MONASH University

Understanding the mechanisms underlying the neuroprotective role of calorie restriction in Parkinson's Disease

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A thesis submitted for the degree of *Doctor of Philosophy* at

Monash University in 2016

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Abstract

Parkinson's Disease (PD) is a debilitating neurological condition classified by a reduction of dopamine in the nigrostriatal region of the brain, resulting in movement disorders. Calorie restriction (CR) has shown to be neuroprotective during PD however, adhering to CR is difficult. In this thesis we attempted to create an alternative option that can mimic CR without having to reduce the amount of calories we consume. Initially we focused on the "hunger hormone" ghrelin, as it is elevated in the plasma during CR and is known to be protective in PD. Ghrelin exists in two distinctive isoforms, each with its own metabolic profile. In PD acyl ghrelin administration is neuroprotective, however, the role of des-acylated ghrelin is unknown. We wanted to identify the relative contributions each isoform plays using the MPTP model of PD. Chronic administration of acyl ghrelin in mice lacking both isoforms of ghrelin (Ghrelin KO) attenuated the MPTP-induced loss on Tyrosine Hydroxylase (TH; marker for dopamine) neuronal number and volume and TH protein concentration in the nigrostriatal pathway. However, injection of acyl ghrelin also elevated plasma des-acylated ghrelin, indicating *in vivo* deacetylation. Next, we chronically administered des-acylated ghrelin to Ghrelin KO mice and observed no neuroprotective effects. The lack of a protective effect was mirrored in Ghrelin-O-Acyltransferase (GOAT) KO mice, which lacks the ability to acylate ghrelin and consequently chronically increases plasma des-acyl ghrelin. Using this information we wanted to determine if acyl ghrelin was responsible for the neuroprotective actions of CR. CR attenuated the MPTP-induced loss of substantia nigra (SN) dopamine neurons and striatal dopamine turnover in Ghrelin WT but not KO mice, demonstrating that ghrelin mediates CR's neuroprotective effect. CR elevated phosphorylated AMPK-activated kinase (AMPK) levels in the SN of WT but not KO mice suggesting that AMPK is a target for ghrelin-induced neuroprotection. Indeed, exogenous acyl ghrelin significantly increased pAMPK in the SN. Genetic deletion of AMPKβ1 and 2 subunits only in dopamine neurons (AMPK KO) prevented ghrelin-induced AMPK phosphorylation and neuroprotection. Hence, ghrelin signaling through AMPK in SN dopamine neurons mediates CR's neuroprotective effects. Next we wanted to recreate the neuroprotective actions of CR with an already safe therapeutic. Metformin is the most commonly used drug to treat type 2 diabetes. It acts via AMPK activation in the periphery to ultimately lower blood glucose levels. Recently Metformin has been shown to be neuroprotective in PD. We wanted to determine if this was due to a direct effect on AMPK activity in dopaminergic neurons. We show that Metformin is neuroprotective in a mouse model of PD by attenuating dopaminergic cell loss and gliosis.

This effect was present in both AMPK WT and KO mice indicating that Metformin's neuroprotective actions are not due to AMPK activation in the SN dopaminergic neurons. Overall, these studies suggest a pathway linking CR with elevated acyl ghrelin which in turn phosphorylates AMPK in dopaminergic neurons to elicit a neuroprotective effect. CR mimetics should focus on AMPK activation in dopaminergic neurons as one potential target for the treatment of PD.

Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

Thesis including published works General Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes 1 original paper published in peer reviewed journals and 3 unpublished publications. The core theme of the thesis is neurodegeneration. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the candidate, working within the department of Physiology under the supervision of Associate Professor Zane Andrews.

(The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research.)

In the case of Chapters 1-4 my contribution to the work involved the following:

Thesis chapter	Publication title	Publication status*	Nature and extent (%) of students contribution
1	Ghrelin is neuroprotective in Parkinson's disease: molecular mechanisms of metabolic neuroprotection	Accepted. Ther Adv Endocrinol Metab. 2013 Feb; 4(1): 25–36.	Review paper. This article formed the framework of the introduction. My contribution 80% with the remainder being editing done by my supervisor.
2	Acylated but not des-acyl ghrelin is neuroprotective in an MPTP mouse model of Parkinson's Disease	Returned for Revision (minor revision required) Journal of Neurochemistry	Contributions **J.A.B** and Z.B.A designed experiments **J.A.B**, V.V.S, M.D. and M.B.L, performed experiments. **J.A.B** and Z.B.A wrote the manuscript. Overall contribution = 90%
3	Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie Restriction in Parkinson's Disease	Returned for Revision (minor revision required)	Contributions **J.A.B**, J.S.D, B.E.K, Z.B.A designed experiments

		Journal of Neuroscience	**J.A.B**, M.B.L, V.V.S, A.T, D.R, S.G, J.E performed experiments. B.E.K, J.S.D, J.E provided materials and animals for the experiments and helped prepare and draft the manuscript. **J.A.B** and Z.B.A wrote the manuscript. Overall contribution = 80%
4	Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons.	Submitted for publication Journal of Neurochemistry	Contributions **J.A.B** and Z.B.A designed experiments **J.A.B**, V.V.S and M.B.L, performed experiments. **J.A.B** and Z.B.A wrote the manuscript Overall contribution = 90%

** e.g. 'published'/ 'in press'/ 'accepted'/ 'returned for revision'*

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student signature: [redacted] **Date: 25.01.2016**

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the student and co-authors' contributions to this work.

Main Supervisor signature: [redacted] **Date: 25.01.16**

List of publications by candidate

- **Bayliss J**, Stark R, Reichenbach A and Andrews ZB**.** Gut Hormones Restrict Neurodegeneration in Parkinson's Disease, *Advanced Understanding of Neurodegenerative Diseases*, 2011 Chapter 12;269-284. Raymond Chuen-Chung Chang (Ed.), ISBN: 978-953-307-529-7, InTech, Available from: http://www.intechopen.com/articles/show/title/gut-hormones-restrict-neurodegeneration-in-parkinson-s-disease
- Kenny R, Cai G, **Bayliss JA**, Clarke M, Choo YL, Miller AA, Andrews ZB, and Spencer SJ. Endogenous ghrelin's role in hippocampal neuroprotection after global cerebral ischemia: does endogenous ghrelin protect against global stroke? American Journal of Physiology: Regulatory, Integrative and Comparative Physiology, 2013 304(11):R980-90
- **Bayliss JA**, Andrews ZB. Ghrelin is neuroprotective in Parkinson's Disease; molecular mechanisms of metabolic neuroprotection. Therapeutic Advances in Endocrinology and Metabolism. 2013 4(1):25-36
- Wu Q, Lemus MB, Stark R, **Bayliss JA**, Reichenbach A, Lockie SH, Andrews ZB. The temporal pattern of cfos activation in hypothalamic, cortical and brainstem nuclei in response to fasting and refeeding in male mice. Endocrinology 2014 155(3):840-53
- **Bayliss JA**, Lemus M, Stark R, Santos V, Thompson A, Rees D, Galic S, Elsworth J, Kemp B, Davies J, Andrews ZB. Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie Restriction in Parkinson's Disease. Journal of Neuroscience, 2016
- **Bayliss JA**, Lemus M, Santos V, Deo M, Andrews ZB. Acylated but not des-acyl ghrelin is neuroprotective in an MPTP mouse model of Parkinson's disease. Journal of Neurochemistry, 2016
- **Bayliss JA**, Lemus M, Santos V, Deo M, Andrews ZB. Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons. Journal of Neurochemistry, 2016 (under revision)

Acknowledgements

It is a pleasure to thank all the people who made this thesis possible.

It is difficult to overstate my gratitude to my supervisor A/Prof Zane Andrews. With his enthusiasm, inspiration, and great efforts to explain things clearly and simply, he helped make this PhD fun for me. Throughout my thesis-writing period, he provided encouragement, sound advice, good teaching, and heaps of good ideas. I could not have imagined having a better advisor and mentor for my PhD.

I would also like to thank my fellow lab-mates, for the stimulating discussions, support, and for all the fun we've had over the years. A special mention to Moyra Lemus for entertaining even the most trivial questions, holding my hand throughout the years, and for being as excited as I was to finally got a positive result. Minh, with you never ending enthusiasm and out of tune singing there was never a dull moment in the lab. The people in this lab kept me sane when Western Blot trials 1 through to 15 didn't work and antibodies decided the stop working. For this I offer my thanks, you all have the patience of a Saint.

Outside of the lab, plenty of people kept me sane and happy. I am indebted to my many student colleagues for providing a stimulating and fun environment to work in. The many Christmas parties and pizza nights reminded me that there is a world outside the lab. I would also like to thank my family; this year would not have been possible without you. Special mention to my sister for proof reading the final thesis.

Lastly, I offer my thanks to all the people who supported me in any way during the completion of this project. Every one of you from the staff at Cinque Lire who fuelled by caffeine addiction to the animal house staff who looked after the wellbeing of my mice were essential for the completion of this thesis. Both mice and caffeine are the only reason this thesis exists.

For all these people and many more, THANK YOU!

Abbreviations

ACC = Acetyl Coenzyme A Carboxylase

ADP = Adenosine Diphosphate

AICAR = 5-Aminoimidazole-4-carboxamide ribonucleotide

AMPK = Adenosine Monophosphate Kinase

APT1 = Acyl-protein thioesterase 1

ATP = Adenosine Triphosphate

BChE = Butyrylcholinesterase

BMI = Body Mass Index

CR = Calorie Restriction

DA = Dopamine

ETC = Electron Transport Chain

GFAP = Glial Fibrillary Acidic Protein

GHSR1a = Growth Hormone Secretagogue Receptor 1a

GLP1 = Glucagon Like Peptide 1

GOAT = Ghrelin O-acyltransferase

IBA1 = Ionised calcium Binding Adaptor Molecule 1

IL-6 / IL-8 = Interleukin 6 or 8

KO = Knockout

LRRK2 = Leucine rich repeat kinase 2

MPTP = 1-methy-4-phenyl-1,2,3,6- tetrahydropyridine

mtDNA = mitochondrial DNA

NEFA = Non Esterified Fatty Acids

PARIS = Parkin interacting substrate

PD = Parkinson’s Disease

PFA = Paraformaldehyde

PGC-1α = Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha

PINK1 = PTEN-induced putative kinase 1

ROS = Reactive Oxygen Species

SN = Substantia Nigra

T2DM = type 2 diabetes mellitus

TG = Triglyceride

TH = Tyrosine Hydroxylase

UCP2 = Uncoupling Protein 2

WT = Wildtype

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Declaration for Thesis Chapter 1

Declaration by candidate

In the case of Chapter 1, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Review paper. This article formed the framework of the introduction. My contribution 80% with the remainder being editing done by my supervisor (A/Prof. Zane Andrews)	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Zane Andrews	Editing	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date: 25.01.16**
Main Supervisor's Signature	[redacted]	**Date: 25.01.16**

Chapter 1 – Introduction

Parkinson's disease (PD) is a common neurodegenerative disease that results in the progressive loss of movement. It is the second most common brain condition in Australia with over 80,000 diagnosed patients at a cost of 6.8 billion dollars per year (Keranen et al., 2003). Strikingly, the number of diagnosed patients continues to rise 3-4% per year causing a rapidly expanding social, medical and financial burden (Keranen et al., 2003).

PD is categorised by a significant (70-80%) reduction in dopamine (DA) levels in the brain. The dopaminergic cell bodies are located in the Substantia Nigra pars compacta (SN) and project to the dorsal striatum where DA is released. The pathology of PD in humans involves an accumulation of Lewy bodies (abnormal gathering of proteins that displaces other cellular components) in the SN and a consequent loss of DA in the striatum. The major protein in Lewy bodies is α-synuclein. Many studies show that α-synuclein is responsible for the pathogenesis of PD including: (1) Some familial forms of PD are a result of α-synuclein mutations (Polymeropoulos et al., 1997, 2011) (2) Triplications of the α-synuclein locus cause PD (Singleton et al., 2003) and (3) Overexpression of α-synuclein in both Drosophila (Feany and Bender, 2000) and transgenic mice (Saha et al., 2000) results in a similar phenotype to PD. The major symptom of PD is motor dysfunction, which is caused by a loss of DA in the striatum. This motor dysfunction includes rigidity, resting tremor, postural instability and bradykinesia or slowness of movement. These symptoms occur only after a significant reduction in DA levels (German et al., 1989). However, other symptoms manifest before the onset of motor dysfunction such as sleeping disturbances, depression and gastrointestinal problems (Starkstein et al., 1991; Edwards et al., 1992). At present the cause of PD remains largely unknown, and only a few monogenic mutations that accelerate the onset of PD are currently known to exist. Hence most cases are considered to be idiopathic and are attributed to a complex relationship between age and environment. However, it is known that metabolic status, more specifically calorie restriction, has positive effects on the number one risk factor for PD, aging.

Beneficial effects of calorie restriction in aging

Calorie restriction (CR) is the most robust, non-genetic intervention to increase lifespan and reduce age-related diseases in a variety of species. In the yeast S. cerevisiae, switching from a standard growth medium to water results in a consistent 2-fold extension in lifespan (Jiang et al., 2000). In the nematode C. elegans, feeding the worms little or no bacteria also increases lifespan (Kaeberlein et al., 2006; Lee et al., 2006). In Drosophila either CR or dilution of food extends longevity in the flies (Partridge et al., 2005). In mice CR extends mean and maximal lifespan and reduces age-related diseases (Weindruch et al., 1986). Collectively, these results show the prolongevity effects of CR in a wide range of organisms, however, in higher order species such as primates the results are less clear. In primates two main studies have looked at the effect of CR on longevity, but with differing results. The University of Wisconsin-Madison study (UW) (Colman et al., 2009) showed enhanced lifespan whereas the National Institute on Aging (NIA) study did not (Mattison et al., 2012). This discrepancy in results is potentially due to the parameters of the ad-libitum control group. The UW study had an elevated sucrose concentration leading to a shortened lifespan in the control group. In contrast the NIA study used a healthier lower sucrose ad-libitum diet. This control group lived longer thus negating any additional benefits of CR. These two studies indicate that a healthy overall diet is potentially comparable to a diet that is high in sugar but involves CR. Despite the differing lifespan outcomes both studies showed enhanced health-span with a reduced risk for diabetes, cardiovascular disease and cancer in the CR groups. Overall, these studies indicate the beneficial effects of CR on health-span and lifespan across various species. There are three main theories as to how CR extends lifespan:

1) Reduced Reactive Oxygen Species (ROS) production

This theory is called the Free Radical Theory of Aging and stipulates that cellular damage is a result of the accumulation of free radical damage over time. In both yeast and Drosophila reducing oxidative damage extends lifespan (Fontana et al., 2010). In mice lifespan extension with anti-oxidants was reported in some (Schriner et al., 2005) but not all studies (Perez et al., 2009). CR in rodents results in lower accrual of tissue oxidative damage and significantly lower generation of mitochondrial free radicals. CR reduces ROS production from complex I of the mitochondrial electron transport chain (ETC) (Gredilla et al., 2001; Lopez-Torres et

al., 2002). Hence, CR has the capacity to extend lifespan by reducing oxidative damage and minimising ROS production.

2) Reduced mtDNA damage

As age increases so does the amount of mutations in mitochondrial DNA (mtDNA). This is due to the proximity to the inner mitochondrial membrane where ROS production occurs. Various tissues from aged individuals have a lower mitochondrial respiratory function compared to young individuals (Cooper et al., 1992; Boffoli et al., 1994). In humans mtDNA deletions increase with age (Corral-Debrinski et al., 1992). In rodents a 40% CR over one year significantly lowers mtDNA damage, free radical generation and oxidative damage (Gredilla et al., 2001). mtDNA damage and ROS levels are thus interrelated because as mtDNA is damaged complex I function will be diminished resulting in increased ROS production. However CR reduces the damaging effects of both of these processes and thus helps to reduce the age related decline in cellular function.

3) Increased mitochondrial function

During aging mitochondrial levels undergo morphological changes in mammals to have an abnormally rounded appearance (Wilson and Franks, 1975). Mitochondrial number also diminishes with age in the liver cells of mice (Herbener, 1976), rats (Stocco and Hutson, 1978) and humans (Sato and Tauchi, 1975), concurrent with diminished mtDNA copy number and mitochondrial protein levels (Stocco and Hutson, 1978). Mitochondrial respiratory capacity is diminished by 40% in the rodent liver in aged (24 months) compared to juvenile animals (3 months) (Stocco et al., 1977). In addition to a decline in number, mass and overall function, the activity of specific mitochondrial complexes also diminish with age. A 22-35% reduction of complex IV was measured in the brain of aging rats (Navarro, 2004). This trend was also observed in humans where a reduced activity of complex IV was found in skeletal muscle, heart and brain of aging subjects (Muller-Hocker, 1989; Curti et al., 1990; Boffoli et al., 1994). Indeed, mitochondria isolated from aged mouse brains showed elevated dysfunctional mitochondria that have about 50% the activity of complex I and IV compared to young mice (Navarro and Boveris, 2007).

One such way to minimize mitochondrial loss and to enhance respiration is by CR. In yeast CR enhances mitochondrial respiration and deletion of cytochrome c abolishes lifespan extension due to CR (Lin et al., 2002). In Drosophila (Zid et al., 2009), mice (Nisoli et al., 2005) and rats (Sreekumar et al., 2002) CR increases mtDNA as well as mitochondrial biogenesis. Indeed, in a six month human trial where subjects were CR muscle punch biopsies showed increased mitochondrial number (Civitarese et al., 2007). Collectively, these studies show the ability of CR to negate the detrimental effects of aging on mitochondrial health.

Aging is associated with a decline in mitochondrial function, characterised by reduced adenosine triphosphate (ATP) production, increased mtDNA mutations and enhanced ROS production, all of which predispose to neurodegenerative disorders. One way to delay / minimise these detrimental processes is by CR. Indeed, CR has shown to be neuroprotective in various neurodegenerative diseases, including PD.

Calorie Restriction and Parkinson's Disease

CR protects against a number of pathological conditions including diabetes, cancer, heart disease and neurodegeneration. Using the MPTP model of PD, an alternate day feeding schedule (where overall calories were reduced by 30-40%) was found to be neuroprotective in mice (Duan and Mattson, 1999). Mice also elicited a neuroprotective response when alternate day feeding was commenced after exposure to MPTP (Holmer et al., 2005). Primates with a chronic overall 30% reduction in food intake were also resistant to MPTP induced neurotoxicity (Maswood et al., 2004). These studies show that CR is beneficial in PD, however the difficulty to adhere to CR necessitates an alternative method to recapitulate the neuroprotective benefits of CR whilst bypassing dietary constraints. Cultured HeLa cells treated with plasma from CR rats caused an increase in mitochondrial bioenergetic capacity, mitochondrial biogenesis and reduced ROS production (Lopez-Lluch et al., 2006). This study indicates that during CR a component in the plasma, i.e. a circulating factor or hormone, is responsible for these beneficial effects.

Ghrelin

Ghrelin is a hormone produced from the stomach and released into the bloodstream, it plays a role in maintaining body weight and adiposity. Pro-ghrelin is synthesised in the X/A –like cells of the stomach. The enzyme Ghrelin-O-acyltransferase (GOAT) acylates proghrelin in the endoplasmic reticulum after which a large proportion is rapidly converted to des-acyl ghrelin in the plasma by Acyl-Protein Thioesterase 1 (APT1) (Satou et al., 2010), see Figure 1.1 GOAT is found in the gastrointestinal tract, predominantly in the stomach (Yang et al., 2008) where it is co-localised with ghrelin producing cells (Sakata et al., 2009). Importantly, ghrelin exists in the plasma in both biologically distinct states, acyl and des-acyl ghrelin and des-acyl ghrelin is the dominant form in the blood (Takagi et al., 2013). Acyl ghrelin binds to the Growth Hormone Secretagogue Receptor 1a (GHSR1a) located in various brain regions including the hypothalamus, hippocampus, SN and olfactory bulb (Mani et al., 2014). However, des-acyl ghrelin acts through an unknown receptor.

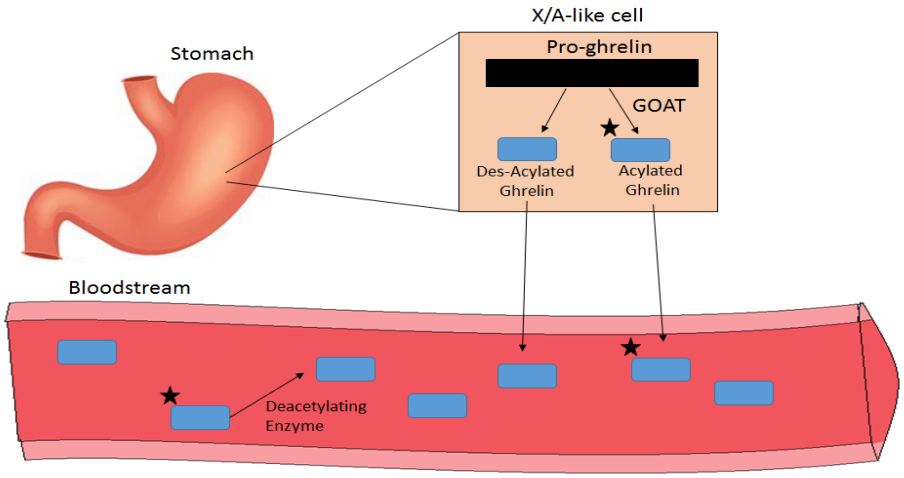

Figure 1.1: Production and relative ratio of acyl and des-acyl ghrelin in the bloodstream. Pro-ghrelin is synthesised in the X/A –like cells of the stomach. It is cleaved into either an unacylated version (des-acyl ghrelin) or with the aid of GOAT, acyl ghrelin. Both isoforms exist in the bloodstream with des-acyl ghrelin being the dominant. In the plasma acyl ghrelin is converted to des-acyl ghrelin via the deacetylating enzyme, APT1.

Acyl ghrelin is important for meal initiation, as plasma acyl ghrelin levels rise with prolonged fasting and promptly fall postprandially. Acyl ghrelin is well known as a modulator of energy homeostasis; however, it also has many other non-metabolic functions. These include enhanced learning and memory through hippocampal synaptic plasticity (Diano et al., 2006), anxiolytic and anti-depressive effects (Lutter et al., 2008) and is neuroprotective in many neurodegenerative diseases including Alzheimer's disease (Gahete et al., 2011), amyotrophic lateral sclerosis (Lee et al., 2012) and PD (Andrews et al., 2009). MPTP selectively targets dopaminergic neurons in the SN to inhibit complex I activity resulting in reduced dopaminergic neuronal number in the SN and reduced DA concentration in the striatum. Acyl ghrelin acts on SN neurons to increase the concentration of tyrosine hydroxylase (TH; rate limiting enzyme in the production of DA) in the midbrain, and attenuates MPTP-induced TH cell loss in the SN, as well as DA turnover in the dorsal striatum (Andrews et al., 2009). This study demonstrated that exogenous acyl ghrelin is neuroprotective, which was also confirmed in genetic models as MPTP reduced striatal DA and decreased SN DA cell number in ghrelin knockout (KO) compared with wild-type (WT) mice. Moreover, this effect was reversed when the ghrelin receptor was re-expressed selectively on catecholaminergic neurons, showing that ghrelin receptor signalling on catecholaminergic neurons was the primary mode of action. Interestingly, this neuroprotection was dependent upon uncoupling protein 2 (UCP2), a mitochondrial protein that plays a role in respiration, ROS production and mitochondrial biogenesis (Andrews et al., 2005a; Andrews et al., 2005b; Conti et al., 2005).

The neuroprotective properties of acyl ghrelin also involve suppressed microglial activation. Microglial activation is associated with phagocytic activity and the release of pro-inflammatory cytokines (Banati et al., 1993). Activated microglia accumulate during various neurotoxic insults including trauma (Davalos et al., 2005), infection (Rock et al., 2004) and neurodegenerative diseases (Dickson et al., 1993) in order to remove damaged neurons before they cause any further damage to surrounding healthy cells. Indeed, acyl ghrelin treatment reduced SN dopamine neuronal death with MPTP treatment, and reduced microglial activation (Moon et al., 2009). Burguillos et al recently illustrated that microglial activation is a consequence of apoptotic caspase 8 and 3/7 signalling (Burguillos et al., 2011) and acyl ghrelin is known to suppress apoptotic pathways via reduced caspase 3 activation and regulation of Bcl-2 and Bax (Jiang et al., 2008; Dong et al., 2009). This reduction in apoptosis occurs in the mitochondria, providing another link between acyl ghrelin and

mitochondrial function. Collectively, these studies imply that acyl ghrelin is neuroprotective by reducing apoptosis and consequent inflammation via reduced caspase-mediated microglial activation, as well as increased mitochondrial biogenesis.

These studies demonstrate that acyl ghrelin is neuroprotective, however, they do not take into account the effect of des-acyl ghrelin. Mice lacking both des-acyl and acyl ghrelin (Ghrelin KO) show enhanced neurodegeneration in a mouse model of PD (Andrews et al., 2009) although in this model it is impossible to ascribe the protective effect to acyl or des-acyl ghrelin. Indeed, in normal mice chronic acyl ghrelin administration is neuroprotective (Andrews et al., 2009), however as acyl ghrelin is readily deacetylated in the plasma the protective effect could be ascribed to acyl ghrelin or des-acyl ghrelin. Thus the question still remains; is des-acyl ghrelin neuroprotective in a mouse model of PD? This is an extremely important question that must be answered before ghrelin can be considered for clinical trials in PD.

There are numerous reports showing that des-acyl ghrelin acts independent of both acyl ghrelin and its receptor; GHSR1a. For example, des-acyl ghrelin is neuroprotective in ischemic brain injury and Alzheimer's Disease. Des-acyl ghrelin administration prevents cell death in cultured neurons exposed to oxygen and glucose deprivation in the presence of a GHSR antagonist (Chung et al., 2008; Hwang et al., 2009). *In vivo*, des-acyl ghrelin administration exhibits a vasodilator response (Ku et al., 2015) and both acyl and des-acyl ghrelin are protective after transient focal ischemia reperfusion (Hwang et al., 2009). In microglia exposed to Amyloid-beta, des-acyl ghrelin counteracted the activation of the pro-inflammatory cytokine interleukin 6 (IL-6), whereas acyl ghrelin had no effect (Bulgarelli et al., 2009).

Collectively these studies imply that elevated levels of acyl, and potentially des-acyl ghrelin, are neuroprotective in PD. Hence, increased circulating levels of plasma ghrelin may provide neuroprotection in PD and reduced plasma ghrelin may predispose individuals to SN DA degeneration.

Metabolic status and ghrelin

During CR acyl ghrelin levels rise and recent studies show that the actions of acyl ghrelin are elevated during negative energy balance, as acyl ghrelin primarily functions to shift an organism from negative to neutral energy balance (Briggs et al., 2011). In addition to promoting food intake after fasting (Salome et al., 2009), acyl ghrelin mediates the anti-depressive and anti-anxiogenic effects of CR (Lutter et al., 2008), as well as helping to maintain blood glucose during CR (Zhao et al., 2010). On the other hand, diet-induced obesity suppresses many of the metabolic actions of acyl ghrelin including food intake and growth hormone secretion (Perreault et al., 2004; Briggs et al., 2010; Zigman et al., 2016). These studies imply that metabolic status plays a fundamental role in the effectiveness and actions of acyl ghrelin in the body, whereby negative energy balance enhances acyl ghrelin's action and diet-induced obesity attenuates acyl ghrelin's action.

Metabolic dysregulation is a risk factor for PD as both obesity and diabetes predispose development of the disease (Abbott et al., 2002), although the correlation with obesity is debated by others (Logroscino et al., 2007). In addition, mouse models of PD show that diet-induced obesity enhances dopaminergic cell loss (Choi et al., 2005). In PD there is a paradoxical relationship between plasma acyl ghrelin and Body Mass Index (BMI). PD patients have lower plasma acyl ghrelin concentrations compared to healthy individuals when matched for BMI (Fiszer et al., 2010), indicating that acyl ghrelin secretion is disrupted in people with PD. Thus, this evidence suggests that metabolic dysfunction associated with diet-induced obesity contributes to the progression of PD. Whether deficits in plasma acyl ghrelin signalling in diet-induced obesity predispose individuals to degeneration is unknown.

Ghrelin modulation of the dopamine receptor

In PD, many therapies, such as DA receptor agonists, are used to maintain optimal DA concentration in the striatum. These therapies act directly on dopaminergic neurons and mimic the endogenous neurotransmitter resulting in enhanced motor control for PD patients. Any agent that increases the activity of this receptor will reduce motor symptoms associated with PD. Recently the dopaminergic receptor DRD2 and the acyl ghrelin receptor GHSR1a have been shown to physically interact and form heterodimers *in vitro* and show co-

localisation in the cortex, SN, ventral tegmental area, as well as the hypothalamus (Jiang et al., 2006).

To determine the role of GHSR1a:DRD2 heterodimers in appetite Kern and colleagues gave mice cabergoline (DRD2 agonist) (Kern et al., 2012). Selective activation of DRD2 receptors on these neurons resulted in anorexia indicating that activation of DRD2 was sufficient to reduce food intake without the need for acyl ghrelin to be present. This effect was dependent upon the GHSR1a:DRD2 receptor interaction (Kern et al., 2012). Although this study focused on food intake, it indicates that in neurons expressing both populations of receptors such as the SN, the GHSR1a receptor can modify dopaminergic signaling. Hence, circulating acyl ghrelin can bind to the GHSR1a receptor on DA neurons, form a heterodimer with the DRD2 receptor and enhance DA release in the striatum resulting in reduced motor symptoms in PD. Further, even in the absence of acyl ghrelin ligand binding, GHSR1a and DRD2 could influence signaling. Therefore, acyl ghrelin may be neuroprotective in PD by directly activating GHSR1a to enhance mitochondrial bioenergetics, reduce mitochondrial apoptosis and microglial activation, as well as by modulating the DRD2 receptor to increase DA release.

