC
22 ratio (Figure 7M&N) in the SN of either AMPK WT or AMPK KO mice. However,
23 MPTP treatment elicited an increase in the pAMPK/AMPK (Figure 7H, K&L) and
24 pACC/ACC ratios (Figure 7O&P) in the striatum of AMPK WT mice, but not AMPK

1 KO mice. The mitophagy proteins PINK1 and Parkin (Data not shown) and the
2 autophagosome marker LC3 II were not significantly different between genotypes
3 and treatment (Figure 7Q-V).

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1 **DISCUSSION**

2 Calorie Restriction (CR) protects against a number of pathological conditions
3 including diabetes, cancer, heart disease and neurodegeneration. In Parkinson's
4 Disease (PD) an alternate-day feeding schedule, where rats consumed 30-40% less
5 calories than ad-libitum controls was neuroprotective post MPTP exposure (Duan
6 and Mattson, 1999). Mice also elicited a neuroprotective response when alternate
7 day feeding was begun after exposure to MPTP (Holmer et al., 2005). Primates with
8 a chronic overall 30% reduction in food intake were also resistant to MPTP induced
9 neurotoxicity (Maswood et al., 2004). These studies prove that CR is beneficial in PD
10 however the difficulty to adhere to CR necessitates an alternative method to
11 recapitulate the neuroprotective benefits of CR whilst bypassing dietary constraints.
12 Evidence from cells treated with serum from CR rats suggests a hormonal factor
13 improves mitochondrial function and cell viability (Lopez-Lluch et al., 2006). We
14 hypothesized that ghrelin may be this hormonal factor, because CR increases
15 plasma acyl ghrelin (Lutter et al., 2008) and ghrelin restricts degeneration in PD
16 (Andrews et al., 2009). In this study we show for the first time that ghrelin mediates
17 the neuroprotective effect of CR in a mouse model of PD by attenuating MPTP-
18 induced loss of TH neurons, TH neuronal volume and dopamine content in the
19 striatum. Further, we show that AMPK in SN dopamine neurons is a molecular target
20 for ghrelin's neuroprotective effects, as deletion of AMPK β 1 & β 2 subunits
21 prevented ghrelin-induced neuroprotection. These results suggest that ghrelin, and
22 its downstream target AMPK, has a potential therapeutic application in the treatment
23 of PD to mimic the neuroprotective effect of CR without the need for strict dietary
24 constraints.

1 Although this is the first study to show that ghrelin mediates the neuroprotective
2 effects of CR in a mouse model of PD, it supports an increasing number of
3 observations that ghrelin restricts the negative consequences of CR or negative
4 energy balance. For example, ghrelin prevents the excessive decline in blood
5 glucose during severe CR (Zhao et al., 2010) and the anxiolytic effects of CR require
6 GHSR signaling (Lutter et al., 2008). A recent study by Macfarlane (McFarlane et al.,
7 2014) shows that adult-ablation of ghrelin secreting cells has no effect on food
8 intake, body weight and fed blood glucose. Only under CR did these mice show
9 deficits in blood glucose. Moreover, CR reduces hippocampal cell death in GHSR
10 WT but not GHSR KO mice (Walker et al., 2015) and CR induces neurogenesis in a
11 GHSR dependent manner (Hornsby et al., 2016). Collectively, these studies show
12 that the major function of ghrelin is to act as a feedback signal of CR (negative
13 energy balance) and maintain physiological and neurological function during this
14 time.

15

16 Our data show that AMPK in SN dopamine neurons is a molecular target of ghrelin
17 during CR to maintain neuronal function. Firstly, metabolic stress (CR) and/or toxic
18 stress (MPTP) promoted AMPK activity in striatal dopamine nerve terminals in
19 Ghrelin WT but not Ghrelin KO. The ability of MPTP to increase AMPK activity is
20 supported by previous studies in mice and cells (Choi et al., 2010). AMPK enhances
21 mitochondrial function and biogenesis (Reznick and Shulman, 2006) as such, we
22 suggest CR-induced AMPK phosphorylation at the nerve terminal promotes neuronal
23 energy metabolism and supports ongoing dopaminergic neuronal activity, which is
24 supported by the reduced striatal DOPAC/dopamine ratio of both CR Ghrelin and
25 AMPK WT but not their respective KO mice. Moreover, AMPK activity diminishes