Intracellular targets of ghrelin

Determining intracellular targets in PD could potentially lead to new therapeutic strategies to reduce disease progression. Genome-wide analysis in identified DA neurons from patients with PD have identified specific gene sets as potential targets. These include defects in mitochondrial electron transport as well as glucose utilization and glucose sensing, all of which are connected to ghrelin (Andrews et al., 2009; Delhanty and van der Lely, 2011). One particular target that was identified was peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), (Zheng et al., 2010) often referred to as the master regulator of mitochondrial biogenesis. Activation of PGC-1α results in increased mitochondrial number and reduced dopaminergic neuronal loss in the MPTP model (St-Pierre et al., 2006; Mudo et al., 2012). Mitochondrial dysfunction is a well-known contributor to the onset of PD (Abou-Sleiman et al., 2006) and mitochondrial interventions may provide a treatment strategy to prevent or reduce neurodegenerative disease progression. A potential target could include PGC-1α or any agent that modulates its activity, for example adenosine monophosphate-

activated kinase (AMPK). AMPK is an energy sensor that promotes mitochondrial biogenesis to optimize cellular function.

Ghrelin and AMPK

In the hypothalamus acyl ghrelin increases AMPK activity (Andrews et al., 2008), whether or not acyl ghrelin increases AMPK in the SN is unknown. We hypothesise that acyl ghrelin is neuroprotective in PD via increased AMPK activity in the SN. AMPK is a sensor of cellular energy that increases ATP production and suppresses energy consumption during cellular stress (Hardie et al., 2012). AMPK increases energy production by regulating mitochondrial function and during chronic energy depletion AMPK is a major regulator of mitochondrial biogenesis in muscle (Bergeron et al., 2001). Consequently, chronic elevation of AMPK by the drug 5-Aminoimidazole-4-carboxamide ribonucleotide (AICAR) up-regulates key mitochondrial enzymes in skeletal muscle, resulting in mitochondrial biogenesis (Winder et al., 2000). Moreover, exercise is a well-documented physiological stimulus for AMPK activation and aerobic exercise is neuroprotective in PD (Sung et al., 2012), suggesting that elevated AMPK could mediate the neuroprotective role of exercise in PD.

Downstream actions of AMPK involve enhanced sirtuin 1 (SIRT1) activity, elevated activity of its downstream target PGC-1α and mitochondrial biogenesis (Canto and Auwerx, 2009). PGC-1α levels increase in response to AMPK activity and are also reduced in AMPKα2 KO mice (Iglesias et al., 2004). Further support for the importance of PGC-1α in preventing neurodegeneration comes from PGC-1α null mice, which are more susceptible to MPTP (St-Pierre et al., 2006). Hence, potential intracellular metabolic targets to reduce neurodegeneration include SIRT1, AMPK and PGC-1α, however, if they are activated in response to acyl or des-acyl ghrelin in dopaminergic neurons is currently unknown, see Figure 1.2. Recent studies in Drosophila support the idea that AMPK mediates the neuroprotective effects of PD. This study used Leucine Rich Repeat Kinase 2 (LRRK2) and Parkin (known mutations that result in PD) null flies to model PD as mutations in either have been linked to early onset PD. Genetic activation of AMPK ameliorated the Parkinsonian phenotype resulting in increased motor control (Ng et al., 2012).

AMPK consists of three subunits with different isoforms (α1, α2, β1, β2, γ1, γ2 and γ3) (Carling et al., 1994). Each subunit plays a key role in overall AMPK activity; the alpha subunit has a catalytic role (Crute et al., 1998) whereas beta and gamma play regulatory roles. Whole-body deletion of α2 results in mild insulin resistance and impaired glucose tolerance (Jorgensen et al., 2004), both these metabolic alterations are features of type 2 diabetes mellitus (T2DM). Muscle specific deletion of beta 1 and beta 2 caused mitochondrial dys-function and insulin resistance (O'Neill et al., 2011). Indeed, activators of AMPK (such as Metformin) are well-known treatments of T2DM and interestingly Metformin reduces the risk for developing PD in a population of diabetics (Wahlqvist et al., 2012). As T2DM is a risk factor for PD we believe that AMPK is a promising target for future research in PD.

AMPK is also involved in autophagy, the process whereby damaged or unnecessary organelles (including mitochondria) are removed from the cell. During negative energy balance, AMPK is phosphorylated, resulting in the inhibition of mTOR to ultimately enhance autophagy.

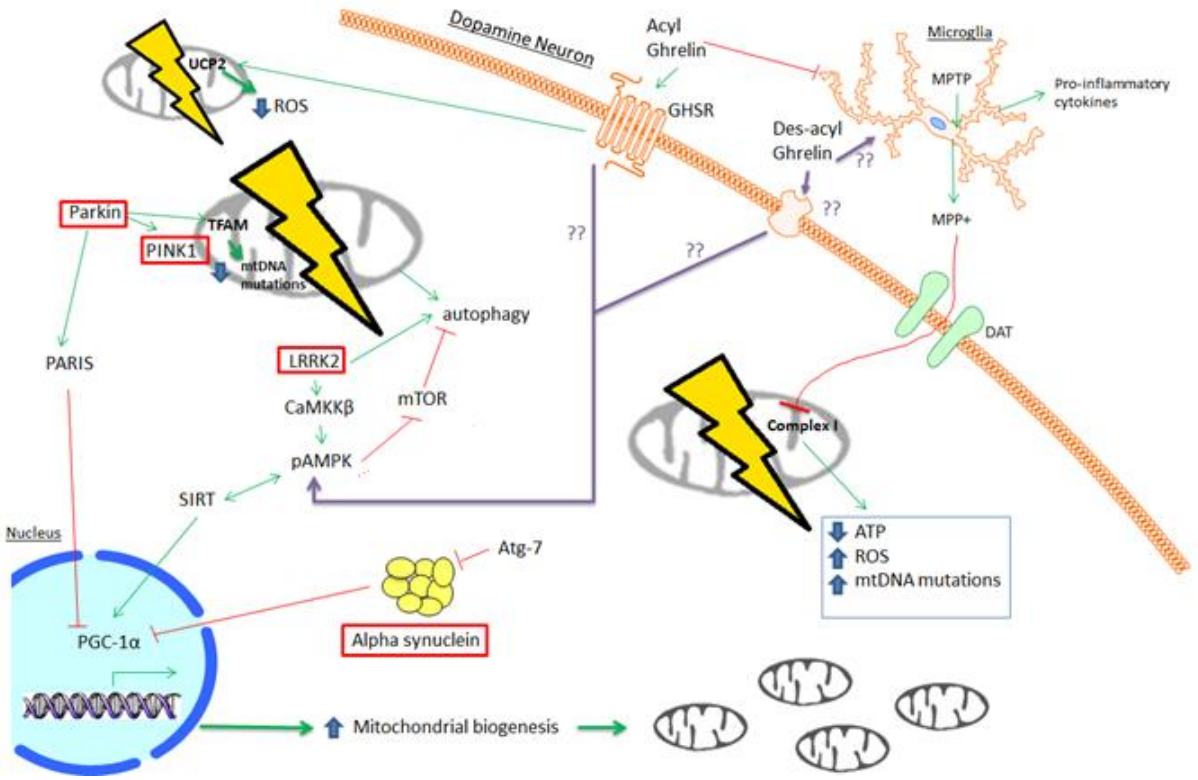

Figure 1.2. Potential ghrelin neuroprotective pathways in PD. MPTP is a model of PD and recapitulates the disease via inhibition of complex I in the mitochondria resulting in increased ROS and reduced ATP production. It also acts on microglia by stimulating pro-inflammatory cytokines to enhance the removal of damaged neurons. MPTP crosses the blood–brain barrier and is converted into its toxic form MPP^+, which enters the DA neuron via the dopamine transporter (DAT), making it selective for DA neurons. Acyl ghrelin reduces the inflammatory component of microglial activation observed in PD and also binds to the GHSR receptor to ultimately enhance mitochondrial biogenesis and reduce oxidative stress via UCP2. Des-acyl ghrelin does not bind to GHSR, instead acting through an unknown receptor. The effect of des-acyl ghrelin on glia is unknown. Both acyl and des-acyl ghrelin may also act via pAMPK to inhibit mTOR to enhance autophagy. Other downstream actions of pAMPK involve enhanced SIRT1 activity, to elevate the activity of its downstream target PGC-1α and increase mitochondrial biogenesis. There are many known genetic mutations linked to PD highlighted in red. Many of these are involved in removal of damaged mitochondria, for example the PINK1/ Parkin pathway. Damaged mitochondria (depicted with a lightning bolt) attract PINK1 around the damaged organelle. This recruits Parkin from the cytosol and initiates mitophagy. Parkin is involved in reducing mtDNA mutations by enhancing the actions of TFAM and is also responsible for maintaining mitochondrial biogenesis by reducing the amount of PARIS in dopaminergic cells. PARIS represses PGC-1α leading to a reduction in mitochondrial biogenesis. Another known gene mutation that results in repression of PGC-1α is alpha synuclein, this is the main pathological hallmark of PD. Another genetic mutation linked to PD is LRRK2 which is important in autophagy. Together, this picture shows a potential role of acyl and des-acyl ghrelin in neuroprotection and also shows a potential role of pAMPK in this process. Further research will be required to determine whether there is a link.

Mitophagy and PD

Problems with mitophagy (the selective degradation of mitochondria) have been linked with PD. Mitochondria are considered the 'powerhouse' of the cell, as they are responsible for aerobic respiration and the conversion of Adenosine Diphosphate (ADP) into the energy rich ATP. As mitochondria are essential in maintaining neuronal function and neuronal metabolism any agent that positively regulates mitochondrial function could be a therapeutic target for the reduction or prevention of neurodegenerative disease progression. There are a multitude of mitochondrial impairments that contribute to PD, including reduced mitochondrial uncoupling (Conti et al., 2005; Andrews et al., 2009), increased free radical production and oxidative stress (Lin and Beal, 2006; St-Pierre et al., 2006), a reduction in the formation of ATP (Mann et al., 1992), impaired calcium buffering (Marongiu et al., 2009) and reduced mitochondrial biogenesis (St-Pierre et al., 2006).

When mitochondrial dysfunction occurs by any mechanism listed above, damage or loss of cellular function occurs. These damaged mitochondria need to be efficiently removed as otherwise mitochondrial dysfunction results in increased oxidative stress, and damage to DNA, proteins and membrane lipids (Lin and Beal, 2006). Damaged mitochondria attract PTEN-induced putative kinase protein 1 (PINK1) around the damaged organelle. This recruits Parkin from the cytosol and initiates ubiquitylation of damaged mitochondria (Vives-Bauza et al., 2010). Ubiquitin then binds and labels mitochondria for destruction surrounding it in an isolation membrane. This membrane fuses with lysosomes where mitochondria are degraded and recycled within the cell (Vives-Bauza et al., 2010). Defects in mitophagy result in early onset hereditary forms of PD (Kitada et al., 1998). These defects result from a mutation that leads to loss of function in either PINK1 or Parkin. If mitophagy is affected, damaged mitochondria accumulate, restricting ATP generating potential and increasing ROS production. This decreases neuronal function and predisposes cells to degeneration. Parkin is also involved in maintaining mitochondrial biogenesis by reducing the amount of Parkin Interacting Substrate (PARIS) in dopaminergic cells. PARIS represses PGC-1α and the PGC-1α target gene NRF-1 by binding to the insulin response sequence in the PGC-1α promoter. Overexpression of PARIS results in dopaminergic cell death in the SN, which is negated when either PGC-1α or Parkin are over-expressed (Shin et al., 2011). This study indicates that Parkin maintains optimal mitochondrial biogenesis via the Parkin–PARIS–PGC-1α

pathway. Collectively these studies show that Parkin is responsible for maintaining optimal mitochondrial function and removal of damaged mitochondria via mitophagy.

Removal of damaged components in a cell (autophagy) has been implicated as one factor contributing to the development of PD. Recently, Friedman and colleagues developed a mouse model that is autophagy-deficient by the selective removal of an essential autophagy gene Atg-7. This gene was deleted selectively in dopaminergic neurons, resulting in mice with late onset locomotor deficits similar to those seen in PD. Whole-brain Atg-7 KO results in presynaptic accumulation of LRRK2 and α-synuclein (Friedman et al., 2012). This study implicates disrupted autophagy in the pathogenesis of idiopathic PD. If autophagy is disrupted α-synuclein is allowed to accumulate in cells potentially resulting in the formation of Lewy bodies, a pathological hallmark of PD. In further support of this, mutations in α-synuclein result in early onset PD. α-synuclein also modulates transcription of PGC-1α, the mastergene of mitochondrial biogenesis (Siddiqui et al., 2012). This study shows that nuclear α-synuclein localization is increased under conditions of oxidative stress *in vivo* and *in vitro*. Another study using SH-SY5Y cells overexpressing the α-synuclein mutation A53T show that α-synuclein localizes at the mitochondrial membrane resulting in oxidative modification of mitochondrial components (Parihar et al., 2008). These studies indicate that accumulation of α-synuclein during PD will not only disrupt cellular functioning but will also reduce the number of mitochondria present and decrease the likelihood of dopaminergic survival. Hence any agent that enhances autophagy to prevent α-synuclein accumulation would be neuroprotective in PD.

Another genetic cause of PD is a defect in the LRRK2 gene where mutations in this gene are associated with both familial and idiopathic PD (Bonifati, 2007). LRRK2 causes the accumulation of autophagic structures when overexpressed in SY5HSY dopaminergic cells or in transgenic mice (Plowey et al., 2008). LRRK2 also interacts with AMPK via activation of CaMKK-beta resulting in the increase in autophagosome formation (Gomez-Suaga et al., 2012). Following MPTP administration, LRRK2 mRNA levels increase (Hurley et al., 2007) and excess LRRK2 accelerates the progression of neuropathic abnormalities such as the formation of Lewy bodies (Lin et al., 2009).

In order to overcome mitochondrial pathology such as oxidative stress and defective mitophagy, there is the need to increase mitochondrial turnover. Turnover is enhanced by mitochondrial biogenesis, the process whereby new mitochondria are formed within a cell

(Suliman et al., 2004). Enhancing mitochondrial biogenesis represents a therapeutic target for future PD research. We propose that acyl ghrelin via increased AMPK activation will attenuate metabolic degeneration by enhancing mitochondrial biogenesis and turnover, increasing autophagy and reducing oxidative stress. Another CR mimetic that also activates AMPK is Metformin.

Metformin

Metformin is a biguanide analogue commonly used for the treatment of T2DM and is generally well tolerated. Metformin reduces blood glucose levels through increased glucose uptake into muscle and a downregulation of key gluconeogenesis genes leading to reduced glucose production in the liver. By lowering blood glucose, IGF-1 and insulin signalling, Metformin creates an environment that is similar to CR and as such many beneficial effects of CR can be reproduced by chronic Metformin treatment. Metformin has been shown to extend median survival by 40% in C. elegans, whilst also prolonging youthful locomotion in a dose-dependent manner (Onken and Driscoll, 2010). In mice Metformin produced approximately a 6% lifespan extension, which was also accompanied by improved locomotor performance (Martin-Montalvo et al., 2013). Indeed, in a human study patients with T2DM on Metformin monotherapy had a longer survival than matched non-diabetic controls (Bannister et al., 2014). As CR is beneficial for PD (Duan and Mattson, 1999) and T2DM (Pi-Sunyer et al., 2007), Metformin has the potential to treat both disease states.

Although used in the treatment of T2DM, Metformin's beneficial effects are not exclusive to the lowering of blood glucose. T2DM patients using Metformin have a reduced risk for the development of cancer independent of Metformin's effects on T2DM (Evans et al., 2005; Gallagher and LeRoith, 2011) and a greater protective effect against strokes (Cheng et al., 2014). In T2DM oxidative stress occurs in various brain regions and Metformin protects against this imbalance (Correia et al., 2008). Metformin is also protective *in vitro* by reducing apoptotic cell death in primary cortical neurons (El-Mir et al., 2008). Previous studies show that Metformin is neuroprotective in PD. *In vitro*, treatment with Metformin reduced the neurotoxicity associated with α-synuclein overexpression (Dulovic et al., 2014). In a Drosophila model of PD, Metformin treatment alleviated dopaminergic dysfunction and mitochondrial abnormalities (Ng et al., 2012). Metformin chronically administered to mice

reduces oxidative stress, dopaminergic degeneration and motor abnormalities associated with MPTP administration (Patil et al., 2014). A retrospective study by Wahlqvist et al. (2012) observed that patients with T2DM had reduced risk of developing PD when they had been taking oral anti-hyperglycemic agents (OAA) such as Metformin. 800,000 Taiwanese patient records were collated into either T2DM or non-T2DM patients and taking OAA therapy or not. It was concluded that T2DM increases the risk PD incidence by 2.2-fold and further enhanced by over 50% by other anti-diabetic medications such as sulfonylurea, however when combined with metformin the risk of developing PD decreased significantly (Wahlqvist et al., 2012). Hence, Metformin treatment has a protective effect in PD. Future research is required to determine if Metformin is neuroprotective due to an enhanced global metabolic profile (i.e. reduction in glucose, IGF-1 and insulin signalling) or a specific downstream activator.

As Metformin has been deemed safe with minimal side effects and is known to rapidly cross the blood brain barrier and disperse into various brain regions (Labuzek et al., 2010), it is an ideal therapeutic for the treatment of PD. Recently, a mechanism of action for how Metformin suppresses gluconeogenesis has been discovered (Madiraju et al., 2014), however, how Metformin is neuroprotective is still unknown. For example, after Metformin treatment the transcription factor SKN/Nrf2 is activated, ultimately increasing the expression of anti-oxidant genes to protect against oxidative damage (Onken and Driscoll, 2010). Metformin has also been shown to inhibit mTOR to enhance mitochondrial function (Dowling et al., 2007; Johnson et al., 2013). Metformin can also activate AMPK by inhibiting complex I of the ETC (Zhou et al., 2001). This results in an increased AMP/ATP ratio and the subsequent activation of AMPK. AMPK acts to increase mitochondrial biogenesis (Canto et al., 2009) and as patients with PD have impaired mitochondrial function, AMPK activation in DA neurons may be responsible for Metformin's protective actions. However, *In vitro* Metformin can scavenge ROS and recently has been shown to block endogenous ROS production in an AMPK independent manner. As excessive ROS production leads to oxidative stress, a protective effect will be observed in both PD and T2D.

To summarise, enhancing AMPK activity to increase mitochondrial function results in neuroprotection in PD and therapeutics such as Metformin that enhance AMPK activity could be a potential therapeutic target for reducing PD progression. However, if the neuroprotective effects of Metformin can be solely attributed to AMPK activation in the SN is still unknown.

Conclusion

CR is perhaps the most robust and reproducible mechanism to enhance lifespan and promote healthy aging. The exact mechanism/s responsible are currently unknown, however, several potential theories include altered stress response pathways, altered signalling pathways (involving SIRT1, PGC1α and AMPK) as well as alterations in metabolic hormones such as ghrelin and insulin. CR places a mild metabolic stress on the body that results in favourable compensatory biological changes to allow the organism to better respond to metabolic stress in the future. This leads to enhanced cognitive function and neuronal plasticity and also works to enhance intracellular mitochondrial health, termed "mitohormesis". During mitohormesis, the mild metabolic stress of CR acts to enhance mitochondrial function and as mitochondrial dysfunction plays a key role in PD progression, the overall effect is neuroprotection during CR. Although CR may control mammalian aging, improving overall health and reducing neurodegeneration, CR is not well tolerated or adhered to in the general population, which requires ~20-40% reduction in calorie intake over decades in order to achieve maximal benefits. Consequently, there is a need to recapitulate these beneficial effects without withholding calories. In this thesis we want to elucidate the mechanism behind how CR is neuroprotective in PD. We hypothesise that acyl ghrelin is neuroprotective during CR by increasing the intracellular mechanisms involved in mitochondrial function, namely AMPK. Further, we wish to re-create the beneficial effects of CR using therapeutic approaches that don't involve minimising caloric intake. The specific aims to be tested are:

1. To determine which isoform of ghrelin is responsible for neuroprotection in a PD model (Chapter 2)
2. To determine the role of ghrelin in the neuroprotective actions of CR in a PD model (Chapter 3)
3. To determine if acyl ghrelin increases AMPK activity to elicit a neuroprotective effect in nigral dopamine neurons (Chapter 3) and
4. To determine if Metformin activates AMPK in nigral dopamine neurons to attenuate neuronal loss in a mouse model of PD (Chapter 4)

Introduction for Chapter 2

In Chapter 2 we have focused on the neuroprotective actions of the metabolic hormone Ghrelin. Ghrelin exists in two distinctive isoforms, acyl and des-acyl ghrelin. In normal mice chronic acyl ghrelin administration is neuroprotective in a mouse model of Parkinson's Disease (Andrews et al., 2009), however as acyl ghrelin is readily deacetylated the protective effect could have been ascribed to acyl ghrelin or des-acyl ghrelin (that was deacetylated in situ). Thus the question still remains; is des acyl ghrelin neuroprotective in mouse models of PD? We attempted to answer this in Chapter 2.

Declaration for Thesis Chapter 2

Declaration by candidate

In the case of Chapter 2, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Myself and ZBA designed the experiment. I performed all laboratory testing and analysis (with the assistance of M.L., M.D. and V.S.), with the exception of the HPLC analysis. In collaboration with my supervisor (A/Prof. Zane Andrews) we wrote the entire manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Moyra Lemus	Assisted with laboratory experiments	
Minh Deo	Assisted with laboratory experiments	
Vanessa V Santos	Assisted with laboratory experiments	
John Elsworth	Performed all HPLC experiments	
Zane Andrews	Assisted with designing of experiments and writing of the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date: 25.01.16**
Main Supervisor's Signature	[redacted]	**Date: 25.01.16**

Chapter 2 - Acylated but not des-acyl ghrelin is neuroprotective in an MPTP mouse model of Parkinson's Disease

Acylated but not des-acyl ghrelin is neuroprotective in an MPTP mouse model of Parkinson's Disease

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Key words: Dopamine, Acylated ghrelin, Des-acylated ghrelin, GOAT, Parkinson's Disease, corticosterone

Abbreviations

DA = Dopamine, GFAP = Glial Fibrillary Acidic Protein, GHSR1a = Growth Hormone Secretagogue Receptor 1a, GLP1 = Glucagon Like Peptide 1, GOAT = Ghrelin-O-Acyltransferase, IBA1 = Ionized Calcium Binding Adaptor Molecule 1, MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NEFA = Non-esterified Fatty Acid, PD = Parkinson's Disease, PFA = Paraformaldehyde, SN = Substantia Nigra, TH = Tyrosine Hydroxylase

Abstract

The gut hormone ghrelin is widely beneficial in many disease states. However, ghrelin exists in two distinctive isoforms, each with its own metabolic profile. In Parkinson's Disease (PD) acylated ghrelin administration is neuroprotective, however, the role of des-acylated ghrelin is unknown. In this study we wanted to identify the relative contribution each isoform plays using the MPTP model of PD. Chronic administration of acylated ghrelin in mice lacking both isoforms of ghrelin (Ghrelin KO) attenuated the MPTP-induced loss on Tyrosine Hydroxylase (TH) neuronal number and volume and TH protein concentration in the nigrostriatal pathway. Moreover, ghrelin reduced the increase in Glial Fibrillary Acidic Protein (GFAP) and Ionized calcium binding adaptor molecule 1 (IBA1) microglia in the substantia nigra. However, injection of acylated ghrelin also elevated plasma des-acylated ghrelin, indicating *in vivo* deacetylation. Next, we chronically administered des-acylated ghrelin to Ghrelin KO mice and observed no neuroprotective effects in terms of TH cell number, TH protein, GFAP and IBA1 cell number. The lack of a protective effect was mirrored in Ghrelin-O-Acyltransferase (GOAT) KO mice, which lacks the ability to acylate ghrelin and consequently chronically increases plasma des-acyl ghrelin. Plasma corticosterone was elevated in GOAT KO mice and with des-acylated ghrelin administration. Overall, our studies suggest that acylated ghrelin is the isoform responsible for *in vivo* neuroprotection and pharmacological approaches that prevent plasma conversion from acyl-ghrelin to des-acyl ghrelin may have clinical efficacy to help slow or prevent the debilitating effects of PD.

Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease characterised by rigidity, tremor and an overall lack of smooth motor control. These symptoms are due to a generalised lack of dopamine secreted from nerve terminals in the striatum. The cell bodies of these dopamine neurons are located in the substantia nigra (SN) and collectively this pathway is known as the nigrostriatal pathway. The cause of this debilitating disease is unknown and for this reason treatment involves symptom management as opposed to halting disease progression.

In recent years, efforts have focussed on discovering endogenous neuroprotective mediators of SN dopamine neurons primarily because treatments have the potential to reach clinical settings faster. An example of this is the gut hormone glucagon-like peptide 1 (GLP1) that controls blood glucose. It was discovered that in addition to regulating glucose homeostasis, GLP1 also targets receptors on SN dopamine neurons to prevent neurodegeneration in mouse models of PD (Bertilsson et al., 2008; Li et al., 2009). Since this initial discovery, the GLP1 agonist Exenatide has proved to be clinical effective to reduce disease progression (Aviles-Olmos et al., 2013).

We have focussed on another gut hormone ghrelin, which also targets the brain to regulate metabolism and neuroprotection. Ghrelin is a hormone produced from the stomach and released into the bloodstream and plays a role in maintaining body weight and adiposity. Importantly, ghrelin exists in the plasma in two biologically distinct states, acyl and des-acyl ghrelin and des-acyl ghrelin is the dominant form in the blood (Takagi et al., 2013). The enzyme Ghrelin-O-acyltransferase (GOAT) acylates proghrelin in the endoplasmic reticulum after which a large proportion is rapidly converted to des-acyl ghrelin in the plasma by Acyl-Protein Thioesterase 1 (APT1) (Satou et al., 2010). GOAT is found in the gastrointestinal tract, predominantly in the stomach (Yang et al., 2008) where it is co-localised with ghrelin producing cells (Sakata et al., 2009). Acylated ghrelin binds to the Growth Hormone Secretagogue Receptor 1a (GHSR1a) receptor located in many different brain regions including the hypothalamus, hippocampus, substantia nigra and olfactory bulb (Mani et al., 2014). However, des-acyl ghrelin acts through an unknown receptor.

Plasma ghrelin levels are elevated just prior to meal initiation and a single injection of acylated ghrelin enhances food intake in both rodents and humans (Wren et al., 2000;

Cummings et al., 2001; Wren et al., 2001). Ghrelin also has many non-metabolic roles including reduced anxiety and stress (Spencer et al., 2012), enhanced learning and memory (Diano et al., 2006) and neuroprotection in PD (Andrews et al., 2009; Moon et al., 2009). Chronic administration of acylated ghrelin is protective in a mouse model of PD (Andrews et al., 2009) an effect mediated through the growth hormone secretagogue receptor (GHSR; acylated ghrelin receptor). Mice lacking both des-acyl and acyl ghrelin (Ghrelin KO) mice showed enhanced neurodegeneration in a mouse model of PD (Andrews et al., 2009) although in this model it is impossible to ascribe the protective effect to acyl or des-acyl ghrelin. Indeed, in normal mice chronic acylated ghrelin administration is neuroprotective (Andrews et al., 2009), however as ghrelin is readily deacetylated the protective effect could have been due to acylated ghrelin or des-acyl ghrelin (that was deacetylated in situ). Thus the question still remains; is des-acyl ghrelin neuroprotective in mouse models of PD? This is an extremely important question that must be answered before ghrelin can be considered for clinical trials on neuroprotective effects in PD.

There are numerous reports showing that des-acyl ghrelin acts independent of both acyl ghrelin and of GHSR. For example, des-acyl ghrelin administration prevents cell death in cultured neurons exposed to oxygen and glucose deprivation in the presence of a GHSR antagonist (Chung et al., 2008; Hwang et al., 2009). In vivo, des-acyl ghrelin administration exhibits a vasodilator response (Ku et al., 2015) and both acylated and des-acyl ghrelin are protective after transient focal ischemia reperfusion (Hwang et al., 2009). In microglia exposed to Amyloid-beta, des-acyl ghrelin counteracted the activation of the pro-inflammatory cytokine IL-6, whereas acylated ghrelin had no effect (Bulgarelli et al., 2009). We hypothesized that elevated des-acyl ghrelin will prevent nigrostriatal degeneration in a mouse model of PD.

Methods

Animals

All experiments were conducted in compliance with the Monash University Animal Ethics Committee guidelines. Mice were kept at standard laboratory conditions with free access to food and water at 23 ^{o}C in a 12 hours light/dark cycle unless otherwise stated. Male Ghrelin and GOAT KO mice (approximately 8-10 weeks old) on a C57/Bl6 background were attained from Regeneron Pharmaceuticals (Tarrytown, NY) and bred in the Monash Animal Services.

Experimental Protocol

GOAT mice, which contain naturally high circulating des-acyl ghrelin levels but negligible acylated ghrelin as described previously (Zhao et al., 2010), were injected with 1-methyl4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) (30mg/kg, i.p.) dissolved in saline over two consecutive days. Control mice received sterile saline. Animals were culled 7 days later and perfused for immunohistochemical analysis or fresh tissue collection for Western Blot or HPLC analysis. The relatively short time frame used with MPTP was chosen based on previous studies indicating an approximately 50% loss in dopaminergic neurons and striatal dopamine using this timeframe (Andrews et al., 2005b; Andrews et al., 2009). Future studies should concentrate on the long-term effects to focus on the behavioural phenotype in these studies.

Ghrelin KO mice that produce neither acylated nor des-acylated ghrelin (Wortley et al., 2004; Wortley et al., 2005) were used to examine the role each distinct form of ghrelin plays in PD progression when each isoform was returned. The use of Ghrelin KO mice allows us to directly assess the impact of exogenous acyl or des-acyl ghrelin without endogenous acyl or des-acyl ghrelin acting as a confounding variable. Mice were treated chronically (14 days) with des-acyl ghrelin (1mg/kg) or acylated ghrelin (1mg/kg). This dose was chosen based upon previous research showing that acylated ghrelin elicits a feeding response (Briggs et al., 2013). To prevent mice from consuming excess calories during this process they were injected during the light phase and food was removed, 6 hours later food was returned. Previous studies (Andrews et al., 2009) indicate that if calories are consumed after injection

of acyl-ghrelin there is no neuroprotective effect observed, presumably because the actions of ghrelin are negated by the consumption of food and associated metabolic feedback of glucose and insulin. After 7 days of treatment mice received MPTP treatment (30mg/kg, i.p.) or sterile saline over two consecutive days. 7 days after these injections mice were sacrificed (45 minutes after final injection) and perfused for immunohistochemistry or fresh tissue collection for Western Blot analysis.

Immunohisochemistry

All mice were deeply anaesthetised and perfused with 0.05% PBS followed by 4% paraformaldehyde (PFA). Brains were collected and post fixed overnight in PFA then transferred into 30% sucrose. After 48 hours in sucrose 30µm coronal sections of the entire substantia nigra were collected and stored in cryoprotectant (30% Glycerol and 20% ethylene glycol in 0.1M PB) at -20°C until staining was performed.

Before the primary antibody was added the tissue was washed thoroughly using 0.1M PB and endogenous peroxidase activity was blocked with 1% H2O2 in 0.1M PB for 15 minutes and washed again. The tissue was then blocked using 4% normal horse serum followed by a secondary mouse blocking step using AffiniPure Goat Anti-Mouse IgG (H+L) (1:200, Jackson ImmunoResearch) to prevent non-specific binding of mouse antibodies in mouse tissue. The sections were then incubated with the primary antibody, either anti-TH (mouse, 1:5000, Milipore) and anti-IBA1 (rabbit, 1:1000, Wako) or anti-TH (mouse, 1:5000, Milipore) and anti-GFAP (rabbit, 1:1000, DAKO), diluted in 0.1M PB + 0.3% Triton-X + 4% normal horse serum and incubated at 4°C overnight. After this incubation the tissue was washed thoroughly and incubated in the corresponding secondary antibody 1:400 goat anti-mouse IgG (H+L) Alexa Fluor 488 (Invitrogen) and 1:400 goat anti-rabbit IgG (H+L) Alexa Fluor 594 (Invitrogen) for fluorescent staining for 90 minutes at room temperature. The tissue was subsequently washed, mounted onto slides and cover slipped using anti-fade media. Specificity of the primary antibody was confirmed with secondary antibody omission.

Stereological investigation of cell number and volume

We used design-based stereology to quantify the number microglia and astrocytes in the SN as well as the number (optical fractionator probe) and volume (nucleator probe) of the TH

neurons. The cells were visualised using a Zeiss microscope with a motorised stage and a MicroFibre digital camera connected to the computer and analysed using the StereoInvestigator software (MicroBrightField, Williston, VT, USA). The entire SN was cut at 30µm (allowing for a 20µm optical dissector within each section) with systematic sampling of every fifth section, the first sample set was chosen at random.

We estimated the total TH cells in the SN by counting a fraction of the total cells using randomly positioned counting frames controlled by StereoInvestigator. Guard zones, which account for section damage and to prevent oversampling error were set at 10% of the thickness of the section. The width (X) and height (Y) of the counting frame was 40.2 µm producing a counting frame area (XY) of 1616 μm^2. The dissector height (Z) was 20 µm to create a dissector volume (XYZ) of 32320 μm^3. To be included as within the counting frame the cells had to touch the green inclusion border and not come into contact with the red exclusion border of the sampling grid. An acceptable cell estimation had a coefficient of error (CE; using the Gunderson method) less than or equal to 0.1 with a smoothness factor m=1. This value is used to estimate sampling precision, which is independent of natural biological variance, whereby the closer the value is to 0 the less uncertainty there is in the estimate.

Concurrent to counting cell number we also measured cell volume using the well-defined nucleator method. The nucleator generates orthogonal lines at the midpoint of the cell whereby markers are placed at the intersection between these lines and the cell boundary. By taking the third power of these measurements the cell was calculated

Western Blot

Fresh tissue samples of the substantia nigra and striatum were processed for western blot analysis. Briefly, tissue was immersed in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100) containing a proteinase inhibitor (Sigma) and sonicated. The samples were subsequently centrifuged (10,000 rpm, 10min, 4^0C) to remove cellular debris and the supernatant collected. Protein concentration was measured using a BCA kit (Pierce, Rockford, IL) according to kit instructions. The samples were then standardised and mixed with Laemmli buffer and boiled for 5 minutes. Samples were loaded onto 10% acrylamide gels and separated by SDS polyacrylamide gel electrophoresis, then transferred to a PVDF membrane. The blot were then blocked for 1 hour in Tris-Buffered Saline Solution containing 0.1% Tween-20 (TBST) and 5% Bovine Serum Albumin (BSA)

and incubated overnight at 4°C in TBST and 5% BSA with the following antibodies: anti-TH (mouse, 1:1000, Milipore) with anti-β actin (rabbit, 1:1000, Abcam) used as a control. After thorough washing in TBST the blots were incubated with their corresponding secondary antibodies: goat anti-mouse IgG (Light Chain Specific) and goat anti-rabbit IgG (H+L)-HRP conjugate (1:10,000, Jackson ImmunoResearch). The blots were then visualised using the chemiluminescence method (ECL, Amersham) using the acquisition and analysis ImageLab Software, version 4.1, Biorad.

High Performance Liquid Chromatography (HPLC)

Using HPLC we identified, separated and quantified dopamine and DOPAC in the striatum. Striatal tissue was rapidly dissected and snap frozen at approximately -70°C. The tissue was then sonicated in 0.4 mL cold 0.1M perchloric acid containing an internal standard. The samples were centrifuged and dopamine, DOPAC and the internal standard in the supernatant were extracted on alumina at pH 8.4, eluted in 0.1M perchloric acid, separated by reverse-phase HPLC and detected using electrochemical detection. The concentration of dopamine and DOPAC was calculated in reference to the internal and external standards. Protein concentration was determined from the centrifuged pellet of each sample using the Lowry method. The concentrations of dopamine and DOPAC are expressed as ng/mg of protein present (mean ± SEM).

Plasma analysis

Trunk Blood was collected at time of death during the light cycle (approximately 45 minutes after injection) and collected into EDTA tubes pre-treated with pefabloc (SC Roche Applied Science, Mannheim, Germany) to achieve a concentration of 1mg/mL. The blood was collected during the light cycle as corticosterone levels are cyclic in nature and levels will be lowest during the light phase (Malisch et al., 2008). This low baseline allowed us to accurately detect any minute differences that resulted from the injection of acylated / des-acylated ghrelin. All samples were collected at the same time (approximately 1400).The blood was subsequently centrifuged and acidified using HCl (final concentration 0.05N). The plasma was analysed for: Acylated ghrelin and Des-acyl ghrelin Enzyme-Linked Immunoassay Kits (Mitsubishi Chemical Medicine; Tokyo, Japan), NEFA (Wako Life

Sciences; CA, USA), Triglycerides (Roche/Hitachi; IN, USA), Corticosterone (Abnova; CA, USA) and Blood glucose (Sigma; Missouri, USA). All measurements were obtained following kit instructions.

Statistical Analysis

All data is represented as Mean ± Standard Error of the Mean (SEM). Two-Way ANOVA with a Bonferroni post hoc was used to determine statistical significance, unless otherwise stated in the Figure Legend. $p<0.05$ was considered statistically significant. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Results

Acylated ghrelin restricts TH loss after MPTP administration

In order to examine the neuroprotective properties of des-acyl ghrelin we first confirmed that acyl ghrelin attenuated SN Dopamine (DA) cell loss in ghrelin KO mice. Administration of acylated ghrelin significantly elevated circulating plasma concentration for acylated (Figure 2.1A) and des-acylated (Figure 2.1B) ghrelin, indicating *in vivo* deacetylation has occurred. There was no significant change in body weight as a result of chronic acylated ghrelin administration (Supplementary Figure 2.1A). Administration of MPTP reduced body weight (Supplementary Figure 2.1A) and elevated Non-Esterified Fatty Acids (NEFA), Triglycerides and Corticosterone (Figure 2.1C, D & R) in the plasma, as well as Ionized calcium binding adaptor molecule 1 (IBA1) and Glial Fibrillary Acidic Protein (GFAP) in the SN. Microglia (IBA1+ cells) and astrocytes (GFAP+ cells) are activated during cellular damage and are responsible for minimising overall dopaminergic cell loss (Kohutnicka et al., 1998). Greater numbers of microglia and astrocytes present indicate a greater amount of cellular damage. Both microglia and astrocytes were significantly elevated post MPTP administration with a significant protective effect of acylated ghrelin on microglial numbers only, although there was a trend for acyl ghrelin to reduced GFAP positive astrocytes (Figure 2.1G & H). This was concomitant with a significant reduction in the number and size of TH neurons (enzyme marker of dopamine neurons) in the SN post MPTP administration, which was significantly attenuated with chronic acylated ghrelin (Figure 2.1E, F & K). Acylated ghrelin also attenuated the MPTP-induced decrease in TH levels in the SN and Striatum (Figure 2.1L-N). HPLC analysis of dopamine and DOPAC revealed a significant reduction with MPTP administration (Figure 2.1O & P). Acylated ghrelin prevented the increase in the DOPAC:DA ratio observed after MPTP administration (Figure 2.1Q). Although these results collectively indicate that acylated ghrelin is neuroprotective in PD, data from Figure 2.1B indicate that injection of acylated ghrelin significantly increases des-acyl ghrelin. Therefore it is reasonable to assume that some neuroprotection may have come from elevated des-acyl ghrelin.

Figure 2.1

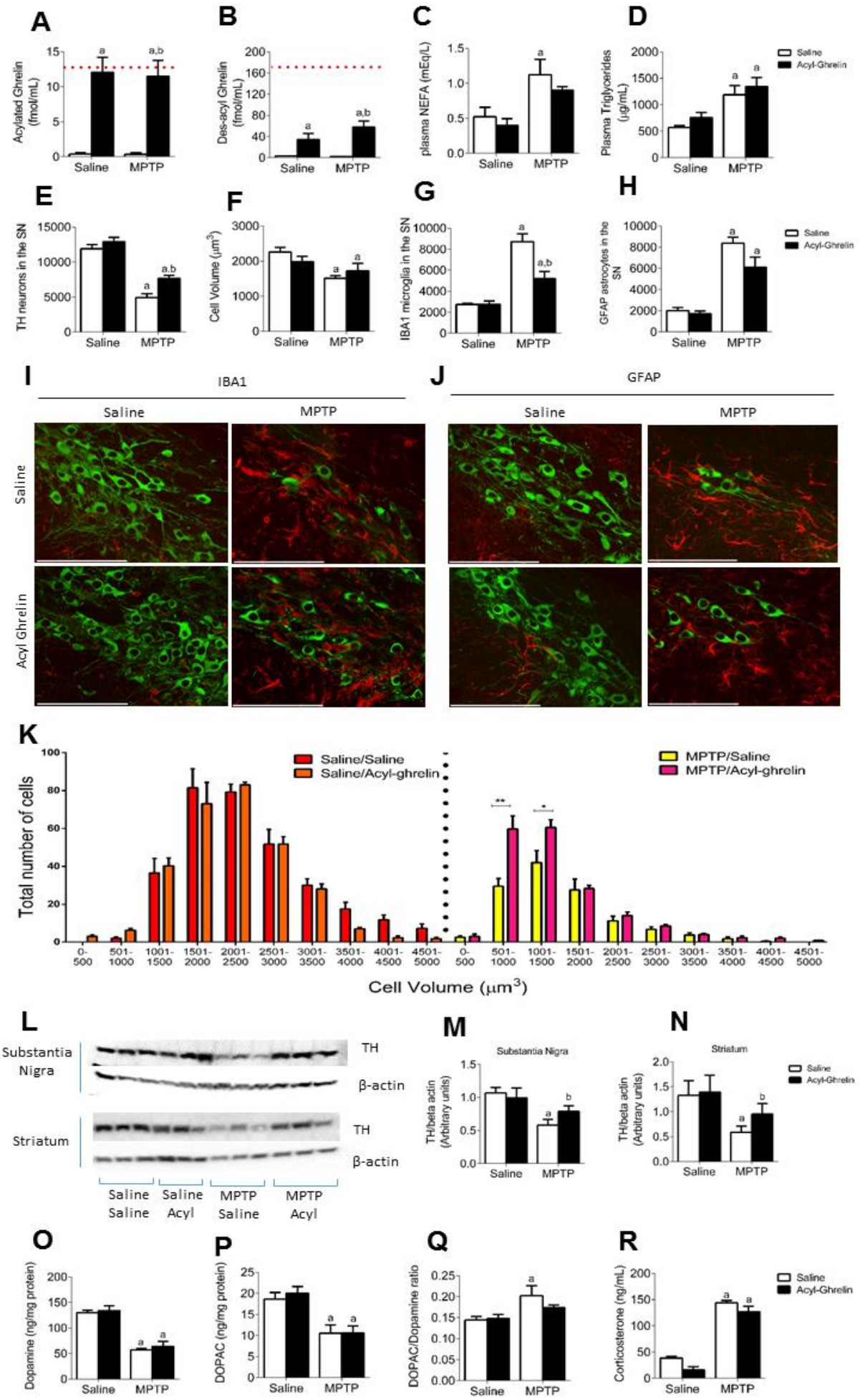

A
Acylated Ghrelin (fmol/mL)
Saline
MPTP
B
Des-acyl Ghrelin (fmol/mL)
C
plasma NEFA (mEq/L)
D
Plasma Triglycerides (µg/mL)
Saline
Acyl-Ghrelin
E
TH neurons in the SN
F
Cell Volume (µm3)
G
IBA1 microglia in the SN
H
GFAP astrocytes in the SN
I
IBA1
J
GFAP
Saline
MPTP
Acyl Ghrelin
K
Saline/Saline
Saline/Acyl-ghrelin
MPTP/Saline
MPTP/Acyl-ghrelin
Total number of cells
Cell Volume (µm3)
L
Substantia Nigra
Striatum
TH
β-actin
Saline Saline
Saline Acyl
MPTP Saline
MPTP Acyl
M
Substantia Nigra
TH/beta actin (Arbitrary units)
N
Striatum
O
Dopamine (ng/mg protein)
P
DOPAC (ng/mg protein)
Q
DOPAC/Dopamine ratio
R
Corticosterone (ng/mL)

Figure 2.1. Neuroprotective effects in Ghrelin KO mice reinstated with acylated ghrelin. **A & B**, Plasma analysis of acyl and des-acyl ghrelin show an elevation in both acylated and des-acyl ghrelin after acylated ghrelin administration. Red dotted line indicates average circulating levels of acyl and des-acyl ghrelin in Wild-type mice. **C & D**, Plasma NEFA and Triglyceride levels are elevated post MPTP administration. **E**, Stereological quantification of TH neurons in the SN showing chronic acylated ghrelin is protective in Ghrelin KO mice. **F**, Overall cell volume showed a significant reduction with MPTP regardless of injection. Stereological quantification of IBA1 (**G**) and GFAP (**H**) shows elevated levels following MPTP, which is attenuated in mice treated with acylated ghrelin. **I + J**, Representative images showing MPTP induced (**I**) microglial and (**J**) astrocyte activation in the SN (green = TH and red = (**I**) IBA1 or (**J**) GFAP). **K**, When TH cells were separated and plotted based on number distribution, mice treated with MPTP and acylated ghrelin had a significant effect on smaller volume (500-1500µm^3) cells compared to those not treated with acylated ghrelin. **L**, Representative Western Blot images of TH and beta actin in the SN and Striatum. Quantification of TH levels in the SN (**M**) and Striatum (**N**) reveals an attenuated loss of TH in MPTP treated with MPTP and acylated ghrelin compared to MPTP alone. **O & P**, MPTP significantly reduced both dopamine and DOPAC with no effect of acylated ghrelin. **Q**, Acylated ghrelin reduced the elevation of the DOPAC:DA ratio in MPTP treated mice compared saline alone. **R**, Plasma corticosterone levels are significantly elevated in response to MPTP regardless of injection. a, significant compared to Saline/saline treated mice and b, significant compared to Saline/MPTP treated mice. * $p<0.05$, ** $p< 0.01$ compared to MPTP / Saline. Data are represented as mean ± SEM (n=4-8, two-way ANOVA,). Scale bar = 100µm.

Chronic des-acyl administration is not neuroprotective in PD

In order to determine the relative neuroprotective potential of des-acyl ghrelin *in vivo* we chronically administered Ghrelin KO mice (lacking both acylated and des-acylated ghrelin) with des-acyl ghrelin. The use of Ghrelin KO mice allowed us to directly assess the impact of the exogenously des-acyl ghrelin peptides without confounding changes in the acyl to des-acyl ghrelin ratio. Plasma analysis shows negligible acylated ghrelin levels and a higher than average circulating plasma des-acyl concentration (Figures 2.2A & B). This indicates that des-acyl ghrelin cannot be converted back to its acylated counterpart and that any observed effects are solely due to elevated des-acyl ghrelin. We analysed various metabolic markers in the plasma and found elevated NEFA (Figure 2.2C) and Triglycerides (Figure 2.2D) with no change in blood glucose (Supplementary Figure 2.1D) in response to MPTP.

MPTP administration reduced dopamine cell number (Figure 2.2E) and volume (Figure 2.2F & K) and elevated IBA and GFAP positive cells (Figures 2.2G & H). However, there was no protective effect of des-acyl ghrelin injection in terms of TH cell loss or gliosis response. TH expression in the SN and Striatum (Figure 2.2L-N) were reduced post MPTP administration with no protective effect of des-acyl ghrelin. HPLC analysis of dopamine and DOPAC revealed a significant reduction with MPTP administration with no effect of des-acyl administration (Figure 2.2O & P). Des-acyl ghrelin did not alter the DOPAC:DA ratio (Figure 2.2Q). Plasma corticosterone was elevated in response to either des-acyl ghrelin administration or MPTP (Figure 2.2R).

Figure 2.2

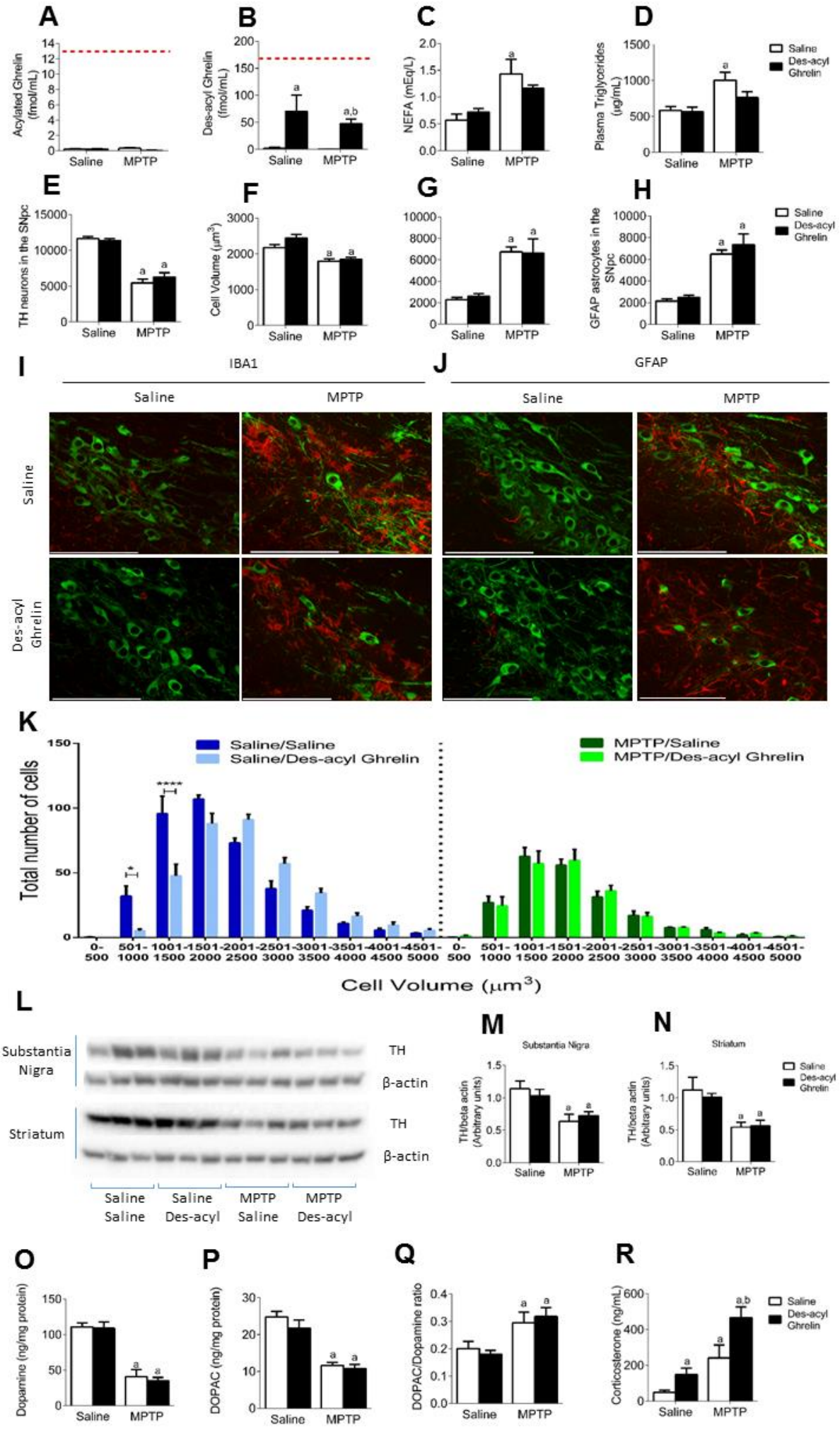

A
Acylated Ghrelin (fmol/mL)
Saline
MPTP
B
Des-acyl Ghrelin (fmol/mL)
C
NEFA (mEq/L)
D
Plasma Triglycerides (µg/mL)
Saline
Des-acyl Ghrelin
E
TH neurons in the SNpc
F
Cell Volume (µm³)
G
H
GFAP astrocytes in the SNpc
I
IBA1
J
GFAP
K
Saline/Saline
Saline/Des-acyl Ghrelin
MPTP/Saline
MPTP/Des-acyl Ghrelin
Total number of cells
Cell Volume (µm³)
L
Substantia Nigra
Striatum
TH
β-actin
Saline Saline
Saline Des-acyl
MPTP Saline
MPTP Des-acyl
M
Substantia Nigra
TH/beta actin (Arbitrary units)
N
Striatum
O
Dopamine (ng/mg protein)
P
DOPAC (ng/mg protein)
Q
DOPAC/Dopamine ratio
R
Corticosterone (ng/mL)

Figure 2.2. No neuroprotective action in Ghrelin KO mice re-instated with des-acyl ghrelin. **A & B**, Plasma analysis of acyl and des-acyl ghrelin show an elevation in des-acyl ghrelin after injection and no change in acylated ghrelin levels. Red dotted line indicates average circulating levels of acyl and des-acyl ghrelin in Wild-type mice. **C & D**, Plasma NEFA and Triglyceride levels are elevated post MPTP administration. **E**, Stereological quantification of TH neurons in the SN showing a significant reduction after MPTP administration but no effect with chronic des-acyl ghrelin. **F**, Overall cell volume showed a significant reduction with MPTP regardless of injection. Stereological quantification of IBA1 (**G**) and GFAP (**H**) shows elevated levels following MPTP, with no difference between chronic saline and chronic des-acyl ghrelin. **I + J**, Representative images showing MPTP induced (**I**) microglial and (**J**) astrocyte activation in the SN (green = TH and red = (**I**) IBA1 or (**J**) GFAP). **K**, TH cell distribution shows no difference comparing MPTP saline and MPTP des-acyl ghrelin. **L**, Representative Western Blot images of TH and beta actin in the SN and Striatum. Quantification of TH levels in the SN (**M**) and Striatum (**N**) reveals a significant loss of TH in MPTP treated mice with no effect of des-acyl ghrelin. **O & P**, MPTP significantly reduced both dopamine and DOPAC with no effect of des- acyl ghrelin. **Q**, MPTP treatment significantly elevated the DOPAC:DA ratio regardless of treatment. **R**, Plasma corticosterone levels are significantly elevated in response to both des-acylated ghrelin and MPTP with a cumulative effect when co-administered. a, significant compared to Saline/saline treated mice and b, significant compared to Saline/MPTP treated mice. * $p < 0.05$, **** $p < 0.0001$ significant compared to Saline / Saline. Data are represented as mean ± SEM (n=6, two-way ANOVA). Scale bar = 100µm.

GOAT mice show no differences in response to MPTP

As shown above des-acylated ghrelin did not attenuated SN TH cell number or cell volume in a mouse model of PD. In order to strengthen these observations, we employed a genetic mouse model that exhibits increased endogenous des-acyl ghrelin without affecting acylated ghrelin throughout entire lifespan. Mice lacking the enzyme GOAT have high levels of des-acyl ghrelin and negligible acylated ghrelin (Figure 2.3A & B) and thereby provide an ideal model to test the role of endogenously high des-acyl ghrelin. We also analysed various metabolic markers in the plasma and found elevated NEFA (Figure 2.3C) and Triglycerides (Figure 2.3D) with no change in blood glucose (Supplementary Figure 2.1F) in response to MPTP. MPTP administration significantly reduced the number and size of TH neurons (Figure 2.3E, F & K) concurrent with elevated GFAP and IBA1 cells in both GOAT WT and KO mice, with no overall effect of genotype (Figure 2.3G & H). These results were mirrored in the analysis of TH protein levels in both the SN and Striatum (Figure 2.3L-N). HPLC analysis of dopamine and DOPAC revealed a significant reduction with MPTP administration with no effect of genotype (Figure 2.3O & P). Genotype did not alter the DOPAC:DA ratio (Figure 2.3Q). Interestingly, corticosterone levels were elevated in GOAT KO and with MPTP indicating a stress response to the elevated levels of des-acyl ghrelin (Figure 2.3R).