1 with age (Reznick et al., 2007) consistent with the age-related neurodegeneration
2 that contributes to the onset of PD. Thus, the ability of CR to maintain AMPK activity
3 in a ghrelin-dependent manner may restrict age-related decline in the nigrostriatal
4 system. This possibility is further strengthened by data showing that plasma ghrelin
5 and ghrelin's function diminishes with age, an effect that can be reversed with CR
6 (Englander et al., 2004; Smith et al., 2007; Sun et al., 2007; Yang et al., 2007;
7 Takeda et al., 2010). Further, PD patients have reduced postprandial plasma ghrelin
8 levels (Unger et al., 2011).

9
10 In cultured dopaminergic neurons both acyl-ghrelin and a ghrelin agonist elicited a
11 robust increase in AMPK activation. Acute acyl-ghrelin injection *in vivo* increased
12 both AMPK and ACC phosphorylation in the SN but not the striatum. This is the first
13 *in vivo* study that shows ghrelin activates AMPK activity in the midbrain, similar to
14 numerous reports showing ghrelin activates AMPK activity in the hypothalamus
15 (Andersson et al., 2004; Kola et al., 2005; Andrews et al., 2008). As noted above,
16 CR drives ghrelin-induced AMPK phosphorylation in the striatum, but not the SN, yet
17 acute ghrelin injection *in vivo* increased AMPK phosphorylation in the SN but not the
18 striatum. We consider this discrepancy may be due to chronically elevated ghrelin
19 vs. an acute ghrelin injection. Chronically high plasma ghrelin, as seen in CR Ghrelin
20 WT mice, activates SN dopamine neurons via the GHSR which then facilitates and
21 propagates AMPK phosphorylation in areas of metabolic need, in this case striatal
22 nerve terminals in order to prevent degeneration. Although acute injection of ghrelin
23 increase pAMPK/AMPK ratio in the SN after 45 minutes, this narrow time frame
24 presumably prevents propagation of AMPK phosphorylation in the striatum. It is

1 important to note that the ghrelin receptor, GHSR, is abundantly expressed in the SN
2 with little or no expression in the striatum (Zigman et al., 2006).

3
4 Importantly, we conclusively demonstrate that AMPK activity in dopamine neurons is
5 necessary for ghrelin-induced neuroprotection in a mouse model of PD. We
6 generated a model in which AMPK β 1 and AMPK β 2 were successfully deleted in
7 DAT expressing neurons. Deletion of both AMPK β 1 and AMPK β 2 in muscle ablated
8 AMPK phosphorylation and lead to impaired glucose homeostasis (O'Neill et al.,
9 2011). Using the model we showed that ghrelin prevents nigrostriatal degeneration in
10 MPTP-treated DAT AMPK WT but not DAT AMPK KO mice, clearly establishing
11 AMPK as a critical molecular mechanism mediating the neuroprotective effects of
12 ghrelin on the nigrostriatal system. Our genetic model also deletes AMPK β 1 and
13 AMPK β 2 in all DAT-cre expressing neurons including populations not associated
14 with PD, such as the hypothalamic and VTA dopamine neurons. However, MPTP
15 predominantly affects SN dopamine neurons (Seniuk et al., 1990; Muthane et al.,
16 1994), which strengthens the specific and important neuroprotective actions of
17 ghrelin on AMPK activity in the SN. We should note that we did not detect a change
18 in pAMPK/AMPK ratio in the SN or striatum of chronic ghrelin treated AMPK WT or
19 KO mice, whereas acute ghrelin injection affected the pAMPK/AMPK ratio in the SN.
20 There are many potential reasons for this including the dosage and time of tissue
21 collection after last injection. However the most plausible reason is due to the tissue
22 collection, since we measured pAMPK/AMPK in a dissected piece of tissue, of which
23 only a small proportion represents SN dopamine neurons. Nevertheless, in response
24 to MPTP treatment AMPK WT mice produced an increase in pAMPK/AMPK ratio in
25 the striatum, which was not observed in AMPK KO. In fact the significant main effect