Figure 2.3

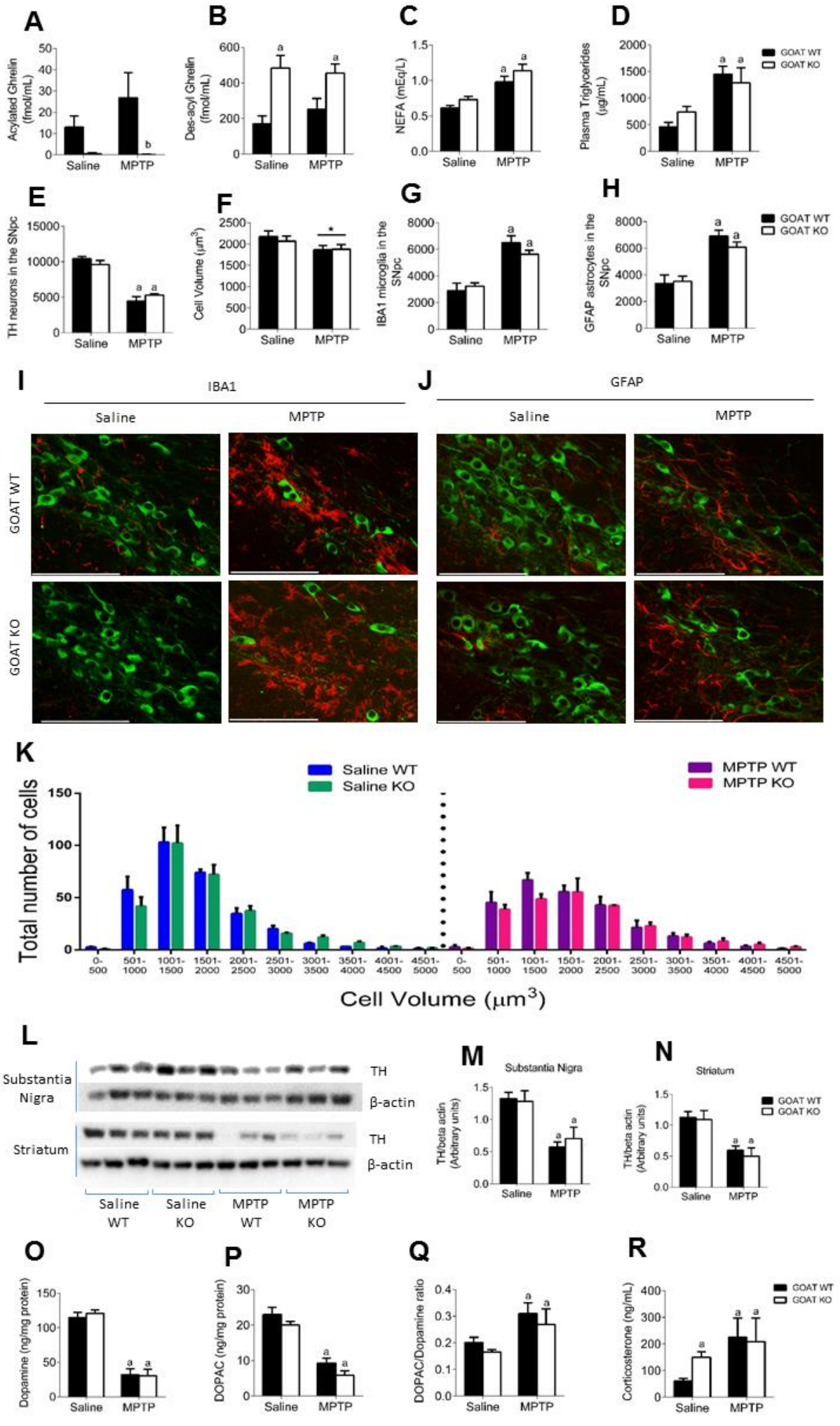
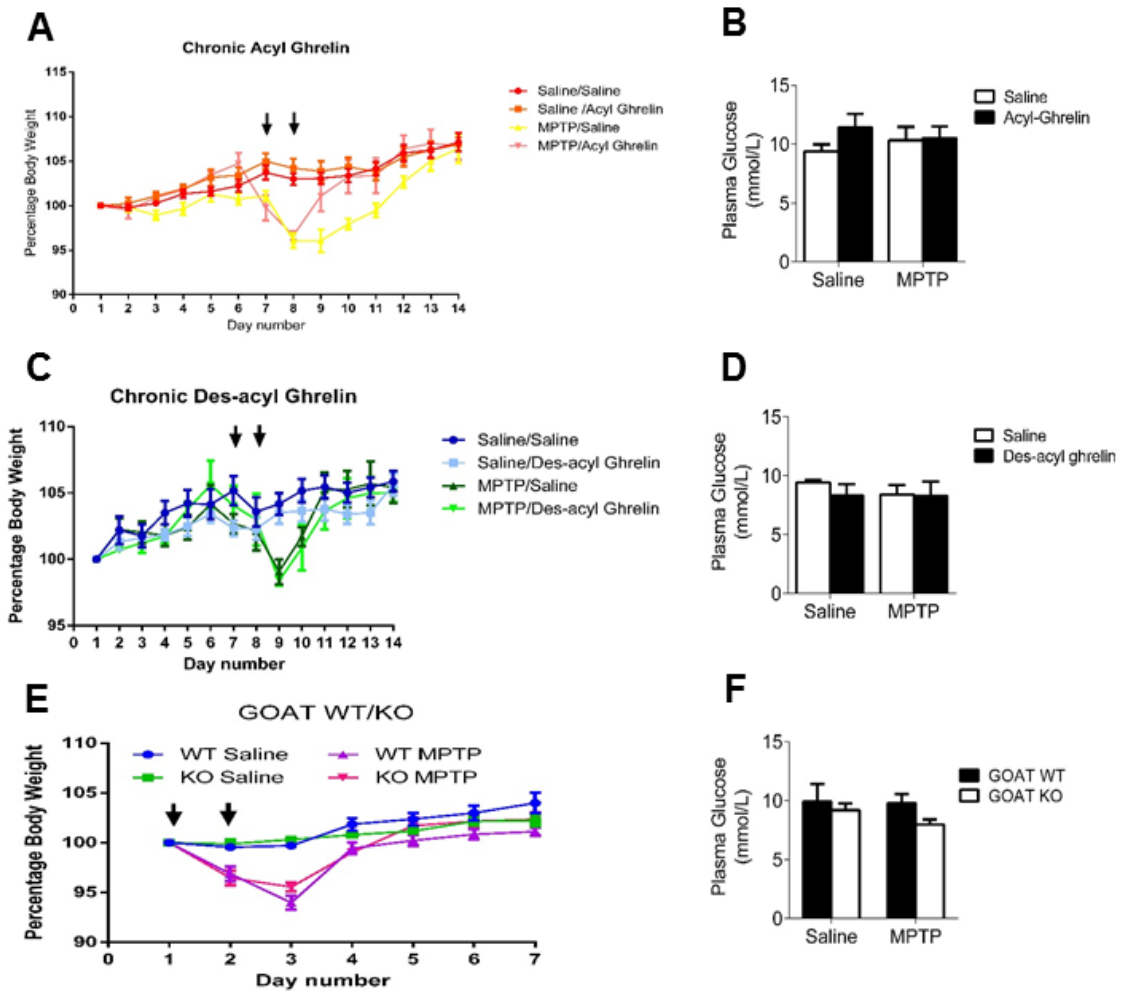

A
Acylated Ghrelin (fmol/mL)
Saline
MPTP
B
Des-acyl Ghrelin (fmol/mL)
C
NEFA (mEq/L)
D
Plasma Triglycerides (µg/mL)
GOAT WT
GOAT KO
E
TH neurons in the SNpc
F
Cell Volume (µm³)
G
IBA1 microglia in the SNpc
H
GFAP astrocytes in the SNpc
I
IBA1
J
GFAP
GOAT WT
GOAT KO
K
Saline WT
Saline KO
MPTP WT
MPTP KO
Total number of cells
Cell Volume (µm³)
L
Substantia Nigra
Striatum
TH
β-actin
Saline WT
Saline KO
MPTP WT
MPTP KO
M
Substantia Nigra
TH/beta actin (Arbitrary units)
N
Striatum
O
Dopamine (ng/mg protein)
P
DOPAC (ng/mg protein)
Q
DOPAC/Dopamine ratio
R
Corticosterone (ng/mL)

Figure 2.3. No significant difference between GOAT WT and KO mice after MPTP exposure. A & B, Plasma analysis of acyl and des-acyl ghrelin show an elevation in des-acyl ghrelin with negligible acylated ghrelin in GOAT KO mice. Both acylated and des-acylated ghrelin are present in GOAT WT mice. C & D, Plasma NEFA and Triglyceride levels are elevated post MPTP administration. E, Stereological quantification of TH neurons in the SN showing no effect of genotype in response to MPTP. F, Overall cell volume showed a significant reduction with MPTP regardless of genotype. Stereological quantification of IBA1 (G) and GFAP (H) shows elevated levels following MPTP, independent of genotype. I + J, Representative images showing MPTP induced (I) microglial and (J) astrocyte activation in the SN (green = TH and red = (I) IBA1 or (J) GFAP). K, TH cell distribution was not different between genotypes. L, Representative Western Blot images of TH and beta actin in the SN and Striatum. Quantification of TH levels in the SN (M) and Striatum (N) reveals a significant loss of TH post MPTP administration with no significant effect of genotype. O & P, MPTP significantly reduced both dopamine and DOPAC with no effect of genotype. Q, MPTP treatment significantly elevated the DOPAC:DA ratio regardless of genotype. R, Plasma corticosterone levels are significantly elevated in response to MPTP and also in saline treated GOAT KO mice. a, significant compared to GOAT WT saline treated mice and b, significant compared to GOAT WT MPTP treated mice. Data are represented as mean ± SEM (n=6-7, two-way ANOVA). Scale bar = 100µm.

Supplementary Figure 2.1



Supplementary Figure 2.1. Body weight and blood glucose graphs in Ghrelin KO mice reinstated with acylated or des-acylated ghrelin and GOAT WT/KO mice. A & B, Ghrelin KO mice chronically treated with acylated ghrelin did not significantly change body weight or blood glucose. C & D, Ghrelin KO mice chronically treated with des-acyl ghrelin did not significantly change body weight or blood glucose. E & F, There was no significant difference between GOAT WT and KO mice in terms of body weight or blood glucose measurements. Data are represented as mean ± SEM (n=8-14, two-way ANOVA, $p<0.05$).

Discussion

Recently, many studies have investigated the beneficial effects of ghrelin administration to combat a multitude of disease including stroke (Liu et al., 2009), epilepsy (Lucchi et al., 2013), encephalomyelitis (Theil et al., 2009) and Alzheimer's disease (Dhurandhar et al., 2013). However, these studies have focused exclusively on the beneficial effects of acylated ghrelin, or simply do not consider des-acyl ghrelin as a potential protective agent. Ghrelin exists in two biologically distinct forms (acylated and des-acylated ghrelin) and research into both of these isoforms is necessary before appropriate therapeutics can be generated. In humans des-acyl ghrelin is the most abundant form where circulating ghrelin is composed of >90% des-acyl ghrelin and less than 10% acylated ghrelin (Patterson et al., 2005). This ratio can be explained by the comparatively shorter half-life of acylated ghrelin (Akamizu et al., 2005) and active deacetylation of acyl ghrelin into des-acyl ghrelin (Tong et al., 2013). Because of the ability of acylated ghrelin to be converted to des-acyl ghrelin in the blood, there is the potential that many physiological actions of acylated ghrelin are in fact because of elevated des-acyl ghrelin.

In this study we wanted to look at the relative contributions of each isoform. In order to do this we chronically injected acyl or des-acyl ghrelin into Ghrelin KO mice. These mice have negligible levels of both acylated and des-acylated ghrelin for their entire lifespan and by re-introducing exclusively one isoform we can determine the effects of one without the interference of the other. The results from chronic injections of acylated ghrelin were consistent with previous literature showing the neuroprotective properties of acyl ghrelin post MPTP administration (Jiang et al., 2008; Andrews et al., 2009; Moon et al., 2009). MPTP administration reduced overall cell number by selectively targeting larger dopaminergic neurons as determined by cell volume analysis. Given the small cell volume and lower membrane capacitance these smaller volume neurons would require less depolarization to reach an action potential threshold. Hence, it is more beneficial and less energy intensive to maintain smaller volume neurons. It is currently unknown how the larger neurons are targeted over smaller cells, this could be due to differential cell types targeted or delayed apoptosis within the smaller volume cells. Further research is required to determine how this process occurs. In this study administration of acyl ghrelin was neuroprotective, however when we measured both acyl and des-acyl ghrelin in the plasma there was an elevation in both acylated and des-acylated ghrelin. Hence, the neuroprotective properties could be due to either

acylated or des-acylated ghrelin. In both human and rat serum acylated ghrelin is readily converted to des-acyl ghrelin within the plasma (De Vriese et al., 2007). However, in Ghrelin KO mice chronic injection of des-acyl ghrelin did not elevate plasma acyl ghrelin levels as acylation occurs intracellularly, within the endoplasmic reticulum, by the enzyme GOAT (Gutierrez et al., 2008; Yang et al., 2008). Hence, the elevated levels of acyl ghrelin was readily deacetylated in the plasma but the elevated des-acyl ghrelin did not affect acyl-ghrelin levels. Although shown experimentally to occur the elevated plasma des-acyl levels could be due to various pathways/feedback loops to minimize excessive circulating acyl-ghrelin levels or potentially to maintain an appropriate des-acyl: acyl ratio (Epelbaum et al., 2010). Further research is required to determine the reason why des-acyl levels are elevated post acyl-ghrelin injection. In this study neuroprotective properties were not seen in mice that were chronically treated with des-acyl ghrelin, indicating that the beneficial effects are solely due to acylated ghrelin. In order to definitively show that des-acyl ghrelin is not neuroprotective we used a genetic approach involving GOAT knockout mice. GOAT acylates ghrelin and thus GOAT KO mice have negligible acylated ghrelin and elevated des-acyl ghrelin throughout their entire lifespan. This allows us to analyse the physiological consequences of a deficiency of ghrelin acylation. Indeed, there was no neuroprotective effect between genotypes in these mice, confirming that des-acyl ghrelin is not neuroprotective in PD.

Interestingly, des-acyl ghrelin can be neuroprotective in *in vitro* settings. Administration of des-acyl ghrelin prevents cell death in cultured neurons during oxygen and glucose deprivation (Chung et al., 2008; Hwang et al., 2009). Des-acyl ghrelin also counteracted the activation of the pro-inflammatory cytokine IL-6 in microglia exposed to amyloid beta (Bulgarelli et al., 2009). *In vivo* studies show a mixed response to des-acyl ghrelin. Recently des-acyl ghrelin, but not acylated ghrelin, has elicited vasodilatory actions in the cerebral endothelium in a model of stroke. This effect was independent of GHSR1a, potentially acting through a novel des-acyl ghrelin receptor in cerebral arteries (Ku et al., 2015). In another study using Ghrelin receptor KO mice, des-acyl ghrelin increased the pilocarpine-evoked seizure threshold, thus acting as an anticonvulsant, in WT but not KO mice (Portelli et al., 2015). In a study examining the role of each isoform during myocardial injury, there was a protective therapeutic action of acylated ghrelin that was significantly weaker than des-acyl ghrelin (Li et al., 2006). This raises the possibility that des-acyl ghrelin maybe protective in some diseases/treatments but not in others. This might reflect differences in expression of the unidentified des-acyl ghrelin receptor, although this requires experimental proof. However

our studies show that in an *in vivo* setting des-acyl ghrelin does not have neuroprotective effects in a mouse model of PD.

Our data shows that chronic des-acylated ghrelin administration elevates the stress hormone, corticosterone. Both age and stress play a role in the development of PD. During ageing cortisol levels are elevated (Deuschle et al., 1998) and this increase has been linked with memory deficits in the elderly (Lupien et al., 1998). Interestingly, cortisol is elevated in PD patients compared to healthy age-matched controls (Hartmann et al., 1997). Either chronic stress or corticosterone administration have detrimental effects on motor control during the reaching test in animals (Metz et al., 2005), indicating an association between corticosterone and abnormal motor behavior. In animal models of depression chronic stress enhances the pro-inflammatory environment (You et al., 2011). Acylated ghrelin has also been linked to the stress axis. Intraperitoneal injection of acylated ghrelin produces a significant dose-dependent increase in serum corticosterone levels one hour after injection (Asakawa et al., 2001). In mice who received chronic immobilization stress there was a stress-related increase in plasma corticosterone and acylated ghrelin levels (Meyer et al., 2014), implying that acylated ghrelin via the actions of corticosterone influence the stress axis. Ghrelin KO mice are more anxious after an acute stress an effect reversed with administration of acyl-ghrelin (Spencer et al., 2012). In these studies, we did not see any effect of chronic acyl ghrelin on plasma cortciosterone, as observed in the studies cited above, in response to MPTP. We believe that this discrepancy could be due to the lack of an acute stress event in these studies or due to the time frame of plasma collection after injection. Compared to saline controls plasma corticosterone was already significantly higher in the MPTP treated group, which may have also limited the ability of acyl ghrelin to further significantly increase corticosterone.

Moreover, these studies do not consider des-acyl ghrelin and as acylated ghrelin is readily deacetylated, these effects in the above studies could be at least partly due to des-acyl ghrelin. Indeed mice receiving chronic des-acyl ghrelin, but not acylated ghrelin, had an elevated corticosterone response, which was further exacerbated when the stressor MPTP was administered. Stress-induced corticosterone release is detrimental to the nigrostriatal system by increasing oxidative stress products (Kim et al., 2005) as well as reducing striatal dopamine levels (Ahmad et al., 2010) making the system more susceptible to degeneration. Collectively our results suggest that elevated levels of des-acyl ghrelin may negate any

neuroprotective effect of des-acyl ghrelin on dopaminergic neurons by the elevation of the stress hormone corticosterone. Indeed, this may explain differences between *in vivo* and *in vitro* studies examining the neuroprotective treatment of des-acyl ghrelin (Chung et al., 2008; Bulgarelli et al., 2009; Hwang et al., 2009). For example, the lack of a physiological response *in vitro*, which includes increased corticosterone, to des-acyl ghrelin treatment may allow experimentally observed neuroprotection. There are certainly other possibilities that could explain the lack of a neuroprotective effect of des-acyl. For example, studies show that the ratio of ghrelin peptides can influence the outcome on energy homeostasis and body composition (Epelbaum et al., 2010). Also, des acyl ghrelin has no effect on microglia or GFAP cell number in the current study, which will also contribute to the lack of effect observed. Inflammation has been researched intensively in PD. Perhaps most relevant, chronic Non-Steroidal Anti-Inflammatory (NSAID) use (including Ibuprofen, Indomethacin, Naproxen and Diflunisal) reduces the risk for the development of PD in a retrospective study (Chen et al., 2003). In our study MPTP-induced neurotoxicity increased microglia and astrocytes number in the nigrostriatal pathway, with a significant protective effect of chronic acylated ghrelin, but not des-acyl administration. This result directly implies that exogenous acyl ghrelin directly reduces gliosis in a mouse model of PD, contributing to the neuroprotective effect. Astrocytes and microglia release deleterious cytokines which results in oxidative damage to proteins, DNA and lipids and impairs dopaminergic neurons in the nigrostriatal pathway. Acylated ghrelin has been shown in many studies to act as an anti-inflammatory agent. Acylated ghrelin inhibits the release of pro-inflammatory cytokines IL-1β, IL-6 and TNFα after LPS administration in peripheral macrophages (Dixit et al., 2004) and T-cells (Waseem et al., 2008) and also reduces the pro-inflammatory responses and NFκB activation in endothelial cells (Li et al., 2004). Exogenous administration of acylated ghrelin in rodents reduces inflammation in Alzheimer's Disease (Dhurandhar et al., 2013), colitis (Gonzalez-Rey et al., 2006) and in mouse dopaminergic neurons acyl-ghrelin attenuates LPS-induced release of IL-6 (Beynon et al., 2013). This is clinically relevant as inflammation is documented in PD patients in post mortem samples (Ouchi et al., 2005) and in vivo PET imaging studies (Ikawa et al., 2011).

Overall, we show that acylated ghrelin is an ideal therapeutic target to reduce PD progression by reducing the gliosis response and attenuating dopaminergic cell loss in the nigrostriatal dopaminergic system. Des-acyl ghrelin on that other hand was not neuroprotective and also induced a heightened stress response by elevating corticosterone levels, which may negate

any neuroprotective potential. Furthermore, our studies suggest pharmacological approaches that prevent plasma conversion from acyl-ghrelin to des-acyl ghrelin may have clinical efficacy to help slow or prevent the debilitating effects of PD.

Contributions

J.A.B and Z.B.A designed experiments

J.A.B, V.V.S, M.D, J.E and M.B.L, performed experiments.

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

Acknowledgements

This work was supported by grants and fellowships from the Australian National Health and Medical Research Council to Z.B.A (546131, 1084344) to Z.B.A and NIH NS056181 to J.E.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Summary for Chapter 2

From Chapter 2 we show that acyl ghrelin is the isoform responsible for neuroprotection in a Parkinson's Disease mouse model (see Figure 2.4). Calorie Restriction is known to elevate circulating levels of acyl ghrelin. In Chapter 3 our aim was to determine if acyl ghrelin was responsible for the neuroprotective actions of Calorie Restriction. Calorie restriction is the most reproducible way to extend lifespan as well as health-span and is also neuroprotective in Parkinson's Disease. We wanted to find out if we could determine the underlying mechanisms responsible for this protective effect so that we could recapitulate this effect without the strict need to adhere to dietary constraints.

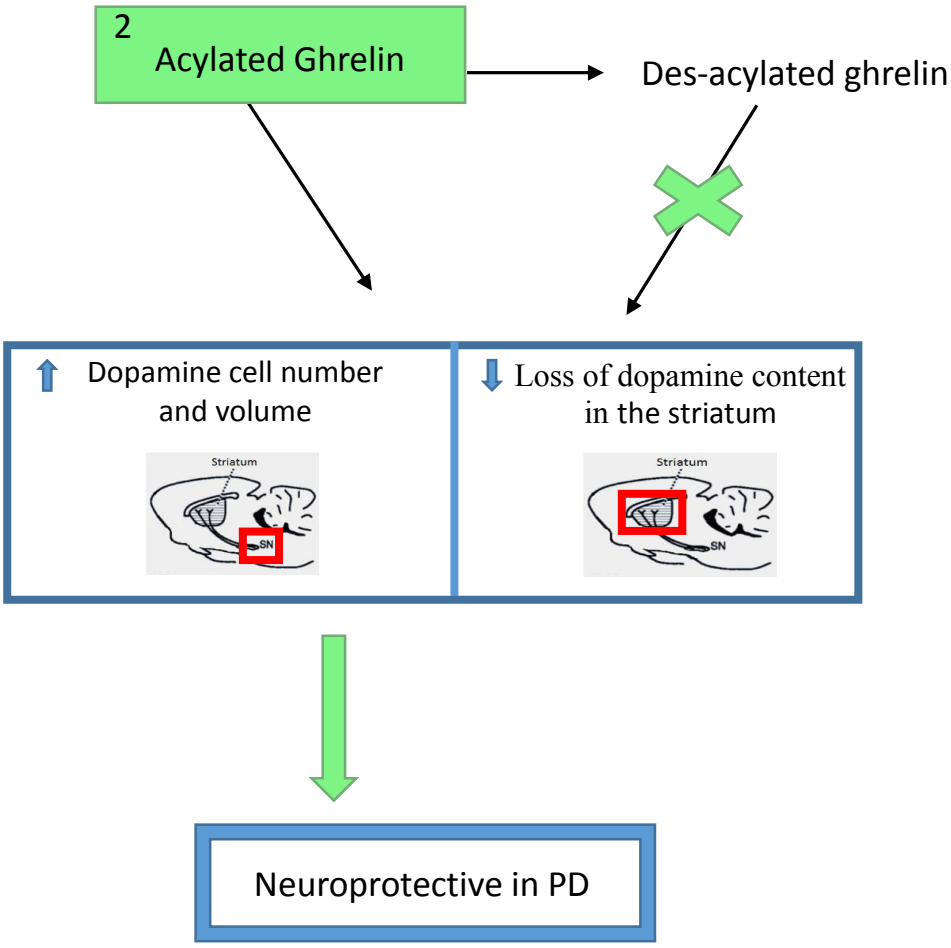


Figure 2.4: Summary of results from Chapter 2. Acylated, not des-acylated ghrelin is neuroprotective in Parkinson's Disease.

Declaration for Thesis Chapter 3

Declaration by candidate

In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Myself and ZBA designed the experiments. I performed the majority of the laboratory testing and analysis (with the assistance of M.L., R.S. and V.S.), with the exception of the HPLC analysis and in vitro experiments. Myself in collaboration with ZBA and BK we wrote the entire manuscript.	80

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Moyra Lemus	Assisted with Laboratory experiments	
Romana Stark	Assisted with Laboratory experiments	
Vanessa V Santos	Assisted with Laboratory experiments	
Aiysha Thompson	Performed in vitro studies	
Daniel Rees	Performed in vitro studies	
Sandra Galic	Assisted with Laboratory experiments	
John Elsworth	Performed HPLC experiments	
Bruce Kemp	Provided materials and animals for the experiments and helped prepare and draft the manuscript	
Jeffrey Davies	Performed in vitro studies; assisted with manuscript editing	
Zane Andrews	Assisted with designing of experiments and writing of the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date: 25.01.16**
Main Supervisor's Signature	[redacted]	**Date: 25.01.16**

Chapter 3 - Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie Restriction in Parkinson's Disease

Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie Restriction in Parkinson's Disease

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Key words: Dopamine, Substantia nigra, AMPK, Ghrelin, Parkinson's Disease, Calorie Restriction

Figures: 7

Abbreviations

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

Abstract

Calorie restriction (CR) is neuroprotective in Parkinson's disease (PD) although the mechanisms are unknown. In this study we hypothesized that elevated ghrelin, a gut hormone with neuroprotective properties, during CR prevents neurodegeneration in an MPTP model of PD. CR attenuated the MPTP-induced loss of substantia nigra (SN) dopamine neurons and striatal dopamine turnover in Ghrelin WT but not KO mice, demonstrating that ghrelin mediates CR's neuroprotective effect. CR elevated phosphorylated AMPK and ACC levels in the SN of WT but not KO mice suggesting that AMPK is a target for ghrelin-induced neuroprotection. Indeed, exogenous ghrelin significantly increased pAMPK in the SN. Genetic deletion of AMPKβ1 and 2 subunits only in dopamine neurons prevented ghrelin-induced AMPK phosphorylation and neuroprotection. Hence, ghrelin signaling through AMPK in SN dopamine neurons mediates CR's neuroprotective effects. We consider targeting AMPK in dopamine neurons may recapitulate neuroprotective effects of CR without requiring dietary intervention.

Introduction

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

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

Ghrelin is synthesized in the stomach where pro-ghrelin is acylated in the endoplasmic reticulum by the enzyme Ghrelin O-acyltransferase (GOAT). Acyl ghrelin is then released into the bloodstream where it crosses the blood-brain barrier and binds to the ghrelin receptor (Growth Hormone Secretagogue Receptor 1a; GHSR1a) in the brain. In addition to its well-known metabolic effects, ghrelin is neuroprotective in models of PD as Ghrelin and GHSR KO mice exhibited significantly greater loss of SN dopaminergic neurons compared to WT controls in an MPTP model of PD (Andrews et al., 2009). The neuroprotective mechanisms include reducing apoptosis and suppressing microglial activation and local inflammatory responses in the SN (Dong et al., 2009; Moon et al., 2009). Moreover, postprandial plasma ghrelin concentrations are lower in human PD patients (Unger et al., 2011), suggesting clinical relevance.

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

Methods

Animals

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

Experimental protocol

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

In the second set of experiments to test the effect of ghrelin administration on neuronal function in the midbrain, we used group housed male C57/Bl6J mice (8-10 weeks old; Monash Animal Services, Victoria, Australia) that had free access to food and water. C57/Bl6J mice were randomly allocated to receive saline, a low dose of ghrelin (5mg/kg) or a high dose of ghrelin (15mg/kg). The mice were injected ip and the food removed from the cage, they were subsequently culled 45 minutes later via decapitation after being deeply anaesthetized, then the brains were dissected and snap frozen (-70^{0}C) for HPLC and western blot analysis.

In order to generate mice with selective deletion of AMPK β1& β2 only in DAT-expressing dopamine neurons, we crossed *Dat*-Cre knock-in mice obtained from Jax Lab [Stock number 006660; B6.SJL-Slc6a3<tm1.1(cre)bkmn>/j] with *Ampk beta 1 subunit* (β1) and *beta 2 subunit* (β2) floxed mice (O'Neill et al., 2011). The resultant offspring (*Dat*-Cre;*Ampk beta* $1^{fl/fl}$;*Ampk beta* $2^{fl/fl}$ designated AMPK KO or *Ampk beta* $1^{fl/fl}$;*Ampk beta* $2^{fl/fl}$ designated AMPK WT) were used as experimental mice. To validate this model, AMPK WT and KO

mice were also bred with cre-dependent loxSTOPlox tdTOMATO reporter mice Stock number 007908; B6;129S6-Gt(ROSA)26Sor$^{tm14(CAG-tdTomato)Hze}$/J). The resultant offspring *Dat*-Cre;tdTomato or *Dat*-Cre;*Ampk beta 1*$^{fl/fl}$;*Ampk beta 2*$^{fl/fl}$;tdTomato mice allow tdTomato visualization of DAT-expressing neurons that have undergone cre recombination. These mice were also used for Fluorescence Activated Cell Sorting (FACS). The *Dat*-Cre;tdTomato were used as AMPK WT mice and *Dat*-Cre;*Ampk beta 1*$^{fl/fl}$;*Ampk beta 2*$^{fl/fl}$;tdTomato mice were used as AMPK KO mice. These mice were culled via inhalation anesthetic and the substantia nigra was collected. The cells were dissociated using papain (Worthington Kit, LK003150) following kit instructions. After collection of approximately 5000 tdTomato cells via FACS sorting using the influx v7 Sorter, the RNA was extracted and PCR was run to determine the presence/absence of AMPKβ1 and 2.

In the third set of experiments to test the effects of ghrelin administration in mice lacking AMPK activation, we group housed AMPK WT and KO mice (8-10 weeks old) with free access to water. The mice were administered ghrelin (1mg/kg) or Saline daily at the beginning of the light cycle for 14 consecutive days. After injections the food was subsequently removed for 6 hours to prevent excess consumption of calories, after this period all mice had free access to food. Previous studies (Andrews et al., 2009) indicate that if calories are consumed after injection of acyl-ghrelin there is no neuroprotective effect observed. On days 7 and 8 mice were injected with Saline or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; 30mg/kg). Mice were culled on day 14 and perfused for immunohistochemical analysis or fresh tissue collection for Western Blot and HPLC analysis.

MPTP administration

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

Immunohistochemistry

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

The sections was washed thoroughly in 0.1M PB and then endogenous peroxidase activity was blocked using 1% H2O2 in 0.1M PB for 15 minutes and washed again. The tissue was then transferred to 4% normal horse serum and 0.3% Triton X-100 in 0.1M PB for one hour, followed by a secondary mouse blocking step using AffiniPure Goat Anti-Mouse IgG (H+L) (1:200, Jackson ImmunoResearch) to prevent non-specific binding of mouse antibodies in mouse tissue. The tissue was then incubated with the primary antibodies, in this case either anti-TH (mouse, 1:5000, Millipore) and anti-IBA1(rabbit, 1:1000, Wako) or anti-GFAP (rabbit, 1:1000, DAKO) for 24 hours at 4^0C. Following the primary antibody incubation the tissue was washed thoroughly and incubated in the secondary antibody goat anti-mouse IgG (H+L) Alexa Fluor 488 (1:400, Invitrogen) and goat anti-rabbit IgG (H+L) Alexa Fluor 594 (1:400, Invitrogen) for fluorescent staining for 90 minutes at room temperature. The tissue was then thoroughly washed and mounted directly onto slides and coverslipped with anti-fade mounting media.

Stereological investigation of cell number and volume.

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

Analysis of blood chemistry

Trunk blood was collected via decapitation from deeply anaesthetised mice and collected into EDTA tubes pre-treated with pefabloc (SC Roche Applied Science, Mannheim, Germany) to achieve a concentration of 1mg/mL. The blood was then briefly centrifuged and the plasma was collected and acidified with HCl (final concentration 0.05N). Plasma ghrelin levels were determined using Active Ghrelin or Des-acyl Ghrelin Enzyme-Linked Immunoassay Kits (Mitsubishi Chemical Medicine, Tokyo, Japan). Active and des-acyl ghrelin were measured according to kit instructions. Plasma Insulin concentration was determined through an in-house ELISA assay.