1 of MPTP to suppress AMPK phosphorylation, independent from ghrelin treatment, in
2 both the SN and striatum of AMPK KO illustrates the important role of AMPK β 1 and
3 AMPK β 2 in SN dopamine neurons to combat cellular stress caused by MPTP.
4 Moreover, CR Ghrelin KO also did not show a compensatory increase in MPTP-
5 induced AMPK phosphorylation in the striatum, further supporting the idea that
6 ghrelin targets AMPK in SN dopamine neurons during CR to prevent degeneration.

7
8 Intriguingly, we noted differential effects of CR and ghrelin treatment on gliosis. In
9 the CR experiment, the microglial response to MPTP was similar in ghrelin WT and
10 KO mice despite the greater TH cell loss in ghrelin KO mice. This is somewhat
11 unexpected given microglia become activated to remove neuronal damage by
12 phagocytosis (Neumann et al., 2009). It is possible that a threshold level of cell loss
13 elicits the same microglial response, perhaps mediated by the release of caspase
14 signal (Burguillos et al., 2011). Moreover, GFAP cell number was increased after
15 MPTP and suppressed in CR mice regardless of genotype. In primates CR elicited a
16 protective effect by limiting astrogliosis in the hippocampus (Sridharan et al., 2013).
17 These results suggest that the effects of CR on gliosis are independent from
18 changes in plasma ghrelin. However, chronic ghrelin-treatment to AMPK WT and
19 AMPK KO mice showed that ghrelin reduced microglia and GFAP in AMPK WT but
20 not AMPK KO mice treated with MPTP. This effect of ghrelin treatment is consistent
21 with in vitro studies that indicate ghrelin directly inhibits glial activation to diminish the
22 inflammatory response (Lee and Yune, 2014). Moreover, that lack of an effect in
23 AMPK KO mice suggests ghrelin acts directly on AMPK in SN dopamine to restrict
24 microglia and GFAP expression, a hypothesis supported by studies showing that

1 AMPK influence gliosis (Lu et al., 2010; Yi et al., 2011; Chen et al., 2014a; Han et
2 al., 2014; Zhou et al., 2014).

3
4 This is the first study to show the important neuroprotective *in vivo* actions of AMPK
5 in dopamine neurons, although a number of studies implicate AMPK as an
6 intracellular energy sensor promoting neuroprotection in models of PD. For example,
7 AMPK attenuates mitochondrial and dopaminergic dysfunction in drosophila models
8 of PD (Ng et al., 2012), and pharmacological activators of AMPK such as
9 Resveratrol (Jin et al., 2008) and Guanidinopropionic acid (Horvath et al., 2011)
10 were neuroprotective in vivo. Metformin treatment in cells overexpressing alpha
11 synuclein, to model PD, also activated AMPK and restricted cell death (Dulovic et al.,
12 2014). However, in vitro studies recently demonstrated that AMPK over-activation
13 has a detrimental effect and promoted alpha synuclein accumulation and inhibited
14 neurite growth (Jiang et al., 2013).

15
16 In conclusion, CR is perhaps the most robust and reproducible mechanism to
17 enhance lifespan and promote healthy aging. The exact mechanism/s that achieve
18 this are currently unknown, however, several theories include altered stress
19 response pathways, altered signaling pathways involving SIRT1, FOXO, UCP2 and
20 AMPK (Andrews, 2010) as well as alterations in metabolic hormones such as ghrelin
21 and insulin. We consider CR induces a mild stress and encourages compensatory
22 metabolic changes that favour improved intracellular mitochondrial health. Although
23 CR promotes metabolic health and reduces neurodegeneration there is a poor
24 compliance in the general population, as it requires ~20-40% reduced calorie intake
25 over years in order to achieve maximal benefits. Consequently, there is a need to

1 recapitulate these beneficial effects without restricting calorie intake. We have
2 discovered a novel pathway where circulating ghrelin, which is elevated during CR,
3 has a protective role in the nigrostriatal system via enhanced AMPK activity. This
4 ghrelin-induced neuroprotection is dependent on AMPK activity in dopamine
5 neurons. Future research should focus on exploiting this pathway to determine the *in*
6 *vivo* neuroprotective effects that restrict neurodegeneration without the need to
7 adhere to strict dietary regimes.

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1 Contributions

2 J.A.B, J.S.D, B.E.K, Z.B.A designed experiments

3 J.A.B, M.B.L, V.V.S, A.T, D.R, S.G, J.E performed experiments.

4 B.E.K, J.S.D, J.E provided materials and animals for the experiments and helped
5 prepare and draft the manuscript.

6 J.A.B and Z.B.A wrote the manuscript.

7

8

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33

1 **Figure 1.** Deletion of Ghrelin negates the protective effect of CR. **A**, CR significantly
2 elevates plasma acylated ghrelin. **B + C**, Overall reduction in plasma insulin levels in
3 response to CR in both genotypes. **D**, Representative images showing MPTP
4 induced TH cell loss in the SN and microglial (IBA) activation. **E & F**, Stereological
5 quantification of TH neurons in the SN showing CR has no significant effect in MPTP
6 treated Ghrelin KO mice (**F**) but is protective in Ghrelin WT mice (**E**). **G & H**,
7 Stereological quantification of IBA1 microglia in the SN shows elevated levels
8 following MPTP treatment but no effect of genotype. **I & J**, Stereological
9 quantification of GFAP in the SN showing that following MPTP administration GFAP
10 levels increased to a lesser extent in Ghrelin WT compared to Ghrelin KO mice **K**,
11 Representative images showing MPTP induced astrocyte (GFAP) activation in the
12 SN (TH = green and GFAP =green. Data are represented as mean \pm SEM (n= 6-10,
13 two-way ANOVA, $p < 0.05$). a, significant compared to saline ad-lib controls, b,
14 significant compared to MPTP ad-lib controls. Scale bar = 50 μ m

15

16 **Figure 2.** Calorie restriction reduces small volume TH cell loss and enhances
17 dopamine turnover in Ghrelin WT but not KO mice. **A**, Overall Cell volume for
18 Ghrelin WT mice showed a significant ($p < 0.05$) effect of MPTP administration but no
19 effect of genotype or diet. **B**, Ghrelin KO mice showed no overall effect of diet,
20 treatment or genotype. The red dotted line represents the average cell volume of
21 Ghrelin WT MPTP treated mice. When the cells were separated based on number
22 and volume distribution as shown in **C & D**, the effect of CR is apparent. **C**, Ghrelin
23 WT have a significant ($p < 0.05$) effect between ad-lib and CR cell volume in smaller
24 (1000-2000 μm^3) cells. There was no significant difference in the Ghrelin KO mice
25 (**D**). **E & H**, CR attenuates striatal DA loss in Ghrelin WT but not Ghrelin KO mice

1 after MPTP administration. **F & I**, MPTP reduced DOPAC with no effect of genotype.
2 **G & J**, CR reduced the elevation of the DOPAC:DA ratio in MPTP treated mice
3 compared to ad lib, in Ghrelin WT but not Ghrelin KO mice. a, significant compared
4 to saline ad-lib controls, b, significant compared to MPTP ad-lib controls.* $p < 0.05$, **
5 $p < 0.01$. Data are represented as mean \pm SEM (n=6-10, two-way ANOVA, $p < 0.05$).