High Performance Liquid Chromatography (HPLC)

We used HPLC to identify, separate and quantify dopamine (DA) and DOPAC concentrations within samples of striatal tissue. Striatal (both sides) tissue was rapidly dissected and snap frozen (approximately -70^{0}C). The samples were then sonicated in 0.4mL cold 0.1M perchloric acid containing internal standard. Following centrifugation, DA DOPAC and internal standard in the supernatant were extracted on alumina at pH 8.4, eluted in 0.1M perchloric acid, separated by reverse-phase HPLC and detected using electrochemical detection. Both dopamine and DOPAC concentrations in the striatum were calculated by reference to the internal standard and external standards. The protein content of each sample was determined from the centrifuged pellet by the Lowry method. The concentrations of DA and DOPAC are expressed as ng/mg of protein present (mean ± SEM)

Western Blot

Whole tissue samples of the SN and Striatum or SN4741 cells were processed for western blot analysis. Briefly, tissue was sonicated in RIPA buffer (50mM Tris.HCl, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X 100) containing a protease inhibitor (Sigma), then centrifuged (10,000 rpm, 10min, 4^{0}C) to remove cell debris and the supernatant was collected. For cell culture studies SN4741 cells were maintained at 37^{o}C in a 5% CO_2 humidified environment in Dulbecco's modified Eagle medium (DMEM, 41965, life technologies) supplemented with 10% fetal bovine serum (FBS), 2mM glutamine, 100U/ml

penicillin and 0.1mg/ml streptomycin, 0.6% glucose. Once cells had reached approximately 90-100% confluency, cells were sub-cultured.

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

An aliquot was then used to identify the amount of protein present in each sample using a BCA kit (Pierce, Rockford, IL) according to kit instructions. The samples concentrations were then standardised and the supernatants were mixed with Laemmli's buffer and boiled for 5 minutes. Samples (20μl) were loaded onto 10% acrylamide gels and separated by SDS polyacrylamide gel electrophoresis. The separated proteins were then transferred from the gel to the PVDF membrane (Biorad). The blots were then blocked for 1 hour in Tris-Buffered Saline Solution containing 0.1% Tween-20 (TBST) and 5% bovine Serum albumin (BSA). The membranes were subsequently incubated overnight at 4^0C in TBST with 5% BSA with either of the following antibodies: TH (1:1000, Milipore), Parkin (1:1000, Santa Cruz), PINK (1:1000, Santa Cruz), LC3B (1:1000, Cell Signaling), pACC (1:1000, Cell Signaling) or pAMPK (1:1000, Cell Signaling), where AMPKα (1:1000, Cell Signaling) antibodies, ACC (1:1000, Cell Signaling) and anti-β actin (1:1000, Abcam) were used as controls. Blots were visualised using the chemiluminescence method (ECL, Amersham) and levels were detected using ImageLab Software, version 4.1, Biorad.

RNA extraction and PCR

After FAC's sorting cells were stored in Qiazol for RNA extraction. Briefly, chloroform was added, samples were centrifuged (12,000g, 15min, 4°c) and supernatant was collected. Isopropanol and glycogen was added and the samples centrifuged (12,000g, 10 minutes, 4°c).

The pellet formed was washed with ethanol (75%) and vortexed. cDNA was synthesized using the iScript cDNA synthesis kit (number 170-8890; Biorad Laboratories). The cDNA collected was combined with Mastermix and primers (either AMPKβ1, AMPKβ2 or GHSR) and exposed to a heat block in the Mastercycler. We used TaqMan Gene Expression Mastermix (Applied Biosystems) and GHSR primers (GHSR forward: GCTGCTCACCGTGATGGTAT and reverse: GCTGCTCACCGTGATGGTAT) as our control. A PCR reaction was required to amplify the AMPKβ1 and AMPKβ2 transcripts from the cDNA. We used nested PCR to enhance accuracy using two PCR reactions involving outer and inner primers (AMPKβ1 outer forward: CCACTCCGAAGAGATCAAGG and reverse: GTGCTGGGTCACAAGAGATG, AMPKβ1 inner forward: CACGACCTGGAAGCGAAT and reverse: CATGTAAGGCTCCTGGTGGT and AMPKβ2 outer forward: GTTATCCGCTGGTCTGAAGG and reverse: CAGCAGCGTGGTGACATACT and AMPKβ2 inner forward: GAGCACCAAGATCCCTCTGA and reverse: GGAAGTAAGGCTGGGTCACA). This process was repeated with inner primers and then visualized in a gel mounting media (agarose gel) and exposed to electrical current (120V) for 25 minutes. The results were viewed using gene snap technology. The specificity of the primers was confirmed using a blast search. Positive control was hypothalamic tissue from C57BL/6 mice and negative control contained no cDNA.

Rotarod

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

Statistical Analysis

All data is represented as Mean ± Standard Error of the Mean (SEM). Two-Way ANOVA with a Bonferroni post hoc test was used to determine statistical significance between treatment and genotype and One-Way ANOVA with a Tukey post hoc test was used to

determine statistical significance between injection groups. Cell lysate analysis used a two-tailed Student's *t*-test. $p<0.05$ was considered statistically significant.

Results

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

Calorie Restriction (CR) significantly elevated acylated (Figure 3.1A) and des-acyl plasma ghrelin in WT, with no detectable levels in KO mice (Appendix 1A-C), confirming reports that CR increases plasma ghrelin. There were no genotypic differences in plasma insulin from ad-lib or CR mice, although CR significantly reduced plasma insulin levels compared to ad-lib mice (significant main effect ad-lib vs. CR) (Figure 3.1B&C). Both body weight and blood glucose measurements exhibited a significant overall reduction in response to CR (Appendix 1D & E).

Ghrelin restricts MPTP-induced nigrostriatal damage during CR

Tyrosine Hydroxylase (TH) neurons

We used the stereological optical fractionator probe to estimate total TH-positive (i.e. dopamine) neurons in the substantia nigra (SN). MPTP administration significantly reduced the number of SN TH neurons in ad-lib and CR Ghrelin WT and KO mice (Figure 3.1D). CR partially attenuated SN TH neuronal loss in Ghrelin WT (Figure 3.1E), however this protective effect was lost in Ghrelin KO mice (Figure 3.1F).

Gliosis

MPTP treatment exhibited a significant elevation in microglia (IBA1+ cells) present in the SN of both genotypes, although CR did not prevent the MPTP-induced increase of Ionized calcium binding adaptor 1 (IBA1) cell number in either Ghrelin WT or Ghrelin KO mice (Figure 3.1G & H). Astrocytes, as represented by Glial Fibrillary Acidic Protein (GFAP) staining, are the most abundant cell type found throughout the central nervous system and play a critical role during cellular damage to minimize overall cell loss (Hailer et al., 2001). Elevated GFAP+ cells in any specified region indicates greater cellular damage in that area. MPTP treatment initiated a significant increase in GFAP cells in both Ghrelin WT and KO ad-lib mice compared to saline controls (Figure 3.1I & J). CR reduced GFAP expression in the SN of both MPTP-treated ghrelin WT and KO mice relative to MPTP ad-lib mice (Figure 3.1I & J), This result indicates that CR restricts GFAP cell expression in the SN, although this does not appear to be directly mediated by ghrelin.

Figure 3.1

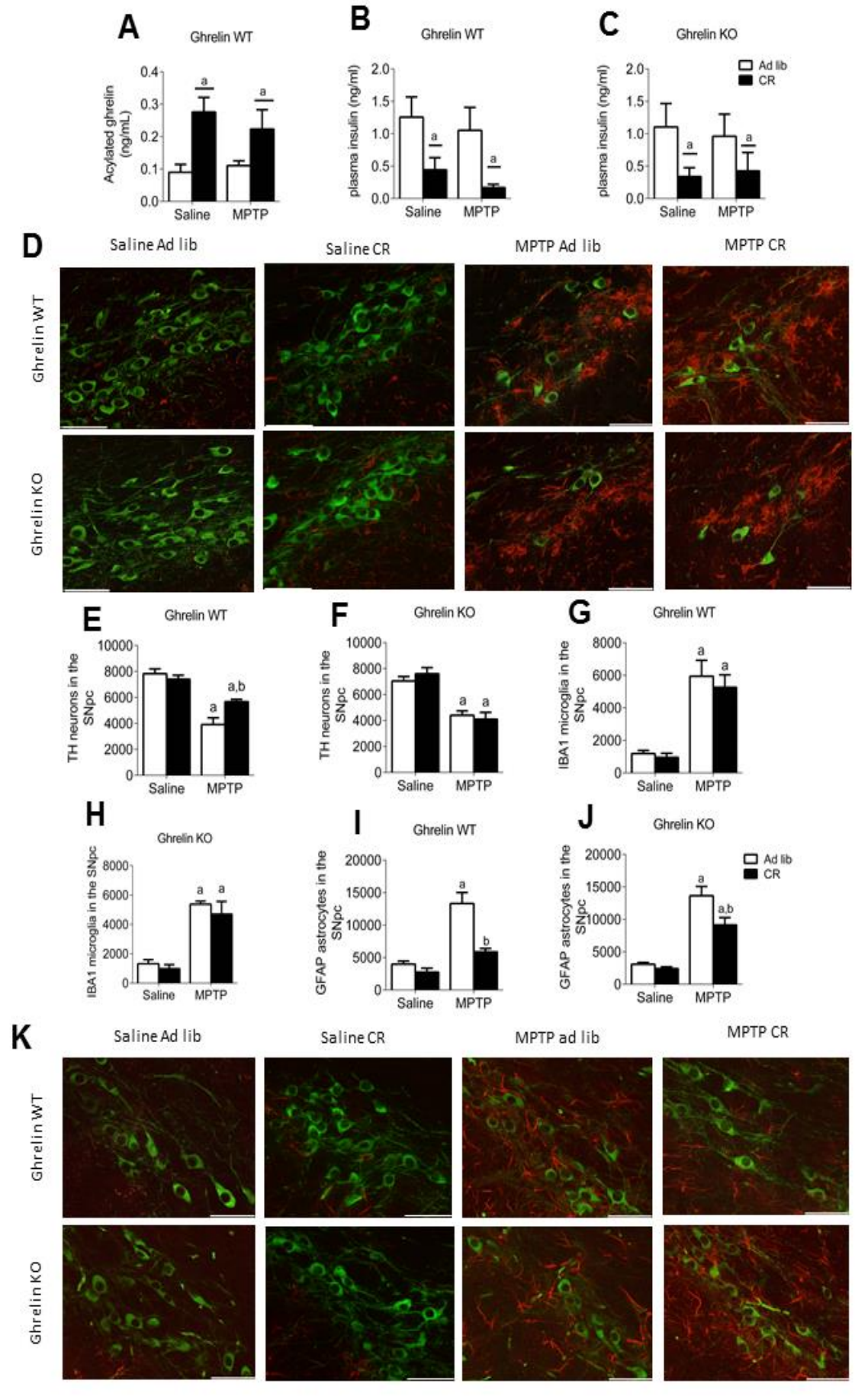

A
Ghrelin WT
Acylated ghrelin (ng/mL)
Saline
MPTP
B
Ghrelin WT
plasma insulin (ng/ml)
C
Ghrelin KO
Ad lib
CR
D
Saline Ad lib
Saline CR
MPTP Ad lib
MPTP CR
Ghrelin WT
Ghrelin KO
E
Ghrelin WT
TH neurons in the SNpc
F
Ghrelin KO
G
Ghrelin WT
IBA1 microglia in the SNpc
H
Ghrelin KO
I
Ghrelin WT
GFAP astrocytes in the SNpc
J
Ghrelin KO
K
Saline Ad lib
Saline CR
MPTP ad lib
MPTP CR

Figure 3.1. Deletion of Ghrelin negates the protective effect of CR. **A**, CR significantly elevates plasma acylated ghrelin. **B + C,** Overall reduction in plasma insulin levels in response to CR in both genotypes. **D**, Representative images showing MPTP induced TH cell loss in the SN and microglial (IBA) activation. **E & F,** Stereological quantification of TH neurons in the SN showing CR has no significant effect in MPTP treated Ghrelin KO mice (**F**) but is protective in Ghrelin WT mice (**E**). **G & H,** Stereological quantification of IBA1 microglia in the SN shows elevated levels following MPTP treatment but no effect of genotype. **I & J**, Stereological quantification of GFAP in the SN showing that following MPTP administration GFAP levels increased to a lesser extent in Ghrelin WT compared to Ghrelin KO mice **K**, Representative images showing MPTP induced astrocyte (GFAP) activation in the SN (TH = green and GFAP =green. Data are represented as mean ± SEM (n= 6-10, two-way ANOVA, $p<0.05$). a, significant compared to saline ad-lib controls, b, significant compared to MPTP ad-lib controls. Scale bar = 50µm

TH cell volume

To accurately measure cell volume we used the nucleator stereological probe. There was a significant main effect for MPTP to reduce average cell volume in Ghrelin WT but not Ghrelin KO (Figure 3.2A & B). We performed a cell volume distribution analysis in order to determine if diet or treatment preferentially affected neuronal number within a certain volume range. MPTP treatment to CR Ghrelin WT mice prevented the loss of TH neurons with volumes between 1000-2000um^3 compared to ad-lib MPTP-treated Ghrelin WT mice (Figure 3.2C). Remarkably, no beneficial effects of CR on TH neuronal cell volume between were observed in Ghrelin KO mice (Figure 3.2D). Thus, ghrelin influences both TH cell number and cell volume distribution during CR.

HPLC analysis

HPLC analysis of dopamine (DA) in the striatum revealed a significant overall ($p<0.05$) reduction with MPTP administration and CR significantly attenuated the loss of dopamine in WT but not KO mice (Figure 3.2E & H). MPTP also significantly reduced DOPAC in the striatum however there was no effect of diet on DOPAC levels regardless of genotype (Figure 3.2F & I). CR also prevented in the increase in the DOPAC:DA ratio observed after MPTP in ghrelin WT ad-lib but not ghrelin KO ad-lib mice (Figure 3.2G & J).

Figure 3.2

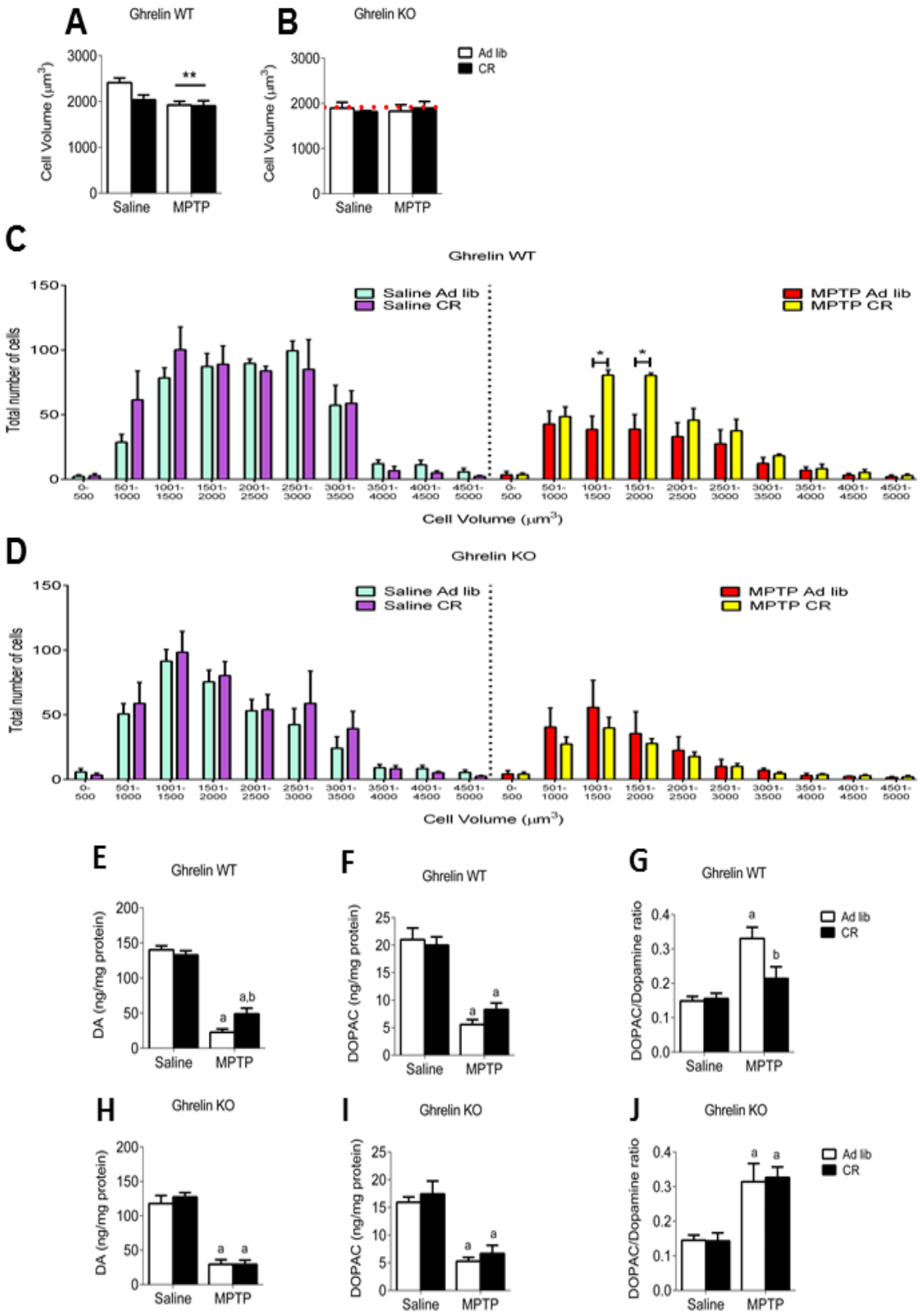

A
Ghrelin WT
Cell Volume (µm3)
3000
2000
1000
0
**
Saline
MPTP
B
Ghrelin KO
Ad lib
CR
C
Ghrelin WT
Saline Ad lib
Saline CR
MPTP Ad lib
MPTP CR
Total number of cells
150
100
50
0
Cell Volume (µm3)
D
Ghrelin KO
E
Ghrelin WT
DA (ng/mg protein)
a
a,b
F
DOPAC (ng/mg protein)
G
DOPAC/Dopamine ratio
H
Ghrelin KO
I
J

Figure 3.2. Calorie restriction reduces small volume TH cell loss and enhances dopamine turnover in Ghrelin WT but not KO mice. **A,** Overall Cell volume for Ghrelin WT mice showed a significant ($p<0.05$) effect of MPTP administration but no effect of genotype or diet. **B,** Ghrelin KO mice showed no overall effect of diet, treatment or genotype. The red dotted line represents the average cell volume of Ghrelin WT MPTP treated mice. When the cells were separated based on number and volume distribution as shown in **C & D**, the effect of CR is apparent. **C**, Ghrelin WT have a significant ($p<0.05$) effect between ad-lib and CR cell volume in smaller (1000-2000 μm^3) cells. There was no significant difference in the Ghrelin KO mice (**D**). **E & H,** CR attenuates striatal DA loss in Ghrelin WT but not Ghrelin KO mice after MPTP administration. **F & I,** MPTP reduced DOPAC with no effect of genotype. **G & J**, CR reduced the elevation of the DOPAC:DA ratio in MPTP treated mice compared to ad lib, in Ghrelin WT but not Ghrelin KO mice. a, significant compared to saline ad-lib controls, b, significant compared to MPTP ad-lib controls.* $p<0.05$, ** $p<0.01$. Data are represented as mean ± SEM (n=6-10, two-way ANOVA, $p<0.05$).

TH protein expression

Reduced dopamine levels in the striatum indicate impaired dopamine synthesis, which is controlled by the rate-limiting enzyme TH. In the SN of Ghrelin WT and KO mice, MPTP administration significantly reduced TH protein expression in both ad-lib fed and CR mice with no protective effect of CR in either genotype (Figures 3.3A, C&D). In the striatum however, CR significantly attenuated the lower TH protein levels in MPTP-treated ghrelin WT but not ghrelin KO mice (Figure 3.3B, E&F). These results highlight that CR has site-specific effects acting to increase TH in the striatum but not the SN. Together, these results corroborate with the TH neuronal counts, cell volume analysis and HPLC DA content results indicating that CR has a protective effect only in Ghrelin WT mice. Overall these results imply that ghrelin is responsible for these protective effects in a mouse model of PD.

Ghrelin influences AMPK activation in the striatum

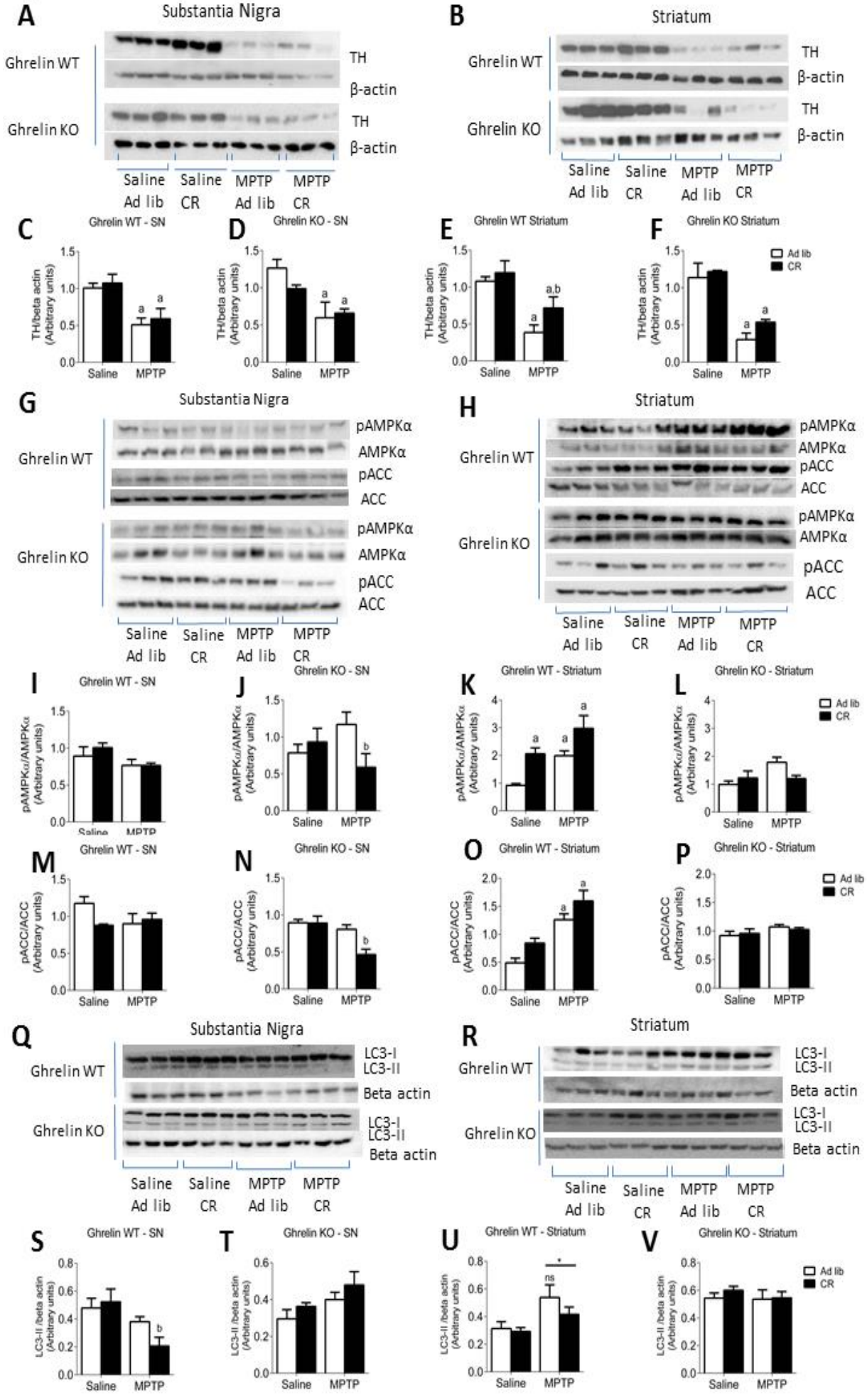
Ghrelin enhances AMPK activity in the hypothalamus (Andrews et al., 2008) and AMPK also increases mitochondrial biogenesis and function in the periphery (Bergeron et al., 2001; Horvath et al., 2011). Thus, we reasoned that the neuroprotective actions of CR induce a ghrelin-dependent increase in AMPK function in SN TH neurons. We found that both metabolic (CR) and chemical (MPTP) stress increased AMPK phosphorylation (pAMPK/AMPK ratio) and subsequent Acetyl CoA Carboxylase (ACC) in the striatum, but not the SN as seen for TH expression, in Ghrelin WT but not Ghrelin KO mice (Figure 3.3G-P). CR in Ghrelin KO MPTP treated mice significantly reduced AMPK and ACC phosphorylation in the SN compared to ghrelin KO MPTP ad-lib mice (Figure 3.3J&N). The maintenance of autophagy is one downstream effect of AMPK activation (Mihaylova and Shaw, 2011), therefore we examined LC3 II, the membrane-bound form of autophagosomes (Kimura et al., 2007). We observed significantly reduced LC3 II in the SN of CR Ghrelin WT mice compared to ad-lib controls (Figure 3.3S), with a significant overall elevation in response to MPTP in striatum (Figure 3.3U). No effect was observed in Ghrelin KO in the SN (Figure 3.3T) or Striatum (Figure 3.3V). The LC3 II results in the SN are inversely related to SN TH cell counts suggesting there is less autophagosome formation required in cells with less MPTP-induced degeneration.

PINK1 and Parkin regulate mitophagy and mutations in PINK and Parkin cause early onset PD, therefore, we also measured the expression of these two proteins in the SN and striatum

from CR and ad-lib Ghrelin WT and KO mice. PINK1 and Parkin expression showed a significant reduction in protein expression post MPTP administration in the striatum with no significant effect of CR or genotype (Appendix 2E-H). There was no change in protein levels in response to metabolic state, MPTP or genotype in the SN (Appendix 2A-D).

Figure 3.3. The protective effect of CR is concomitant with striatal dopamine and elevated pAMPK, an effect not observed in Ghrelin KO mice. **A & B,** Representative Western Blot images of MPTP induced reduction in TH levels in the SN and Striatum. **C & D,** Quantification of TH levels in Ghrelin WT and KO mice showed that MPTP significantly ($p<0.05$) reduced TH expression in the SN. **E & F,** Quantification of TH levels in the Striatum revealed that MPTP significantly ($p<0.05$) reduced TH expression, this effect was rescued in CR Ghrelin WT mice but not in KO mice. **G & H,** Representative Western Blot images of pAMPK, AMPK, pACC and ACC levels in the SN and Striatum after either ad-libitum or CR paradigms followed by MPTP or saline treatment. **I & M,** Quantification of pAMPK/AMPK and pACC/ACC levels in the SN reveals no effect in Ghrelin WT mice however, in KO mice there was a significant ($p<0.05$) reduction between MPTP ad-lib and MPTP CR groups (**J & N**), showing that CR KO mice could not adapt appropriately to MPTP-induced cell degeneration. **K & L,** MPTP and CR individually increased striatal pAMPK/AMPK in Ghrelin WT mice but not in Ghrelin KO mice, as no change from baseline with either MPTP or CR was observed. **O & P**, MPTP-induced an increase in striatal pACC/ACC in Ghrelin WT but not Ghrelin KO mice, mimicking the effects seen with pAMPK/AMPK. **Q & R**, Representative western blots for LC3 I and LC3 II in the SN and Striatum of Ghrelin WT and KO mice. **S**, LC3 II in the SN is significantly reduced in Ghrelin WT mice after MPTP treatment, however this was not observed in Ghrelin KO mice **(T)**. **U & V**, There was no effect of CR on LC3 II in the striatum from Ghrelin WT and KO mice. However, there was a significant main effect of MPTP to increase LC3 II in WT but not KO mice. a, significant compared to saline controls, b, significant compared to a low dose of Ghrelin. * $p<0.05$, ** $p<0.01$. Data are represented as mean ± SEM (n=5-7, one-way ANOVA, $p<0.05$).

Figure 3.3


A
Substantia Nigra
Ghrelin WT
Ghrelin KO
TH
β-actin
Saline Ad lib
Saline CR
MPTP Ad lib
MPTP CR
B
Striatum
C
Ghrelin WT - SN
D
Ghrelin KO - SN
E
Ghrelin WT Striatum
F
Ghrelin KO Striatum
TH/beta actin (Arbitrary units)
Ad lib
CR
Saline
MPTP
G
Substantia Nigra
H
Striatum
pAMPKα
AMPKα
pACC
ACC
I
Ghrelin WT - SN
J
Ghrelin KO - SN
K
Ghrelin WT - Striatum
L
Ghrelin KO - Striatum
pAMPKα/AMPKα (Arbitrary units)
M
Ghrelin WT - SN
N
Ghrelin KO - SN
O
Ghrelin WT - Striatum
P
Ghrelin KO - Striatum
pACC/ACC (Arbitrary units)
Q
Substantia Nigra
R
Striatum
LC3-I
LC3-II
Beta actin
S
Ghrelin WT - SN
T
Ghrelin KO - SN
U
Ghrelin WT - Striatum
V
Ghrelin KO - Striatum
LC3-II /beta actin (Arbitrary units)

Exogenous ghrelin influences the phosphorylation of AMPK and ACC

In order to support the notion that increased endogenous ghrelin is the critical to CR-induced neuroprotection, we examined the effects of exogenous acyl-ghrelin on AMPK and ACC phosphorylation both *in vivo* and *in vitro*. The addition of either acyl-ghrelin or the ghrelin agonist JMV2894 increased AMPK activation in cultured dopaminergic cell line SN4741 (Figure 3.4A-D).

For *in vivo* studies, we injected acyl-ghrelin ip at 2 different doses (low: 5mg/kg and high: 15mg/kg). The high dose of acyl-ghrelin significantly increased AMPK and ACC phosphorylation in the SN (Figure 3.4E, G&H). However, there was no significant difference in the striatum in response to either a low or high dose of acyl-ghrelin (Figure 3.4F, I&J). This is in contrast to the effect of CR on AMPK activation, as we observed a significant difference in the striatum but not the SN.

Injection of ip acyl-ghrelin at a high dose significantly increased TH expression in both the SN and Striatum (Figure 3.4K-M). Moreover, ip acyl-ghrelin increased LC3 II in the SN but not the striatum (Figure 3.4N-P). There was no change in PINK1 expression in either the SN or the striatum (Appendix 3A & C). Parkin expression remained unchanged in the SN, however, in the striatum there was a significant increase with a high dose of acyl-ghrelin (Appendix 3B & D). These results indicate that peripheral acyl-ghrelin injection affects AMPK and ACC phosphorylation, as well as TH, Parkin and LC3 II protein expression in the nigrostriatal system.