6
7 **Figure 3.** The protective effect of CR is concomitant with striatal dopamine and
8 elevated pAMPK, an effect not observed in Ghrelin KO mice. **A & B**, Representative
9 Western Blot images of MPTP induced reduction in TH levels in the SN and
10 Striatum. **C & D**, Quantification of TH levels in Ghrelin WT and KO mice showed that
11 MPTP significantly ($p < 0.05$) reduced TH expression in the SN. **E & F**, Quantification
12 of TH levels in the Striatum revealed that MPTP significantly ($p < 0.05$) reduced TH
13 expression, this effect was rescued in CR Ghrelin WT mice but not in KO mice. **G &**
14 **H**, Representative Western Blot images of pAMPK, AMPK, pACC and ACC levels in
15 the SN and Striatum after either ad-libitum or CR paradigms followed by MPTP or
16 saline treatment. **I & M**, Quantification of pAMPK/AMPK and pACC/ACC levels in
17 the SN reveals no effect in Ghrelin WT mice however, in KO mice there was a
18 significant ($p < 0.05$) reduction between MPTP ad-lib and MPTP CR groups (**J & N**),
19 showing that CR KO mice could not adapt appropriately to MPTP-induced cell
20 degeneration. **K & L**, MPTP and CR individually increased striatal pAMPK/AMPK in
21 Ghrelin WT mice but not in Ghrelin KO mice, as no change from baseline with either
22 MPTP or CR was observed. **O & P**, MPTP-induced an increase in striatal
23 pACC/ACC in Ghrelin WT but not Ghrelin KO mice, mimicking the effects seen with
24 pAMPK/AMPK. **Q & R**, Representative western blots for LC3 I and LC3 II in the SN
25 and Striatum of Ghrelin WT and KO mice. **S**, LC3 II in the SN is significantly

1 reduced in Ghrelin WT mice after MPTP treatment, however this was not observed in
2 Ghrelin KO mice (**T**). **U & V**, There was no effect of CR on LC3 II in the striatum
3 from Ghrelin WT and KO mice. However, there was a significant main effect of
4 MPTP to increase LC3 II in WT but not KO mice. a, significant compared to saline
5 controls, b, significant compared to a low dose of Ghrelin. * p<0.05, ** p<0.01. Data
6 are represented as mean \pm SEM (n=5-7, one-way ANOVA, p<0.05).

7

8 **Figure 4.** Exogenous ghrelin elevates TH and AMPK activation **A**, Representative
9 Western Blot images of cultured dopaminergic neurons shows an increase in
10 pAMPK levels in response to acyl ghrelin, JMV2894 (ghrelin agonist) or oligomycin
11 treatment. Quantification of pAMPK/AMPK levels reveals a significant increase in
12 response to acyl ghrelin (**B**), JMV2894 (**C**) and oligomycin (**D**) treatment. **E & F**,
13 Representative Western Blot images of pAMPK, AMPK, pACC, ACC levels in the SN
14 and Striatum. **G & H**, Quantification of the pAMPK/AMPK and pACC/ACC in the SN
15 (**G**) in response to a high dose of ghrelin reveals a significant elevation in response
16 to the high dose of ghrelin. **I & J**, Quantification of pAMPK/AMPK and pACC/ACC in
17 the striatum reveals no change between saline ghrelin doses. **K**, Representative
18 Western Blot images of TH levels in the SN and Striatum. Quantification of TH levels
19 in the SN (**L**) and Striatum (**M**) show that ip ghrelin significantly increases TH
20 expression in response to a high dose of ghrelin. Representative Western Blot
21 images of LC3 II expression in the SN and Striatum (**N**). Quantification of LC3 II
22 revealed high dose caused a significant increase in the SN (**O**) but not the striatum
23 (**P**). a, significant compared to saline/saline controls, b, significant compared to
24 saline/MPTP controls. Data are represented as mean \pm SEM (n=6-8, two-way
25 ANOVA, p<0.05).