Figure 3.4

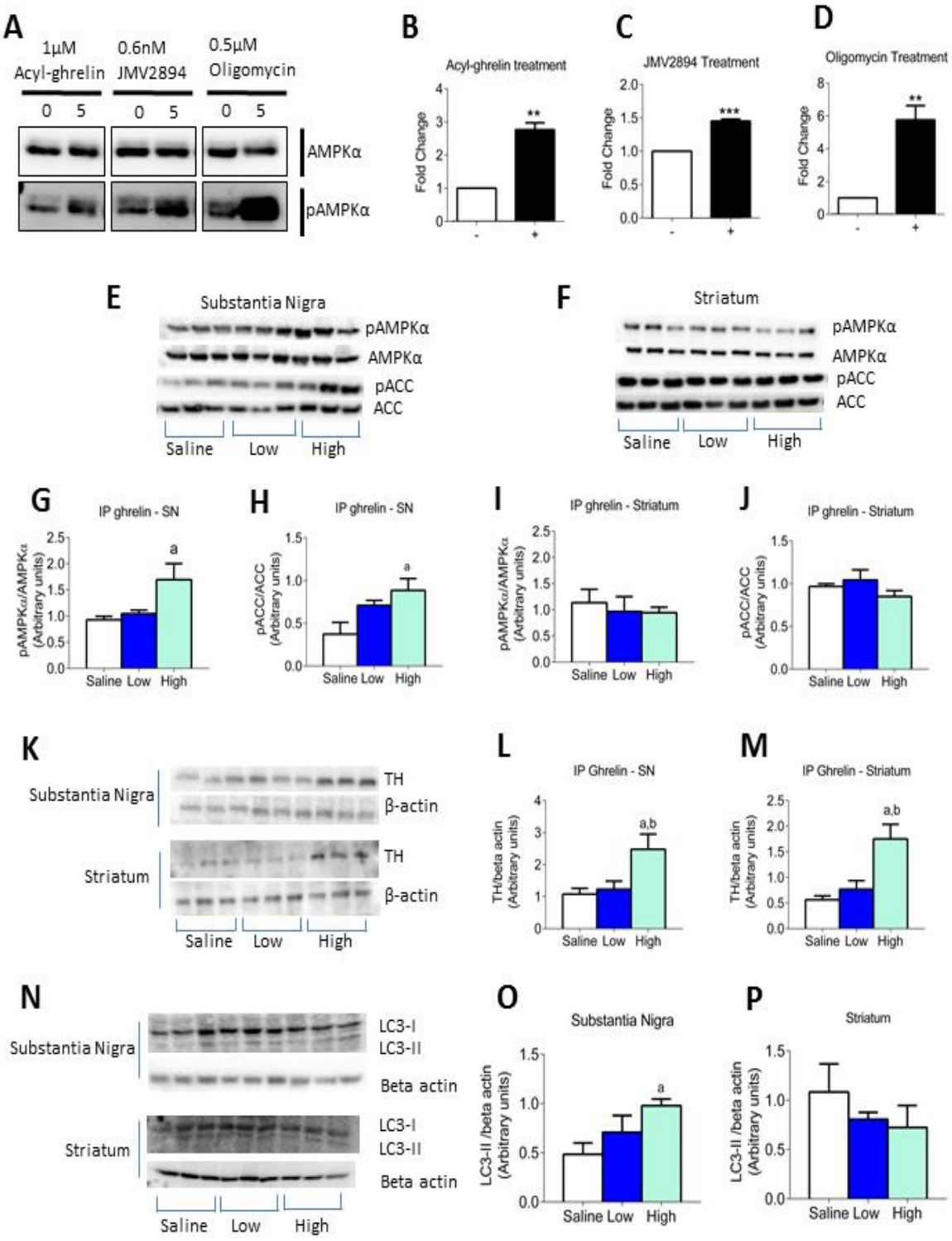

A
1μM Acyl-ghrelin
0.6nM JMV2894
0.5μM Oligomycin
0 5
0 5
0 5
AMPKα
pAMPKα
B
Acyl-ghrelin treatment
Fold Change
**
C
JMV2894 Treatment
Fold Change
***
D
Oligomycin Treatment
Fold Change
**
E
Substantia Nigra
pAMPKα
AMPKα
pACC
ACC
Saline Low High
F
Striatum
pAMPKα
AMPKα
pACC
ACC
Saline Low High
G
IP ghrelin - SN
pAMPKα/AMPKα (Arbitrary units)
a
Saline Low High
H
IP ghrelin - SN
pACC/ACC (Arbitrary units)
a
Saline Low High
I
IP ghrelin - Striatum
pAMPKα/AMPKα (Arbitrary units)
Saline Low High
J
IP ghrelin - Striatum
pACC/ACC (Arbitrary units)
Saline Low High
K
Substantia Nigra
TH
β-actin
Striatum
TH
β-actin
Saline Low High
L
IP Ghrelin - SN
TH/beta actin (Arbitrary units)
a,b
Saline Low High
M
IP Ghrelin - Striatum
TH/beta actin (Arbitrary units)
a,b
Saline Low High
N
Substantia Nigra
LC3-I
LC3-II
Beta actin
Striatum
LC3-I
LC3-II
Beta actin
Saline Low High
O
Substantia Nigra
LC3-II /beta actin (Arbitrary units)
a
Saline Low High
P
Striatum
LC3-II /beta actin (Arbitrary units)
Saline Low High

Figure 3.4. Exogenous ghrelin elevates TH and AMPK activation **A**, Representative Western Blot images of cultured dopaminergic neurons shows an increase in pAMPK levels in response to acyl ghrelin, JMV2894 (ghrelin agonist) or oligomycin treatment. Quantification of pAMPK/AMPK levels reveals a significant increase in response to acyl ghrelin (**B**), JMV2894 (**C**) and oligomycin (**D**) treatment. **E & F**, Representative Western Blot images of pAMPK, AMPK, pACC, ACC levels in the SN and Striatum. **G & H**, Quantification of the pAMPK/AMPK and pACC/ACC in the SN (**G**) in response to a high dose of ghrelin reveals a significant elevation in response to the high dose of ghrelin. **I & J**, Quantification of pAMPK/AMPK and pACC/ACC in the striatum reveals no change between saline ghrelin doses. **K,** Representative Western Blot images of TH levels in the SN and Striatum. Quantification of TH levels in the SN (**L**) and Striatum (**M**) show that ip ghrelin significantly increases TH expression in response to a high dose of ghrelin. Representative Western Blot images of LC3 II expression in the SN and Striatum (**N**). Quantification of LC3 II revealed high dose caused a significant increase in the SN (**O**) but not the striatum (**P**)..a, significant compared to saline/saline controls, b, significant compared to saline/MPTP controls. Data are represented as mean ± SEM (n=6-8, two-way ANOVA, $p<0.05$).

Exogenous ghrelin requires AMPK in dopamine neurons to elicit neuroprotection

To prove that ghrelin-induced neuroprotection requires AMPK activation in SN dopamine neurons, we generated a novel mouse line in which AMPK activation was disabled in dopaminergic neurons. These mice were generated by cross breeding *Dat*-Cre mice with *Ampk beta 1*$^{fl/fl}$; *Ampk beta 2*$^{fl/fl}$ mice in order to generate AMPK WT and AMPK KO mice. AMPK β1 & β2 are regulatory subunits required for AMPK activity (O'Neill et al., 2011). To determine the specificity of the knockout, we bred AMPK WT and KO with Rosa26loxSTOPlox tdTomato reporter mice to generate *Dat-Cre;tdTomato* and *Dat-Cre;Ampk beta 1*$^{fl/fl}$*;Ampk beta 2*$^{fl/fl}$*;tdTomato*. TH and tdTomato co-expression in the SN was >90% (Figure 3.5A-C), indicating cre recombination had occurred in >90% of SN TH neurons. Deletion of AMPKβ1 and AMPKβ2 in dopamine neurons was confirmed by FACS of tdTomato-labelled neurons from midbrain dissections from *Dat-Cre;tdTomato* and *Dat-Cre;Ampk beta 1*$^{fl/fl}$*;Ampk beta 2*$^{fl/fl}$*;tdTomato* cells and nested PCR for AMPKβ1 and AMPKβ2 (Figure 3.5D). Positive bands for both AMPKβ1 and AMPKβ2 were observed in AMPK WT but not AMPK KO mice (Figure 3.5D). As a positive control, nested PCR for GHSR was performed to confirm the presence of the ghrelin receptor in both AMPK WT and KO mice (Figure 3.5D).

In order to show that ghrelin elicits neuroprotection in a mouse model of PD, we chronically administered acyl-ghrelin to DAT AMPK WT & KO mice. In AMPK WT mice, acyl-ghrelin administration significantly attenuated TH cell loss in MPTP-treated mice (Figure 3.5E & F). This effect was abolished in the AMPK KO mice (Figure 3.5G). Acyl-ghrelin reduced IBA1+ cell number in AMPK WT MPTP treated (Figure 3.5H), however no significant effect was observed in AMPK KO mice (Figure 3.5I). A similar pattern was observed with GFAP cells, in which acyl-ghrelin reduced GFAP cell number in AMPK WT but not AMPK KO mice (Figure 3.5J & K).

Despite the attenuated TH cell loss in the acyl ghrelin-treated AMPK WT mice there was no overall change in cell volume or distribution (Figure 3.6A-D). HPLC analysis of dopamine and DOPAC in the striatum revealed that acyl-ghrelin attenuated the MPTP-induced loss of dopamine and prevented the MPTP-induced rise in the DOPAC/dopamine ratio in AMPK WT but not AMPK KO mice (Figure 3.6E-J). Changes in motor behaviour were determined using an accelerating Rotarod by measuring latency to fall. There was no overall change between Saline or Ghrelin treated AMPK WT and KO mice without MPTP treatment (Figure

3.6K & L). When pretreated with saline and given MPTP there was no effect of genotype (Figure 3.6M) however, there was a protective effect of ghrelin administration prior to MPTP in AMPK WT but not KO mice (Figure 3.6N). Collectively, these experiments highlight that ghrelin activates AMPK in SN dopamine neurons, restricts dopaminergic cell loss, maintains striatal dopamine concentrations and promotes locomotor behaviour after MPTP treatment to provide a neuroprotective effect.

Figure 3.5. Ghrelin activates AMPK to elicit neuroprotection in an AMPK-dependent manner. **A**, DAT CRE mice crossed with the tdTomato line shows a >90% co-localisation (**B**) between TH (green) and tdTomato (red) neurons; scale bar = 100µm. **C**, Representative tiled image showing TH (green) and tdTomato (red) where each tile represents a 20x image. **D**, tdTomato labelled TH neurons were sorted via FACs to show the selective deletion of AMPKβ1 and AMPKβ2 in AMPK WT but not AMPK KO mice. Product size for AMPKβ1 = 386kb, AMPKβ2 = 395kb. The ghrelin receptor (GHSR) is unaffected by deletion of AMPKβ1 and AMPKβ2 in SN TH neurons. **E**, Representative images showing TH neurons from AMPK WT and KO mice after chronic ghrelin treatment. **F & G**, Stereological quantification of TH neurons from AMPK WT (**F**) and KO (**G**) mice shows a protective effect of ghrelin treatment in WT but not KO mice. **H,** Stereological quantification of IBA1 microglia in the SN shows that ghrelin suppresses IBA1 cells relative to saline controls following MPTP treatment, however this is not observed in AMPK KO mice (**I**). **J & K**, Stereological quantification of GFAP in the SN shows that ghrelin attenuates the MPTP-induced increase in GFAP cell numbers in AMPK WT (**J**) but not AMPK KO (**K**) mice. **L**, Representative images showing MPTP induced astrocyte (GFAP) activation in the SN (TH = green and GFAP = red). a, significant compared to saline/saline controls, b, significant compared to saline/MPTP controls. Data are represented as mean ± SEM (n=6-8, two-way ANOVA, $p<0.05$). Scale bar = 100 µm.

Figure 3.5

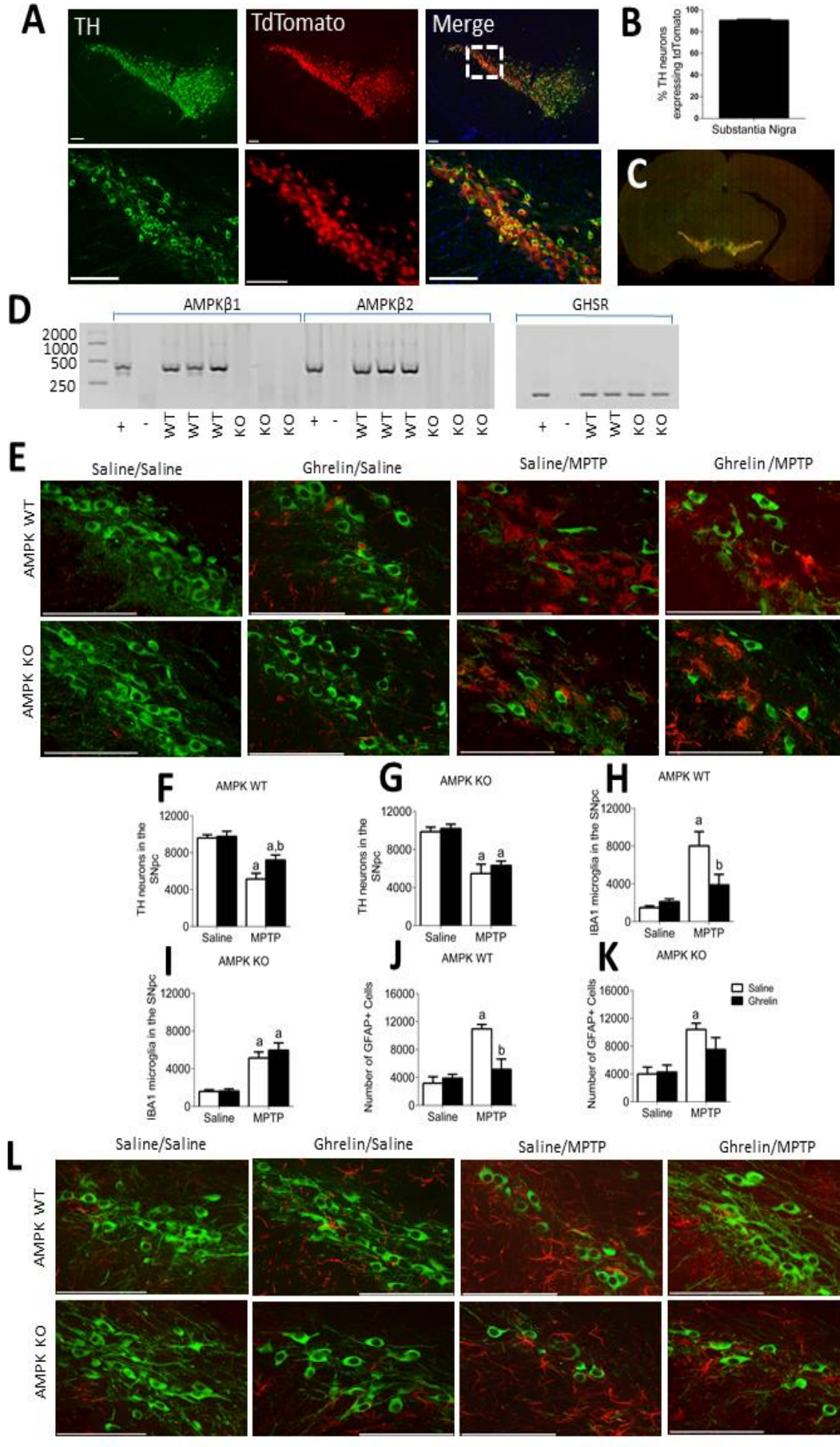

A
TH
TdTomato
Merge
B
% TH neurons expressing tdTomato
Substantia Nigra
C
D
AMPKβ1
AMPKβ2
GHSR
2000
1000
500
250
+ - WT WT WT KO KO KO + - WT WT WT KO KO KO
+ - WT WT KO KO
E
Saline/Saline
Ghrelin/Saline
Saline/MPTP
Ghrelin /MPTP
AMPK WT
AMPK KO
F
AMPK WT
TH neurons in the SNpc
Saline
MPTP
G
AMPK KO
H
AMPK WT
IBA1 microglia in the SNpc
I
AMPK KO
J
AMPK WT
Number of GFAP+ Cells
K
AMPK KO
Saline
Ghrelin
L
Saline/Saline
Ghrelin/Saline
Saline/MPTP
Ghrelin/MPTP
AMPK WT
AMPK KO

Figure 3.6

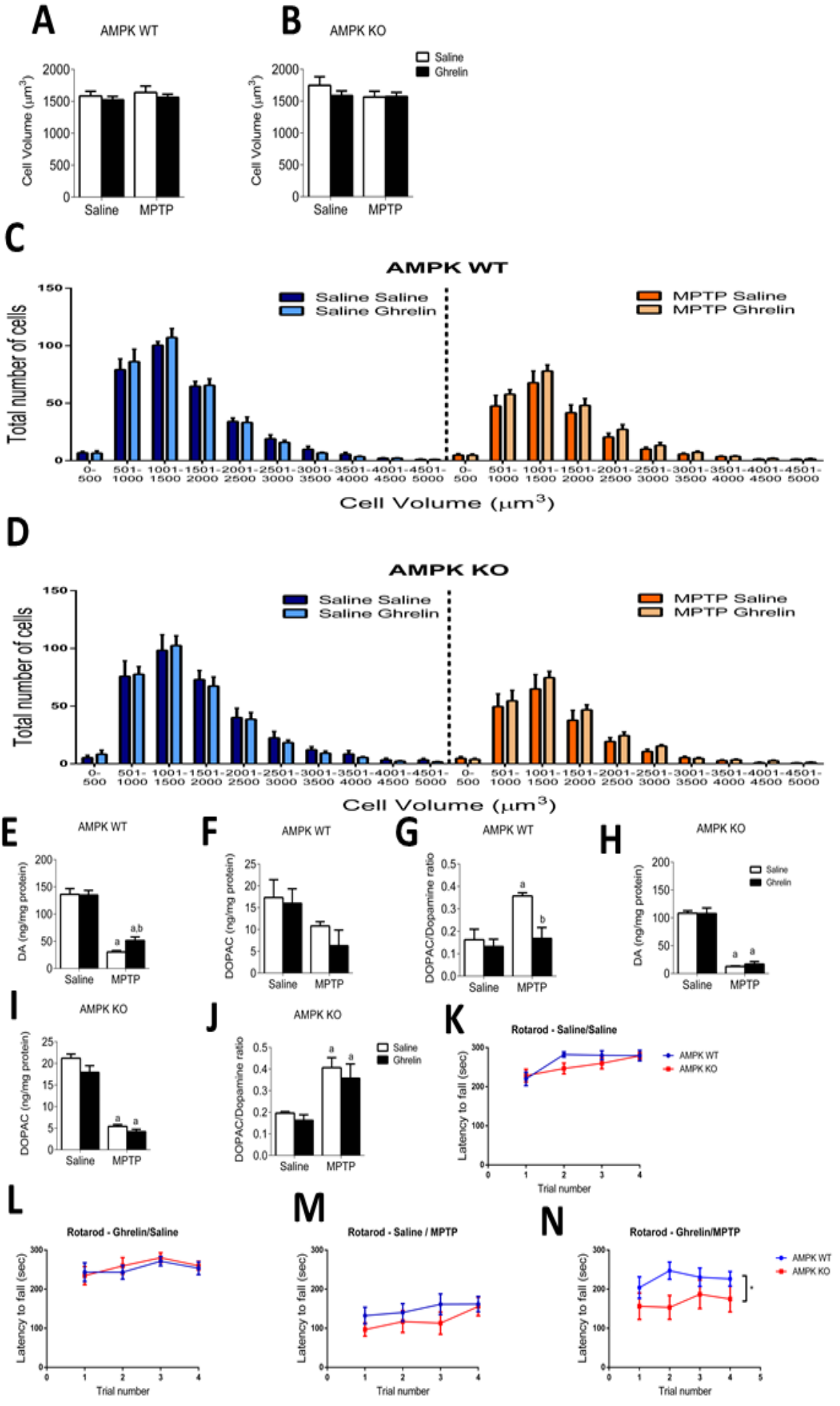
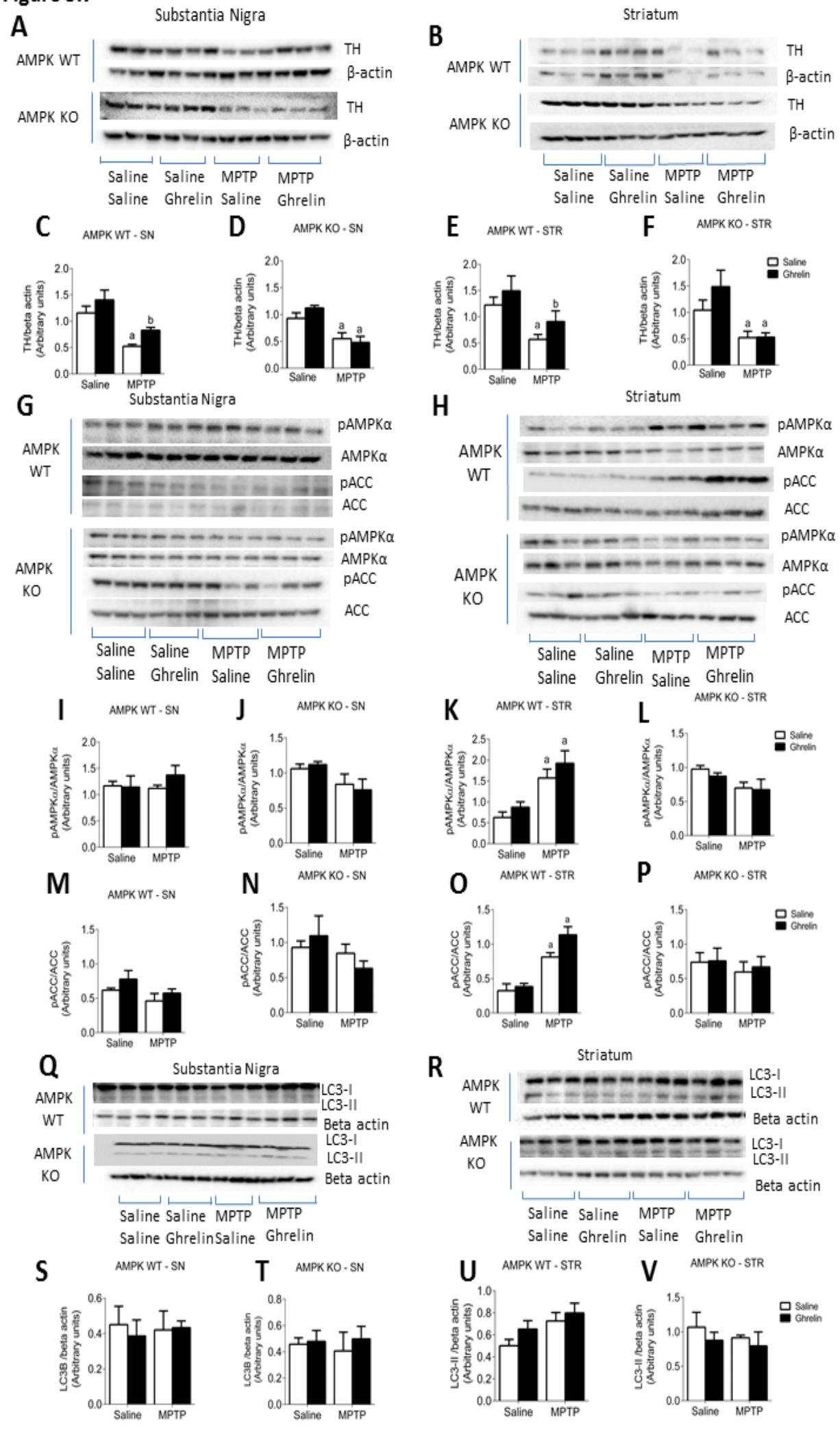

A
AMPK WT
Cell Volume (µm³)
2000
1500
1000
500
0
Saline
MPTP
B
AMPK KO
Saline
Ghrelin
Cell Volume (µm³)
2000
1500
1000
500
0
Saline
MPTP
C
AMPK WT
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
Total number of cells
150
100
50
0
0-500
501-1000
1001-1500
1501-2000
2001-2500
2501-3000
3001-3500
3501-4000
4001-4500
4501-5000
Cell Volume (µm³)
D
AMPK KO
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
Total number of cells
150
100
50
0
0-500
501-1000
1001-1500
1501-2000
2001-2500
2501-3000
3001-3500
3501-4000
4001-4500
4501-5000
Cell Volume (µm³)
E
AMPK WT
DA (ng/mg protein)
200
150
100
50
0
a
a,b
Saline
MPTP
F
AMPK WT
DOPAC (ng/mg protein)
25
20
15
10
5
0
Saline
MPTP
G
AMPK WT
DOPAC/Dopamine ratio
0.5
0.4
0.3
0.2
0.1
0.0
a
b
Saline
MPTP
H
AMPK KO
Saline
Ghrelin
DA (ng/mg protein)
200
150
100
50
0
a
a
Saline
MPTP
I
AMPK KO
DOPAC (ng/mg protein)
25
20
15
10
5
0
a
a
Saline
MPTP
J
AMPK KO
Saline
Ghrelin
DOPAC/Dopamine ratio
0.5
0.4
0.3
0.2
0.1
0.0
a
a
Saline
MPTP
K
Rotarod - Saline/Saline
AMPK WT
AMPK KO
Latency to fall (sec)
300
200
100
0
0
1
2
3
4
Trial number
L
Rotarod - Ghrelin/Saline
Latency to fall (sec)
300
200
100
0
0
1
2
3
4
Trial number
M
Rotarod - Saline / MPTP
Latency to fall (sec)
300
200
100
0
0
1
2
3
4
Trial number
N
Rotarod - Ghrelin/MPTP
AMPK WT
AMPK KO
*
Latency to fall (sec)
300
200
100
0
0
1
2
3
4
5
Trial number

Figure 3.6. Chronic ghrelin injection enhances dopamine turnover and behavioural outcomes in AMPK WT but not KO mice. **A & B**, Overall cell volume showing no reduction in response to genotype or treatment. When cells were separated based on number and volume distribution as shown in **C & D** there was no overall effect of genotype or treatment. HPLC data show that ghrelin significantly attenuates the MPTP-induced decrease in striatal dopamine concentration in AMPK WT but not AMPK KO mice (**E & H**). MPTP reduced DOPAC with no effect of genotype (**F & I**). Ghrelin treatment significantly attenuates the MPTP-induced increase in the DOPAC/Dopamine ration in AMPK WT but not AMPK KO mice (**G & J**). **K – N**, Behavioural analysis showing latency to fall on an accelerating rotarod. **K & L**, no difference in latency to fall in mice not exposed to MPTP. In mice given MPTP, latency to fall is not effected by genotype in mice pre-treated with saline (**M**). However, in mice pre- treated with ghrelin there is a significant protective effect in AMPK WT but not KO as evidence by increased latency to fall (**N**). a, significant compared to saline/saline controls, b, significant compared to saline/MPTP controls. Data are represented as mean ± SEM (n=6-12, two-way ANOVA, $p<0.05$).

We previously showed that ip ghrelin can elicit an increase in the pAMPK/AMPK and pACC/ACC ratio in SN (Figure 3.4G&H) and that chronic ghrelin treatment to AMPK WT, but not AMPK KO is neuroprotective in a mouse model of PD. In further support of this neuroprotection, ghrelin treatment attenuated the MPTP-induced loss of TH in both the SN and striatum of AMPK WT but not AMPK KO mice (Figure 3.7A-F). To determine if chronic ghrelin differentially affected AMPK and subsequent ACC phosphorylation, we measured the pAMPK/AMPK and pACC/ACC ratio in AMPK WT and KO mice in response chronic daily ghrelin injections. There was no effect of chronic ghrelin treatment on the pAMPK/AMPK (Figure 3.7G, I&J) or the pACC/ACC ratio (Figure 3.7M&N) in the SN of either AMPK WT or AMPK KO mice. However, MPTP treatment elicited an increase in the pAMPK/AMPK (Figure 3.7H, K&L) and pACC/ACC ratios (Figure 3.7O&P) in the striatum of AMPK WT mice, but not AMPK KO mice. The mitophagy proteins PINK1 and Parkin (Appendix 4A-H) and the autophagosome marker LC3 II were not significantly different between genotypes and treatment (Figure 3.7Q-V).

Figure 3.7. Chronic ghrelin injections increase nigrostriatal TH expression and AMPK activation in an AMPK-dependent manner. **A & B**, representative Western Blot images of the SN (**A**) and Striatum (**B**) showing TH levels. In both the SN and the Striatum there is a significant protective effect of ghrelin administration on TH levels in AMPK WT mice (**C & E**) that is absent in AMPK KO mice (**D & F**). **G & H**, Representative Western Blot images showing pAMPK, AMPK, pACC and ACC levels in the SN (**G**) and striatum (**H**). There was no significant change in the pAMPK/AMPK (I & J) or pACC/ACC (M & N) ratio in the SN of AMPK WT or AMPK KO mice in response to MPTP or ghrelin. MPTP-induced an increase in the pAMPK/AMPK and pACC/ACC ratio AMPK WT mice (**K & O**) but not AMPK KO mice (**L & P**). In the Striatum MPTP induced an increase in the pAMPK/AMPK and pACC/ACC ratio AMPK WT mice (**K & O**) but not AMPK KO mice (**L & P**). **Q & R**, Representative Western Blot images of LC3 II expression in the SN (**Q**) and Striatum (**R**). There was no significant effect of MPTP or ghrelin administration on LC3-II levels in the SN (**S & T**) or Striatum (**U & V**). a, significant compared to saline/saline controls, b, significant compared to saline/MPTP controls. Data are represented as mean ± SEM (n=6-8, two-way ANOVA, $p<0.05$).

Figure 3.7


A
Substantia Nigra
AMPK WT
TH
β-actin
AMPK KO
TH
β-actin
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
B
Striatum
AMPK WT
TH
β-actin
AMPK KO
TH
β-actin
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
C
AMPK WT - SN
TH/beta actin (Arbitrary units)
Saline
MPTP
D
AMPK KO - SN
TH/beta actin (Arbitrary units)
Saline
MPTP
E
AMPK WT - STR
TH/beta actin (Arbitrary units)
Saline
MPTP
F
AMPK KO - STR
TH/beta actin (Arbitrary units)
Saline
Ghrelin
Saline
MPTP
G
Substantia Nigra
AMPK WT
pAMPKα
AMPKα
pACC
ACC
AMPK KO
pAMPKα
AMPKα
pACC
ACC
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
H
Striatum
AMPK WT
pAMPKα
AMPKα
pACC
ACC
AMPK KO
pAMPKα
AMPKα
pACC
ACC
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
I
AMPK WT - SN
pAMPKα/AMPKα (Arbitrary units)
Saline
MPTP
J
AMPK KO - SN
pAMPKα/AMPKα (Arbitrary units)
Saline
MPTP
K
AMPK WT - STR
pAMPKα/AMPKα (Arbitrary units)
Saline
MPTP
L
AMPK KO - STR
pAMPKα/AMPKα (Arbitrary units)
Saline
Ghrelin
Saline
MPTP
M
AMPK WT - SN
pACC/ACC (Arbitrary units)
Saline
MPTP
N
AMPK KO - SN
pACC/ACC (Arbitrary units)
Saline
MPTP
O
AMPK WT - STR
pACC/ACC (Arbitrary units)
Saline
MPTP
P
AMPK KO - STR
pACC/ACC (Arbitrary units)
Saline
Ghrelin
Saline
MPTP
Q
Substantia Nigra
AMPK WT
LC3-I
LC3-II
Beta actin
AMPK KO
LC3-I
LC3-II
Beta actin
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
R
Striatum
AMPK WT
LC3-I
LC3-II
Beta actin
AMPK KO
LC3-I
LC3-II
Beta actin
Saline Saline
Saline Ghrelin
MPTP Saline
MPTP Ghrelin
S
AMPK WT - SN
LC3B /beta actin (Arbitrary units)
Saline
MPTP
T
AMPK KO - SN
LC3B /beta actin (Arbitrary units)
Saline
MPTP
U
AMPK WT - STR
LC3-II /beta actin (Arbitrary units)
Saline
MPTP
V
AMPK KO - STR
LC3-II /beta actin (Arbitrary units)
Saline
Ghrelin
Saline
MPTP

Discussion

Calorie Restriction (CR) protects against a number of pathological conditions including diabetes, cancer, heart disease and neurodegeneration. In a Parkinson's Disease (PD) model an alternate-day feeding schedule, where rats consumed 30-40% less calories than ad-libitum controls was neuroprotective post MPTP exposure (Duan and Mattson, 1999). Mice also elicited a neuroprotective response when alternate day feeding was begun after exposure to MPTP (Holmer et al., 2005). Primates with a chronic overall 30% reduction in food intake were also resistant to MPTP induced neurotoxicity (Maswood et al., 2004). These studies prove that CR is beneficial in a primate PD model however the difficulty to adhere to CR necessitates an alternative method to recapitulate the neuroprotective benefits of CR whilst bypassing dietary constraints. Evidence from cells treated with serum from CR rats suggests a hormonal factor improves mitochondrial function and cell viability (Lopez-Lluch et al., 2006). We hypothesized that ghrelin may be this hormonal factor, because CR increases plasma acyl ghrelin (Lutter et al., 2008) and ghrelin restricts degeneration in PD (Andrews et al., 2009). In this study we show for the first time that ghrelin mediates the neuroprotective effect of CR in a mouse model of PD by attenuating MPTP-induced loss of TH neurons, TH neuronal volume and dopamine content in the striatum. Further, we show that AMPK in SN dopamine neurons is a molecular target for ghrelin's neuroprotective effects, as deletion of AMPK β1 & β2 subunits prevented ghrelin-induced neuroprotection. These results suggest that ghrelin, and its downstream target AMPK, has a potential therapeutic application in the treatment of PD to mimic the neuroprotective effect of CR without the need for strict dietary constraints.

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

function of ghrelin is to act as a feedback signal of CR (negative energy balance) and maintain physiological and neurological function during this time.

Our data show that AMPK in SN dopamine neurons is a molecular target of ghrelin during CR to maintain neuronal function. Firstly, metabolic stress (CR) and/or toxic stress (MPTP) promoted AMPK activity in striatal dopamine nerve terminals in Ghrelin WT but not Ghrelin KO. The ability of MPTP to increase AMPK activity is supported by previous studies in mice and cells (Choi et al., 2010). AMPK enhances mitochondrial function and biogenesis (Reznick and Shulman, 2006) as such, we suggest CR-induced AMPK phosphorylation at the nerve terminal promotes neuronal energy metabolism and supports ongoing dopaminergic neuronal activity, which is supported by the reduced striatal DOPAC/dopamine ratio of both CR Ghrelin and AMPK WT but not their respective KO mice. Moreover, AMPK activity diminishes with age (Reznick et al., 2007) consistent with the age-related neurodegeneration that contributes to the onset of PD. Thus, the ability of CR to maintain AMPK activity in a ghrelin-dependent manner may restrict age-related decline in the nigrostriatal system. This possibility is further strengthened by data showing that plasma ghrelin and ghrelin's function diminishes with age, an effect that can be reversed with CR (Englander et al., 2004; Smith et al., 2007; Sun et al., 2007; Yang et al., 2007; Takeda et al., 2010). Further, PD patients have reduced postprandial plasma ghrelin levels (Unger et al., 2011). Another nuance to this thesis which will need to be addressed in future studies will be the desensitisation of the ghrelin receptors with long-term calorie restriction, however one would predict that, if GHSR desensitisation occurred, therefore would be a further increase in plasma acyl ghrelin to overcome this, in a similar manner to insulin release from pancreatic beta cells during early stages of insulin resistant diabetes.

In cultured dopaminergic neurons both acyl-ghrelin and a ghrelin agonist elicited a robust increase in AMPK activation. Acute acyl-ghrelin injection *in vivo* increased both AMPK and ACC phosphorylation in the SN but not the striatum. This is the first *in vivo* study that shows ghrelin activates AMPK activity in the midbrain, similar to numerous reports showing ghrelin activates AMPK activity in the hypothalamus (Andersson et al., 2004; Kola et al., 2005; Andrews et al., 2008). As noted above, CR drives ghrelin-induced AMPK phosphorylation in the striatum, but not the SN, yet acute ghrelin injection *in vivo* increased AMPK

phosphorylation in the SN but not the striatum. We consider this discrepancy may be due to chronically elevated ghrelin vs. an acute ghrelin injection. Chronically high plasma ghrelin, as seen in CR Ghrelin WT mice, activates SN dopamine neurons via the GHSR which then facilitates and propagates AMPK phosphorylation in areas of metabolic need, in this case striatal nerve terminals in order to prevent degeneration. Although acute injection of ghrelin increase pAMPK/AMPK ratio in the SN after 45 minutes, this narrow time frame presumably prevents propagation of AMPK phosphorylation in the striatum. It is important to note that the ghrelin receptor, GHSR, is abundantly expressed in the SN with little or no expression in the striatum (Zigman et al., 2006).

Importantly, we conclusively demonstrate that AMPK activity in dopamine neurons is necessary for ghrelin-induced neuroprotection is a mouse model of PD. We generated a model in which AMPKβ1 and AMPKβ2 were successfully deleted in DAT expressing neurons. Deletion of both AMPKβ1 and AMPKβ2 in muscle ablated AMPK phosphorylation and lead to impaired glucose homeostasis (O'Neill et al., 2011). Using the model we showed that ghrelin prevents nigrostriatal degeneration in MPTP-treated DAT AMPK WT but not DAT AMPK KO mice, clearly establishing AMPK as a critical molecular mechanism mediating the neuroprotective effects of ghrelin on the nigrostriatal system. Our genetic model also deletes AMPKβ1 and AMPKβ2 in all DAT-cre expressing neurons including populations not associated with PD, such as the hypothalamic and VTA dopamine neurons. However, MPTP predominantly affects SN dopamine neurons (Seniuk et al., 1990; Muthane et al., 1994), which strengthens the specific and important neuroprotective actions of ghrelin on AMPK activity in the SN. We should note that we did not detect a change in pAMPK/AMPK ratio in the SN or striatum of chronic ghrelin treated AMPK WT or KO mice, whereas acute ghrelin injection affected the pAMPK/AMPK ratio in the SN. There are many potential reasons for this including the dosage and time of tissue collection after last injection. However the most plausible reason is due to the tissue collection, since we measured pAMPK/AMPK in a dissected piece of tissue, of which only a small proportion represents SN dopamine neurons. Nevertheless, in response to MPTP treatment AMPK WT mice produced an increase in pAMPK/AMPK ratio in the striatum, which was not observed in AMPK KO. In fact the significant main effect of MPTP to suppress AMPK phosphorylation, independent from ghrelin treatment, in both the SN and striatum of AMPK KO illustrates the important role of AMPKβ1 and AMPKβ2 in SN dopamine neurons to

combat cellular stress caused by MPTP. Moreover, CR Ghrelin KO also did not show a compensatory increase in MPTP-induced AMPK phosphorylation in the striatum, further supporting the idea that ghrelin targets AMPK in SN dopamine neurons during CR to prevent degeneration.

Intriguingly, we noted differential effects of CR and ghrelin treatment on gliosis. In the CR experiment, the microglial response to MPTP was similar in ghrelin WT and KO mice despite the greater TH cell loss in ghrelin KO mice. This is somewhat unexpected given microglia become activated to remove neuronal damage by phagocytosis (Neumann et al., 2009). It is possible that a threshold level of cell loss elicits the same microglial response, perhaps mediated by the release of caspase signal (Burguillos et al., 2011). Moreover, GFAP cell number was increased after MPTP and suppressed in CR mice regardless of genotype. In primates CR elicited a protective effect by limiting astrogliosis in the hippocampus (Sridharan et al., 2013). These results suggest that the effects of CR on gliosis are independent from changes in plasma ghrelin. However, chronic ghrelin-treatment to AMPK WT and AMPK KO mice showed that ghrelin reduced microglia and GFAP in AMPK WT but not AMPK KO mice treated with MPTP. This effect of ghrelin treatment is consistent with in vitro studies that indicate ghrelin directly inhibits glial activation to diminish the inflammatory response (Lee and Yune, 2014). Moreover, that lack of an effect in AMPK KO mice suggests ghrelin acts directly on AMPK in SN dopamine to restrict microglia and GFAP expression, a hypothesis supported by studies showing that AMPK influence gliosis (Lu et al., 2010; Yi et al., 2011; Chen et al., 2014a; Han et al., 2014; Zhou et al., 2014).

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

In conclusion, CR is perhaps the most robust and reproducible mechanism to enhance lifespan and promote healthy aging. The exact mechanism/s that achieve this are currently unknown, however, several theories include altered stress response pathways, altered signalling pathways involving SIRT1, FOXO, UCP2 and AMPK (Andrews, 2010) as well as alterations in metabolic hormones such as ghrelin and insulin. We consider CR induces a mild stress and encourages compensatory metabolic changes that favour improved intracellular mitochondrial health. Although CR promotes metabolic health and reduces neurodegeneration there is a poor compliance in the general population, as it requires ~20-40% reduced calorie intake over years in order to achieve maximal benefits. Consequently, there is a need to recapitulate these beneficial effects without restricting calorie intake. We have discovered a novel pathway where circulating ghrelin, which is elevated during CR, has a protective role in the nigrostriatal system via enhanced AMPK activity. This ghrelin-induced neuroprotection is dependent on AMPK activity in dopamine neurons. Future research should focus on exploiting this pathway to determine the *in vivo* neuroprotective effects that restrict neurodegeneration without the need to adhere to strict dietary regimes.

Contributions

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

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

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

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

Acknowledgements

This work was supported by grants and fellowships from the Australian National Health and Medical Research Council to Z.B.A (546131, 1084344) and B.E.K; the Australian Research Council to Z.B.A (FT100100966); and NIH NS056181 to J.H. Supported in part by the Victorian Government's Operational Infrastructure Support Program (B.E.K.) and a Monash University Fellowship to Z.B.A.

Summary for Chapter 3

In Chapter 3 we demonstrate that acylated ghrelin is responsible for the neuroprotection observed during Calorie Restriction and that acylated ghrelin activates AMPK in the Substantia Nigra to elicit these neuroprotective actions (see Figure 3.8). Hence, we wanted to determine if we can recapitulate the neuroprotective actions of AMPK activation without the need to adhere to strict dietary constraints or chronic administration of acylated ghrelin. An interesting candidate to that will enhance AMPK activity (in the periphery) with minimal side effects is Metformin. In Chapter 4 our aim was to determine if Metformin was neuroprotective during PD through the activation of AMPK in dopaminergic neurons.

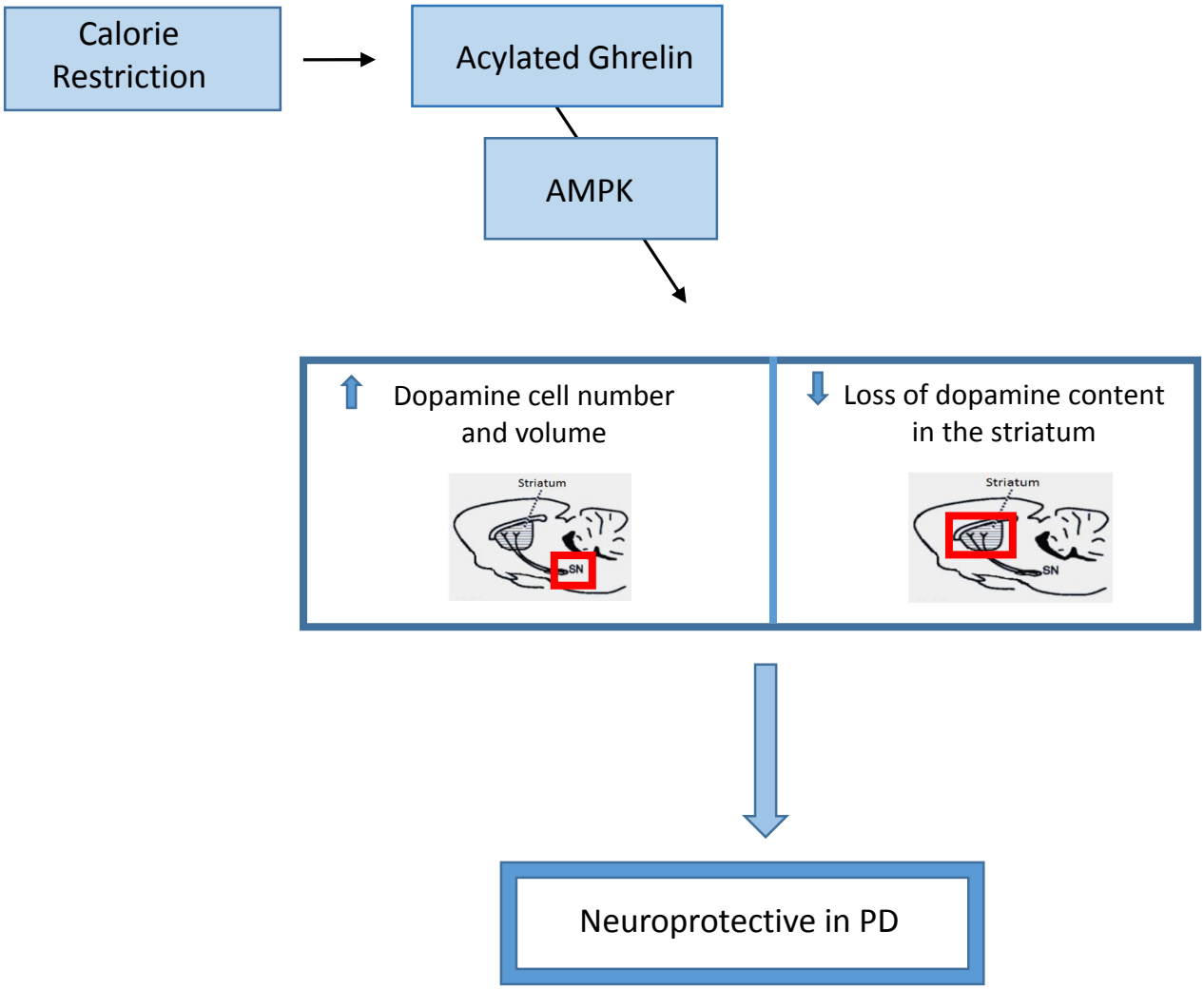


Figure 3.8: Summary for Chapter3. Calorie restriction results in acylated ghrelin mediated AMPK activation, ultimately leading to neuroprotection in PD

Declaration for Thesis Chapter 4

Declaration by candidate

In the case of Chapter 4, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Myself and ZBA designed the experiments. I performed all laboratory testing and analysis (with the assistance of M.L., M.D. and V.S.), with the exception of the HPLC analysis. In collaboration with my supervisor (A/Prof. Zane Andrews) we wrote the entire manuscript.	90

The following co-authors contributed to the work. If co-authors are students at Monash University, the extent of their contribution in percentage terms must be stated:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Moyra Lemus	Assisted with Laboratory experiments	
Minh Deo	Assisted with Laboratory experiments	
Vanessa V Santos	Assisted with Laboratory experiments	
John Elsworth	Performed HPLC experiments	
Zane Andrews	Assisted with designing of experiments and writing of the manuscript	

The undersigned hereby certify that the above declaration correctly reflects the nature and extent of the candidate's and co-authors' contributions to this work.

Candidate's Signature	[redacted]	**Date: 25.01.16**
Main Supervisor's Signature	[redacted]	**Date: 25.01.16**

Chapter 4 - Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons.

Metformin prevents nigrostriatal dopamine degeneration independent of AMPK activation in dopamine neurons.

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Key words: Dopamine, Metformin, AMPK, Parkinson's Disease

Abbreviations

DA = Dopamine, GFAP = Glial Fibrillary Acidic Protein, GLP1 = Glucagon Like Peptide 1, IBA1 = Ionized Calcium Binding Adaptor Molecule 1, Met = Metformin, MPTP = 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, NEFA = Non-esterified Fatty Acid, PD = Parkinson's Disease, PFA = Paraformaldehyde, SN = Substantia Nigra, STR = Striatum, TH = Tyrosine Hydroxylase

Abstract

Metformin is a widely prescribed drug used to treat type-2 diabetes, although recent studies show it has wide ranging effects to treat other diseases. Animal and retrospective human studies indicate that Metformin treatment is neuroprotective in Parkinson's Disease (PD), although the neuroprotective mechanism is unknown, numerous studies suggest the beneficial effects on glucose homeostasis may be through AMPK activation. In this study we tested whether or not AMPK activation in dopamine neurons was required for the neuroprotective effects of metformin in PD. We generated transgenic mice in which AMPK activity in dopamine neurons was ablated by removing AMPK beta 1 and beta 2 subunits from dopamine transporter expressing neurons. These AMPK WT and KO mice were then chronically exposed to Metformin in the drinking water and exposed to MPTP, a mouse model of PD. Chronic Metformin treatment significantly attenuated the MPTP-induced loss of Tyrosine Hydroxylase (TH) neuronal number and volume and TH protein concentration in the nigrostriatal pathway. Additionally, Metformin treatment prevented the MPTP-induced elevation of the DOPAC:DA ratio regardless of genotype. Metformin also prevented MPTP induced gliosis in the Substantia Nigra. These neuroprotective actions were independent of genotype and occurred in both AMPK WT and AMPK KO mice. Overall, our studies suggest that Metformin's neuroprotective effects are not due to AMPK activation in dopaminergic neurons and that more research is required to determine how metformin acts to restrict the development of PD.

Introduction

Parkinson's Disease (PD) is the second most common neurodegenerative disease affecting an estimated 4.1 to 4.6 million people worldwide in 2005, a number projected to double by the year 2030 (Dorsey et al., 2007). Symptoms including tremor, postural instability and bradykinesia are due to a reduction of dopamine in the dopaminergic nigrostriatal pathway in the brain. The dopamine cell bodies are located in the substantia nigra (SN) and project to the striatum. PD is considered idiopathic however many risk factors occur to increase incidence rates such as genetic factors, pesticide exposure and recently several studies have shown a greater incidence in patients who have diabetes (Sandyk, 1993; Hu et al., 2007; Driver et al., 2008). Intriguingly, there is a correlation between the incidence of diabetes preceding PD development in individuals (D'Amelio et al., 2009), indicating that glucose intolerance may be a precipitating factor in the development of PD. Indeed, this is true in other neurological diseases where individuals with Type 2 Diabetes (T2D) are at risk of developing mild cognitive impairment, dementia or Alzheimer's Disease (Peila et al., 2002; Pasquier et al., 2006). If glucose intolerance is an early event or precipitating factor in neurological conditions, then current therapeutic approaches to treat diabetes may offer insights into the pathogenesis of neurological disease, such as PD. In support of this concept, retrospective epidemiological study showed that Metformin-inclusive sulfonylurea therapy reduced the risk of PD occurrence in patients with T2D in a Taiwanese population (Wahlqvist et al., 2012).

Metformin is a buigiunide analogue commonly used for the treatment of T2D and is generally well tolerated. By lowering blood glucose, IGF-1 and insulin signalling, Metformin creates an environment that is similar to calorie restriction (CR) and as such many beneficial effects of CR can be reproduced by chronic Metformin treatment. Metformin has been shown to extend median survival by 40% in C. elegans, whilst also prolonging youthful locomotion in a dose-dependent manner (Onken and Driscoll, 2010). In mice Metformin produced approximately a 6% lifespan extension, which was also accompanied by improved locomotor performance (Martin-Montalvo et al., 2013). Indeed, in a human study patients with T2D with Metformin monotherapy had a longer survival than matched non-diabetic controls (Bannister et al., 2014). These studies collectively imply not only enhanced lifespan but also healthspan with Metformin treatment. Metformin treatment also reduces the incidence of

many age related diseases by reducing cancer incidence (Evans et al., 2005), stroke risk (Cheng et al., 2014), enhancing neurogenesis (Wang et al., 2012) as well as the traditional lowering of blood glucose. As CR is beneficial for PD (Duan and Mattson, 1999) and T2D (Pi-Sunyer et al., 2007), Metformin has the potential to treat both disease states.

Previous studies show that Metformin is neuroprotective in PD. In vitro, treatment with Metformin reduced the neurotoxicity associated with alpha synuclein overexpression (Dulovic et al., 2014). In a Drosophila model of PD, Metformin treatment alleviated dopaminergic dysfunction and mitochondrial abnormalities (Ng et al., 2012). Metformin chronically administered to mice reduces oxidative stress, dopaminergic degeneration and motor abnormalities associated with MPTP (a mouse model for PD) administration (Patil et al., 2014). Hence, Metformin treatment has a protective effect in PD animal models. As Metformin has been deemed safe with minimal side effects and is known to rapidly cross the blood brain barrier and disperse into various brain regions (Labuzek et al., 2010), it is an ideal therapeutic for the treatment of PD. Recently, a mechanism of action for how Metformin suppresses gluconeogenesis has been discovered (Madiraju et al., 2014), however, how Metformin is neuroprotective is still unknown. For example, after Metformin treatment the transcription factor SKN/Nrf2 is activated, ultimately increasing the expression of anti-oxidant genes to protect against oxidative damage (Onken and Driscoll, 2010). Metformin has also been shown to inhibit mTOR to enhance mitochondrial function (Dowling et al., 2007; Johnson et al., 2013). Metformin can also activate AMPK by inhibiting complex I of the ETC (Zhou et al., 2001). This results in an increased AMP/ATP ratio and the subsequent activation of AMPK. AMPK acts to increase mitochondrial biogenesis (Canto et al., 2009) and as patients with PD have impaired mitochondrial function, suggesting that AMPK activation in dopamine neurons may be responsible for Metformin's protective actions.

Peripheral activation of AMPK has been shown to be protective in PD. In cells overexpressing alpha synuclein AMPK becomes activated to restrict cell death (Dulovic et al., 2014). In a Drosophila model of PD, loss of AMPK activity exacerbated neuronal loss and its associated phenotypes (Ng et al., 2012). Resveratrol, an AMPK activator is neuroprotective in a rodent model of PD (Jin et al., 2008). In response to MPTP AMPK is phosphorylated and inhibition of AMPK by Compound C enhanced MPTP-induced cell death

(Choi et al., 2010). However, it is unknown whether AMPK activation in dopaminergic neurons is causative or correlative in the pathogenesis of PD. Many studies have highlighted the beneficial effects of peripheral AMPK activators in PD (Jin et al., 2008; Horvath et al., 2011; Bayliss and Andrews, 2013) however, these neuroprotective actions could be due to indirect actions and may not involve AMPK directly in dopamine neurons or could be due to an overall enhanced peripheral profile.

As both Metformin treatment and AMPK activation protect against PD, we hypothesised that Metformin would activate AMPK in dopaminergic neurons to prevent degeneration. We aimed to determine if Metformin's neuroprotective effects can be attributed to the activation of AMPK in the SN dopamine neurons.

Methods

Animals

All experiments were conducted in accordance with Monash University Animal Ethics Committee guidelines. Mice were maintained under standard laboratory conditions with free access to food and water. Temperature was maintained at 23°C with a 12 hour light/dark cycle. Mice were 8-10 weeks old and group housed.

To generate mice with a selective deletion for AMPKβ1 & β2 only in DAT-expressing neurons, we crossed Dat-Cre knock-in mice from Jax Lab Lab [Stock number 006660; B6.SJL-Slc6a3<tm1.1(cre)bkmn>/j] with AMPK beta 1 (β1) and beta 2 (β2) floxed mice (O'Neill et al., 2011). The resultant offspring (*Dat*-Cre;*Ampk beta* $1^{fl/fl}$;*Ampk beta* $2^{fl/fl}$ designated AMPK KO or *Ampk beta* $1^{fl/fl}$;*Ampk beta* $2^{fl/fl}$ designated AMPK WT) were used as experimental mice. Experimental validation of mice was performed previously (Bayliss et al., 2016b).

Experimental Design

Mice were randomly allocated to be treated with water or water containing Metformin (100mg/kg/day) for the duration of the experiment (27 days). Metformin was given in water to maintain a constant levels of Metformin in the bloodstream and sustained activation of AMPK as opposed to a single dose which is cleared rapidly from the system. Previous literature in stroke models using 100mg/kg/day of Metformin showed increased AMPK activation in the brain with this dose (Li et al., 2010). On days 20 and 21 mice received an injection of either saline or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) (30mg/kg dissolved in saline). Mice were culled on day 27 and either perfused for immunohistochemical analysis or fresh tissue collection for western blot and HPLC analysis.

Immunohistochemistry

All mice were deeply anaesthetized then perfused with 0.05M PBS followed by 4% Paraformaldehyde (PFA) to fix the tissue. Brains were stored in PFA overnight then transferred to 30% sucrose solution. Every fifth coronal section (30µm) was collected and

stored in cryoprotectant (30% Ethylene Glycol, 20% Glycerol in 0.1M PB) at -20°C. The tissue was stained with either anti-TH & anti-IBA1 or anti-TH & anti-GFAP. The sections were washed thoroughly with 0.1M PB and endogenous peroxidase activity was blocked with 1% H_2O_2 in 0.1M PB for 15 minutes and washed again. The tissue was blocked with 4% normal horse serum and 0.3% Triton X-100 in 0.1M PB for one hour and then blocked again with AffiniPure Goat Anti-Mouse IgG (H+L) (Jackson ImmunoResearch, 1:200) to prevent non-specific binding due to mouse tissue being stained with mouse antibodies. The tissue was then incubated with the primary antibody, in this case anti-TH (mouse, 1:5000, Milipore) and anti-IBA1 (rabbit, 1:1000, Wako) or anti-GFAP (rabbit, 1:1000, DAKO) for 24 hours at 4°C. After the incubation the sections were thoroughly washed and incubated with the fluorescent secondary antibodies Goat anti-mouse IgG (H+L) AlexaFluor 488 (1:400, Invitrogen) and Goat anti-rabbit IgG (H+L) AlexaFluor 594 (1:400, Invitrogen) for 90 minutes at room temperature. The tissue was subsequently washed, mounted onto slides and cover-slipped using anti-fade mounting media.

Stereological analysis of cell number and volume

We used design-based stereology to quantify the number of microglia (IBA1 stain), astrocytes (GFAP stain) and the number and volume of dopamine neurons (TH stain) in the Substantia Nigra. Using the StereoInvestigator software (MicroBrightField, Williston, VT, USA) we analysed cell number (optical fractionator probe) and cell volume (nucleator probe). To visualise the cells we used a Zeiss microscope with a motorised stage and a MicroFibre digital camera connected to the computer.

Analysis of blood chemistry

Trunk blood from deeply anesthetised decapitated mice was collected into EDTA tubes pre-treated with pefabloc (SC Roche Applied Science, Mannheim, Germany) to achieve a concentration of 1mg/mL. The blood was centrifuged and the plasma was acidified with HCl (final concentration 0.05N). Plasma metabolites were measured following kit instructions. Active Ghrelin or Des-Acyl Ghrelin Enzyme-Linked Immunoassay Kits (Mitsubishi Chemical Medicine, Tokyo, Japan), NEFA (Wako Life Sciences; CA, USA), Triglycerides (Roche/Hitachi; Indianopolis, USA), Corticosterone (Abnova; CA, USA) and Blood glucose

(Sigma; Missouri, USA). Plasma insulin concentration was determine through an in-house ELISA assay.