1 **Figure 5.** Ghrelin activates AMPK to elicit neuroprotection in an AMPK-dependent
2 manner. **A**, DAT CRE mice crossed with the tdTomato line shows a >90% co-
3 localisation (**B**) between TH (green) and tdTomato (red) neurons; scale bar =
4 100 μ m. **C**, Representative tiled image showing TH (green) and tdTomato (red)
5 where each tile represents a 20x image. **D**, tdTomato labelled TH neurons were
6 sorted via FACs to show the selective deletion of AMPK β 1 and AMPK β 2 in AMPK
7 WT but not AMPK KO mice. Product size for AMPK β 1 = 386kb, AMPK β 2 = 395kb.
8 The ghrelin receptor (GHSR) is unaffected by deletion of AMPK β 1 and AMPK β 2 in
9 SN TH neurons. **E**, Representative images showing TH neurons from AMPK WT and
10 KO mice after chronic ghrelin treatment. **F & G**, Stereological quantification of TH
11 neurons from AMPK WT (**F**) and KO (**G**) mice shows a protective effect of ghrelin
12 treatment in WT but not KO mice. **H**, Stereological quantification of IBA1 microglia in
13 the SN shows that ghrelin suppresses IBA1 cells relative to saline controls following
14 MPTP treatment, however this is not observed in AMPK KO mice (**I**). **J & K**,
15 Stereological quantification of GFAP in the SN shows that ghrelin attenuates the
16 MPTP-induced increase in GFAP cell numbers in AMPK WT (**J**) but not AMPK KO
17 (**K**) mice. **L**, Representative images showing MPTP induced astrocyte (GFAP)
18 activation in the SN (TH = green and GFAP = red). a, significant compared to
19 saline/saline controls, b, significant compared to saline/MPTP controls. Data are
20 represented as mean \pm SEM (n=6-8, two-way ANOVA, p<0.05). Scale bar = 100 μ m.

21

22 **Figure 6.** Chronic ghrelin injection enhances dopamine turnover and behavioural
23 outcomes in AMPK WT but not KO mice. **A & B**, Overall cell volume showing no
24 reduction in response to genotype or treatment. When cells were separated based
25 on number and volume distribution as shown in **C & D** there was no overall effect of

1 genotype or treatment. HPLC data show that ghrelin significantly attenuates the
2 MPTP-induced decrease in striatal dopamine concentration in AMPK WT but not
3 AMPK KO mice (**E & H**). MPTP reduced DOPAC with no effect of genotype (**F & I**).
4 Ghrelin treatment significantly attenuates the MPTP-induced increase in the
5 DOPAC/Dopamine ration in AMPK WT but not AMPK KO mice (**G & J**). **K – N**,
6 Behavioural analysis showing latency to fall on an accelerating rotarod. **K & L**, no
7 difference in latency to fall in mice not exposed to MPTP. In mice given MPTP,
8 latency to fall is not effected by genotype in mice pre-treated with saline (**M**).
9 However, in mice pre- treated with ghrelin there is a significant protective effect in
10 AMPK WT but not KO as evidence by increased latency to fall (**N**). a, significant
11 compared to saline/saline controls, b, significant compared to saline/MPTP controls.
12 Data are represented as mean \pm SEM (n=6-12, two-way ANOVA, p<0.05).

13

14 **Figure 7.**

15 Chronic ghrelin injections increase nigrostriatal TH expression and AMPK activation
16 in an AMPK-dependent manner. **A & B**, representative Western Blot images of the
17 SN (**A**) and Striatum (**B**) showing TH levels. In both the SN and the Striatum there is
18 a significant protective effect of ghrelin administration on TH levels in AMPK WT
19 mice (**C & E**) that is absent in AMPK KO mice (**D & F**). **G & H**, Representative
20 Western Blot images showing pAMPK, AMPK, pACC and ACC levels in the SN (**G**)
21 and striatum (**H**). There was no significant change in the pAMPK/AMPK (I & J) or
22 pACC/ACC (M & N) ratio in the SN of AMPK WT or AMPK KO mice in response to
23 MPTP or ghrelin. MPTP-induced an increase in the pAMPK/AMPK and pACC/ACC
24 ratio AMPK WT mice (**K & O**) but not AMPK KO mice (**L & P**). In the Striatum MPTP
25 induced an increase in the pAMPK/AMPK and pACC/ACC ratio AMPK WT mice (**K**

1 **& O)** but not AMPK KO mice (**L & P**). **Q & R**, Representative Western Blot images of
2 LC3 II expression in the SN (**Q**) and Striatum (**R**). There was no significant effect of
3 MPTP or ghrelin administration on LC3-II levels in the SN (**S & T**) or Striatum (**U &**
4 **V**). a, significant compared to saline/saline controls, b, significant compared to
5 saline/MPTP controls. Data are represented as mean \pm SEM (n=6-8, two-way
6 ANOVA, p<0.05).

7