Western Blot

Fresh tissue was collected of the SN and Striatum and snap frozen in liquid nitrogen. Tissue was then sonicated in RIPA buffer (50mM Tris, 150mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100) containing a protease inhibitor (Sigma), then centrifuged (10,000 rpm, 10 minutes, 4°C) to remove cell debris and the supernatant was collected. The protein concentration was measured using a BCA kit (Pierce, Rockford, IL, USA) according to kit instructions. The samples concentrations were then standardised and the supernatants were mixed with Laemmli's buffer and boiled for 5 minutes. Samples were loaded onto 10% acrylamide gels and separated by SDS polyacrylamide gel electrophoresis, the separated proteins were then transferred from the gel to a PVDF membrane (Biorad). The blots were bocked for 1 hour in Tris-Buffered Saline Solution containing 0.1% Tween-20 (TBST) and 5% Bovine Serum Albumin (BSA). The membrane were incubated overnight at 4°C in TBST with 5% BSA containing one of the following antibodies: anti-TH (1:1000, Milipore), anti-beta actin (1:1000, Abcam), anti-AMPKα (1:1000, Cell signalling) or anti-pAMPK (1:1000, Cell Signaling). Blots were visualised using chemiluminescence (ECL, Amersham) and levels of proteins were detected using ImageLab Software, version 4.1, Biorad.

High Performance Liquid Chromatography (HPLC)

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

Oral Glucose Tolerance Test (oGTT) and Insulin Tolerance Test (ITT)

Mice were fasted for 4 hours prior to first blood measurement. We used short term fasting as this better studies insulin action in the physiological context (Ayala et al., 2010). Blood glucose was measured with ACCU-CHEK Active (Roche DiagnosticsGnH, Tokyo, Japan) and then a bolus of 200µl of 25% D-glucose solution was given and measurements were taken at time points 15, 30, 60 and 90 minutes after bolus. The mice were allowed to recover for 1 week until the ITT was performed. For the ITT the same protocol was followed except the mice were injected with 5µl of insulin in 5mL saline (injected 200µl).

Statistical Analysis

All data are represented as Mean ± Standard Error of the Mean (SEM). Two-Way ANOVA with a Bonferroni post hoc test was used to determine statistical significance between treatments. $P<0.05$ was considered statistically significant.

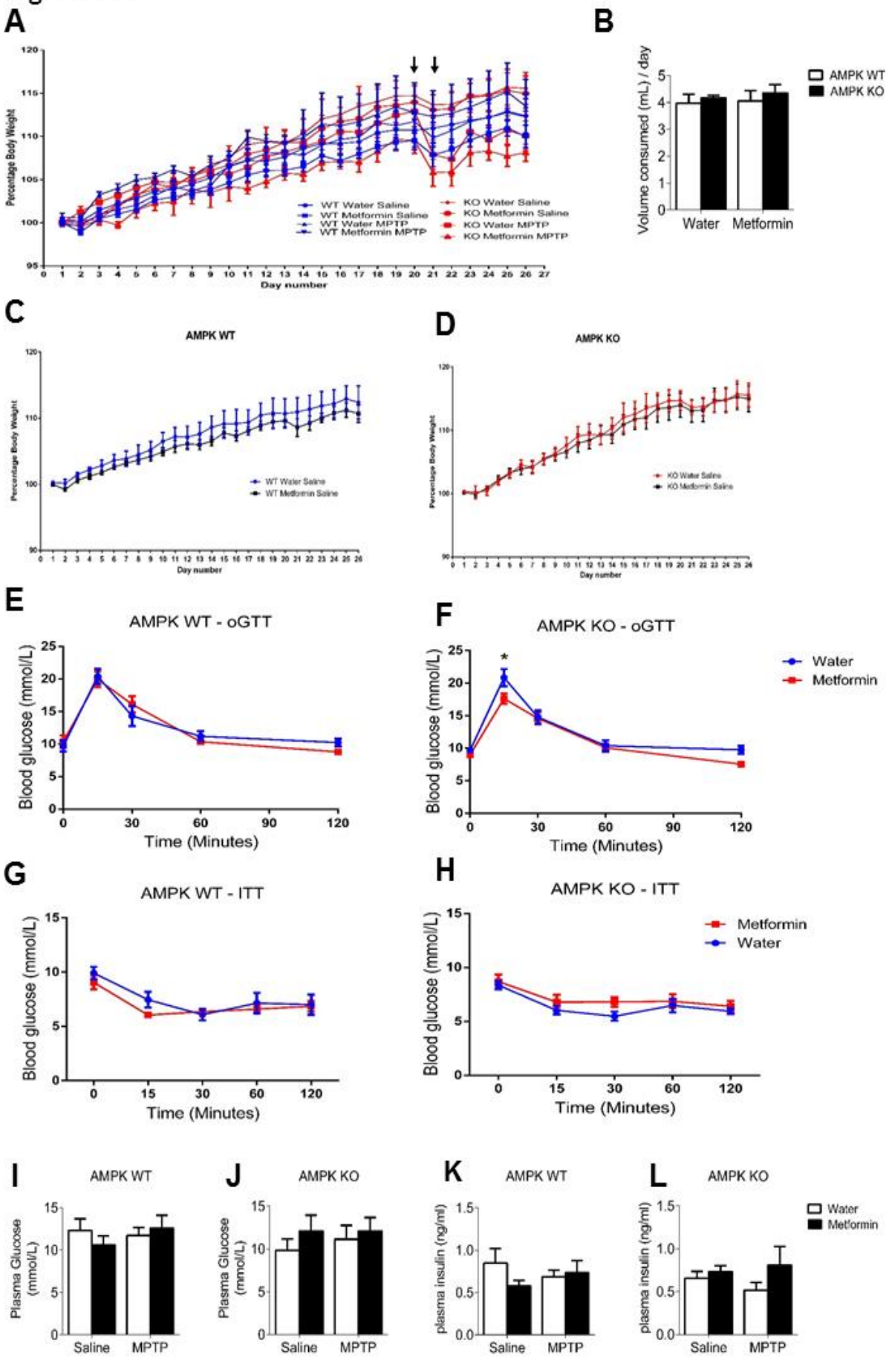
Results

Physiological characterisation of AMPK KO mice

DAT AMPK WT and KO were exposed to either normal drinking water or water with Metformin and then injected with MPTP to induce degeneration in Substantia Nigra (SN) dopamine neurons, as a mouse model for PD. There were no genotype differences in body weight (Figure 4.1A, C & D), blood glucose (Figure 4.1I & J) or insulin levels (Figure 4.1K & L). Because Metformin is known to promote insulin sensitivity (McIntyre et al., 1991) we determined if Metformin in the drinking water affected clearance of glucose during an oral Glucose Tolerance Test (oGTT) and Insulin Tolerance Test (ITT). In AMPK WT mice there was no difference between mice chronically exposed to Metformin compared to tap water alone in both oGTT (Figure 4.1E) and the ITT (Figure 4.1G). However, in AMPK KO mice Metformin significantly increased glucose clearance (Figure 4.1F) at the 15-minute time-point, with no difference in the ITT (Figure 4.1H). This indicates that AMPK activation in dopamine neurons promotes peripheral glucose clearance during an oGTT

Figure 4.1. Body Weight and blood glucose measurements in AMPK WT and KO mice. **A – D**, throughout the experiment there was no difference in body weight or volume of water consumed comparing genotype or treatment. Arrows indicate MPTP injections. During an oGTT Metformin treatment does not alter glucose clearance in AMPK WT mice (**E**), but AMPK KO mice (**F**) clear a bolus of glucose faster when treated with Metformin. Insulin sensitivity is not altered during an ITT in AMPK WT (**G**) or KO (**H**) mice treated with Metformin. **I – L**, Plasma analysis of insulin and glucose levels in trunk blood show no differences between genotype and treatment. * = $p<0.05$. Data are represented as mean ± SEM (n=8-10, two-way ANOVA, $p<0.05$).

Figure 4.1


A
Percentage Body Weight
Day number
WT Water Saline
WT Metformin Saline
WT Water MPTP
WT Metformin MPTP
KO Water Saline
KO Metformin Saline
KO Water MPTP
KO Metformin MPTP
B
Volume consumed (mL) / day
Water
Metformin
AMPK WT
AMPK KO
C
AMPK WT
D
AMPK KO
E
AMPK WT - oGTT
Blood glucose (mmol/L)
Time (Minutes)
F
AMPK KO - oGTT
Water
Metformin
G
AMPK WT - ITT
H
AMPK KO - ITT
Metformin
Water
I
AMPK WT
Plasma Glucose (mmol/L)
Saline
MPTP
J
AMPK KO
K
AMPK WT
plasma insulin (ng/ml)
L
AMPK KO
Water
Metformin

Plasma analysis showed a significant main effect of MPTP to increase Triglycerides (Figure 4.2A & B), Non-Esterified Fatty Acids (NEFA) (Figure 4.2C & D) and corticosterone (Figure 4.2E & F) levels, which occurred with a concurrent reduction in body weight (Figure 4.1A) indicating the stress placed on these mice. To confirm the inability of AMPK to become phosphorylated in the AMPK KO mice we performed protein analysis in the SN and Striatum. In both the SN and striatum AMPK WT mice had an elevated pAMPK/AMPK ratio after MPTP exposure (Figure 4.2I & K). AMPK KO mice exhibited no significant change in the pAMPK/AMPK ratio in response to MPTP in either the SN (Figure 4.2J) or Striatum (Figure 4.2L) implying an inability to be phosphorylated in response to a toxic insult as a protective mechanism, as observed previously (Bayliss et al., 2016b).

Metformin prevents MPTP-induced nigrostriatal damage independent of genotype

MPTP significantly reduced Tyrosine Hydroxylase (TH, a dopamine marker) protein expression, as measured by western blot in both the SN and Striatum in both AMPK WT and KO mice (Figure 4.2M-R) an effect that was attenuated by metformin in both AMPK WT and KO mice in the Striatum (Figure 4.2Q & R) but not in the SN (Figure 4.2O & P). In support of this data, HPLC analysis of dopamine (Figure 2S & V) and DOPAC (Figure 4.2T & W) in the striatum revealed a significant overall reduction with MPTP administration in both AMPK WT and KO mice with no effect of Metformin treatment. However, Metformin treatment prevented the increase in the DOPAC:DA ratio observed after MPTP in both AMPK WT and KO mice (Figure 4.2U & X). These results indicate that Metformin has site-specific protective effects in the Striatum.

Figure 4.2

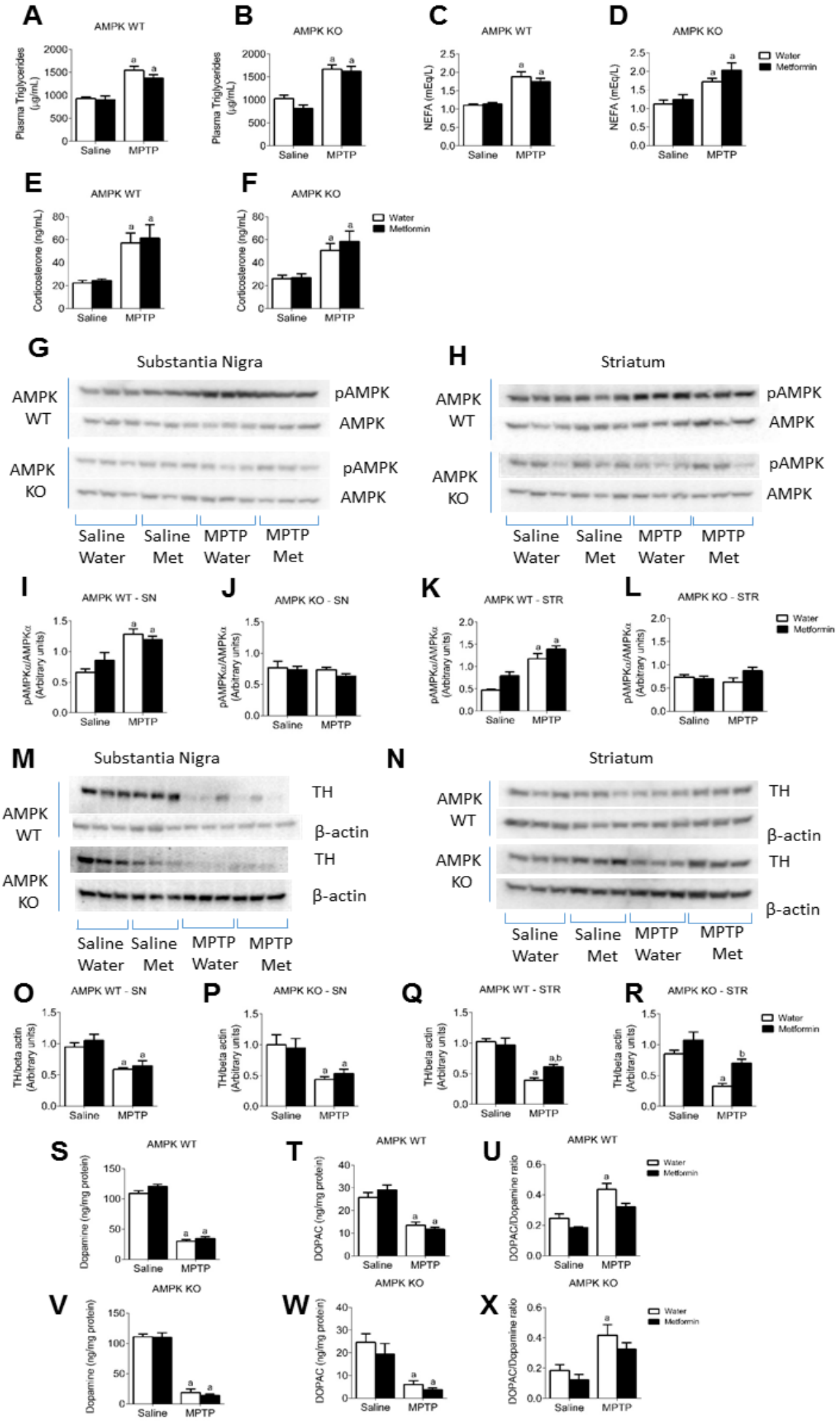

A
AMPK WT
Plasma Triglycerides (μg/mL)
Saline
MPTP
B
AMPK KO
C
AMPK WT
NEFA (mEq/L)
D
AMPK KO
Water
Metformin
E
AMPK WT
Corticosterone (ng/mL)
F
AMPK KO
G
Substantia Nigra
AMPK WT
AMPK KO
pAMPK
AMPK
Saline Water
Saline Met
MPTP Water
MPTP Met
H
Striatum
I
AMPK WT - SN
pAMPKα/AMPKα (Arbitrary units)
J
AMPK KO - SN
K
AMPK WT - STR
L
AMPK KO - STR
M
Substantia Nigra
TH
β-actin
N
Striatum
O
AMPK WT - SN
TH/beta actin (Arbitrary units)
P
AMPK KO - SN
Q
AMPK WT - STR
R
AMPK KO - STR
S
AMPK WT
Dopamine (ng/mg protein)
T
AMPK WT
DOPAC (ng/mg protein)
U
AMPK WT
DOPAC/Dopamine ratio
V
AMPK KO
Dopamine (ng/mg protein)
W
AMPK KO
DOPAC (ng/mg protein)
X
AMPK KO
DOPAC/Dopamine ratio

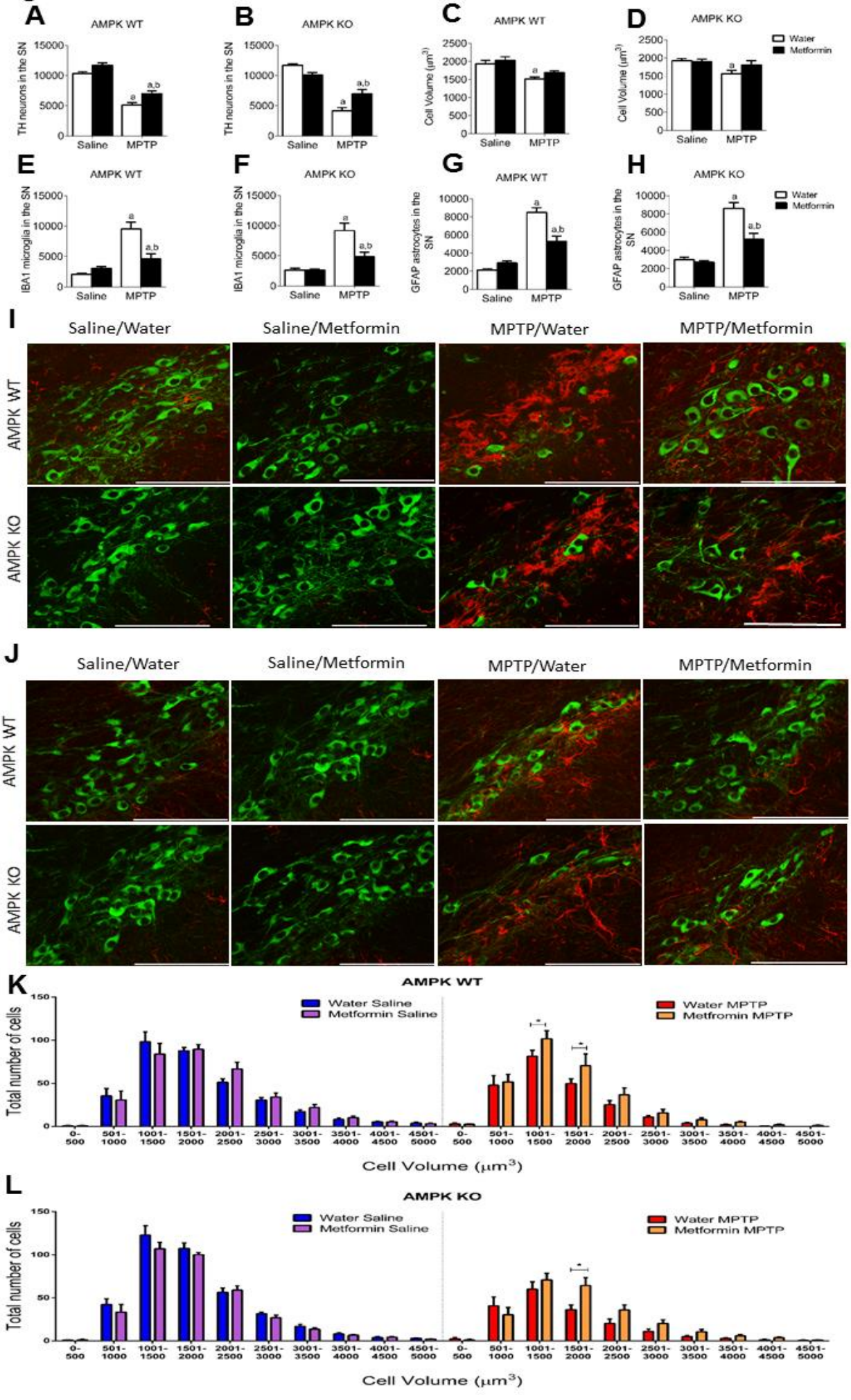
Figure 4.2. Metformin is neuroprotective in AMPK WT and KO mice. Plasma Triglycerides (**A & B**), NEFA (**C & D**) and corticosterone (**E & F**) are elevated in response to MPTP. Representative Western Blot images of pAMPK/AMPK in the SN (**G**) and Striatum (**H**). Protein analysis of the pAMPK/AMPK ratio showing no elevation in response to MPTP in the SN (**J**) and Striatum (**L**) in AMPK KO but an elevation in both the SN and Striatum of AMPK WT mice (**I & K**). Representative images of TH levels in the SN (**M**) and Striatum (**N**). In the SN there is a significant reduction in TH levels in both AMPK WT (**O**) and KO (**P**) in response to MPTP. In the Striatum Metformin elicits a neuroprotective effect in MPTP treated AMPK WT (**Q**) and KO (**R**) mice. MPTP reduced dopamine and DOPAC in both AMPK WT (**S & T**) and AMPK KO (**V & W**) mice. Metformin reduced the elevation of the DOPAC:DA ratio in MPTP treated mice compared to water alone, in AMPK WT and KO mice (**U & X**) a, significant compared to water/saline treated mice and b, significant compared to water/MPTP treated mice. Data are represented as mean ± SEM (n=7-9, two-way ANOVA, $p<0.05$).

MPTP administration significantly reduced the number and volume of TH positive SN neurons in both AMPK WT and KO mice. Metformin attenuated the loss of neurons in AMPK WT (Figure 3A) and KO mice (Figure 4.3B) and also prevented a reduction in cell volume in both genotypes (Figure 4.3C&D), with the majority of the beneficial effect occurring in smaller cells between 1000-2000µm^3 (Figure 4.3E&F). This protective effect was also accompanied by reduced gliosis. MPTP administration significantly elevated microglia (IBA1+ cells) and astrocytes (GFAP+ cells) in the SN, which indicates greater cellular damage in that region. MPTP significantly elevated IBA1 (Figure 4.3G-I) and GFAP (Figure 4.3J-L) in both AMPK WT and KO mice. Metformin treatment blunted the gliosis response to MPTP in both AMPK WT and KO mice, indicating a protective effect of Metformin regardless of genotype (Figure 4.1H-K).

Together, the TH cell number, cell volume and protein expression in the Striatum data indicate that Metformin elicits a protective effect in an MPTP mouse model of PD independent of AMPK action in the dopaminergic neurons.

Figure 4.3. Metformin preserves cell number and volume after MPTP exposure, independent of genotype. Stereological quantification of TH levels in the SN shows a protective effect of Metformin after MPTP exposure in both AMPK WT (**A**) and KO (**B**) mice. Overall cell volume shows a significant reduction after MPTP exposure in water treatment but not with Metformin treatment in AMPK WT (**C**) and KO (**D**). When TH cells were separated and plotted based on volume distribution, mice treated with MPTP and Metformin had a significant effect on smaller volume (1000-2000µm^3) cells compared to those not treated with Metformin in both AMPK WT (**E**) and KO (**F**) mice. **G**, Representative image showing MPTP induced microglial activation in the SN (green = TH, red = IBA1). Stereological quantification of IBA1 (**H & I**) and GFAP (**J & K**) showing increased numbers after MPTP with a significant protective effect of Metformin in both AMPK WT and KO. **L**, Representative images showing MPTP induced astrocytic activation in the SN (green = TH and red = GFAP). a, significant compared to Water/saline treated mice and b, significant compared to Water/MPTP treated mice. * = $p<0.05$. Data are represented as mean ± SEM (n=8-10, two-way ANOVA, $p<0.05$).

Figure 4.3


A
AMPK WT
TH neurons in the SN
Saline
MPTP
B
AMPK KO
C
AMPK WT
Cell Volume (µm³)
D
AMPK KO
Water
Metformin
E
AMPK WT
IBA1 microglia in the SN
F
AMPK KO
G
AMPK WT
GFAP astrocytes in the SN
H
AMPK KO
I
Saline/Water
Saline/Metformin
MPTP/Water
MPTP/Metformin
AMPK WT
AMPK KO
J
K
AMPK WT
Water Saline
Metformin Saline
Water MPTP
Metfromin MPTP
Total number of cells
Cell Volume (µm³)
L
AMPK KO

Discussion

Metformin has been a major therapeutic option for the treatment of T2D since 1958 in the UK and 1995 in the USA. It is the most commonly prescribed drug in the treatment of T2D in clinical use. Recently, the use of Metformin to treat diseases other than T2D has increased. Metformin prevents the development of renal (Pilmore, 2010), hepatic (Mazza et al., 2012), cardiac (Bhamra et al., 2008; El Messaoudi et al., 2011) and neurological conditions (Li et al., 2012). Indeed, Alzheimers Disease (AD) is closely associated with impaired insulin signalling and glucose metabolism and is often referred to as "type 3 diabetes" (de la Monte and Wands, 2008). There is a correlation between insulin resistance and an elevated risk for AD development (Matsuzaki et al., 2010) and studies conducted in obese leptin-resistant mice showed that Metformin attenuated AD-like neuropathology and biological markers (Li et al., 2012). These studies collectively imply that Metformin has the ability to negate any elevated risk for AD development in the susceptible patients who already have T2D. However, it should be noted in a population based study following >7000 people taking Metformin (or other antidiabetic drugs) there was no elevated risk for developing AD (Imfeld et al., 2012).

Metformin has recently been shown to have neuroprotective effects in PD patients. A recent epidemiological study illustrated that the incident Parkinson's risk in patients with pre-existing diabetes treated with sulfonylureas increased by 57%, which was prevented when co-medicated with Metformin (Wahlqvist et al., 2012). We show that Metformin treatment is neuroprotective by attenuating dopamine number and volume loss, reducing gliosis, restricting TH protein loss and enhancing dopamine turnover in the striatum. This study highlights the neuroprotective potential of Metformin to reduce the risk for developing PD and although the evidence for neuroprotection is clear in animal and cell-based models (El-Mir et al., 2008; Adeyemi et al., 2013; Patil et al., 2014), the mechanism underlying this effect is not.

One of Metformin's mechanisms of action in terms of insulin sensitivity is to promote AMPK activation as shown in cells (Zhou et al., 2001) and tissues such as the liver (Zhou et al., 2001) and muscle (Musi et al., 2002). In our studies we clearly demonstrated that metformin

reduces degeneration of the nigrostriatal system in a mouse model of PD, however these protective effects are not dependent on AMPK activation in dopamine neurons. We recently used this novel mouse line to show that ghrelin increases AMPK in dopamine neurons, which is responsible for the neuroprotective actions of calorie restriction in PD (Bayliss et al., 2016b). These results and the impaired striatal AMPK phosphorylation in AMPK KO mice in this study highlights the validity of using this mouse model to explore whether Metformin requires AMPK activation to prevent degeneration in a mouse model of PD.

AMPK activation attenuates dopaminergic dysfunction in a drosophila model of PD (Ng et al., 2012). Other activators of AMPK including Resveratrol (Jin et al., 2008) and ghrelin (Bayliss et al., 2016a; Bayliss et al., 2016b) are neuroprotective in vivo. Overexpression of alpha synuclein in cells (as a model of PD) activates AMPK in order to restrict cell death (Dulovic et al., 2014). Although AMPK activation is neuroprotective in PD and metformin induces direct protective effects through AMPK in other disease states such stroke (Jiang et al., 2014), our studies show that metformin doesn't activate AMPK in dopamine neurons to prevent degeneration in a mouse model of PD.

There are many other potential mechanisms through which Metformin can act. For example Metformin inhibits apoptosis in neuronal cortical cells (El-Mir et al., 2008), prevents oxidative stress-related cellular death (Chakraborty et al., 2011) and plays an inhibitory role on inflammatory transcription factor NF-kB (Hirsch et al., 2013). In mice exposed to Metformin there was reduced superoxide leakage in the mitochondria, indicating greater efficiency of mitochondrial complexes (Martin-Montalvo et al., 2013). As complex I activity is diminished in PD patients (Schapira et al., 1990) this enhanced mitochondrial efficiency coupled with reduced oxidative stress could be responsible for the neuroprotective actions of Metformin. Metformin also activates Sirtuins (SIRTs) and PGC-1α. SIRTs are responsible for a variety of cellular processes including enhancing mitochondrial function, cellular metabolism, gluconeogenesis as well as aging (Finkel et al., 2009). There are increased levels and activity of SIRTs in the livers of Metformin treated mice (Caton et al., 2010). SIRT activation improves mitochondrial function and extends lifespan (Mitchell et al., 2014). Indeed, lifespan is increased in mice overexpressing SIRT1 (Bordone et al., 2007) and decreased in SIRT1 KO (Boily et al., 2008). Another potential target that Metformin could

act through is PGC-1α, a transcriptional regulator involved in mitochondrial biogenesis. Metformin has been shown to increase PGC-1α protein expression in the liver (Aatsinki et al., 2014) and skeletal muscle (Suwa et al., 2006). PGC-1α deficient mice are more susceptible to MPTP-induced dopaminergic neuronal loss (St-Pierre et al., 2006). In mice, overexpression of PARIS, which represses the expression of PGC-1α, results in selective degeneration of substantia nigra dopaminergic neurons (Shin et al., 2011). In humans polymorphisms of PGC-1α are associated with early onset PD (Clark et al., 2011). Collectively, these studies show that Metformin's neuroprotective actions could be due to many other agents that enhance mitochondrial function independent of AMPK activation.
Metformin can increase circulating levels of the gut hormone GLP-1 to help control blood glucose levels (Mulherin et al., 2011). GLP1 also has receptors on SN dopaminergic neurons and prevents neurodegeneration in a mouse model of PD (Bertilsson et al., 2008; Li et al., 2009). This elevation could be responsible for the protective actions of Metformin and raises the possibility that the neuroprotective actions of metformin are secondary to changes in peripheral metabolism.

Many studies have shown the protective actions of Metformin in different disease states however, the neuroprotective mechanism of metformin in PD remains unknown. It potentially exhibits disease specific actions in different tissues. Although it is possible that Metformin is neuroprotective in other disease states such as stroke and Alzheimer's Disease via the actions of AMPK, we show that in a mouse model of PD Metformin's neuroprotective actions are independent to AMPK activation in dopaminergic neurons. Further research is required to determine the exact neuroprotective mechanism of action of Metformin however, some potential options involve indirect effects on metabolism including elevated GLP-1 secretion or direct effects of SIRT1 and PGC-1alpha in SN dopamine neurons.

Contributions

J.A.B and Z.B.A designed experiments

J.A.B, V.V.S, M.D., J. E. and M.B.L, performed experiments.

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

Acknowledgements

This work was supported by grants and fellowships from the Australian National Health and Medical Research Council to Z.B.A (546131, 1084344) and a Monash University Fellowship to Z.B.A.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Summary for Chapter 4

In Chapter 4 we show that Metformin is neuroprotective in a mouse model of PD. Metformin attenuated dopamine cell loss, gliosis and enhanced dopamine turnover. These neuroprotective effects were independent of AMPK activity in dopaminergic neurons.

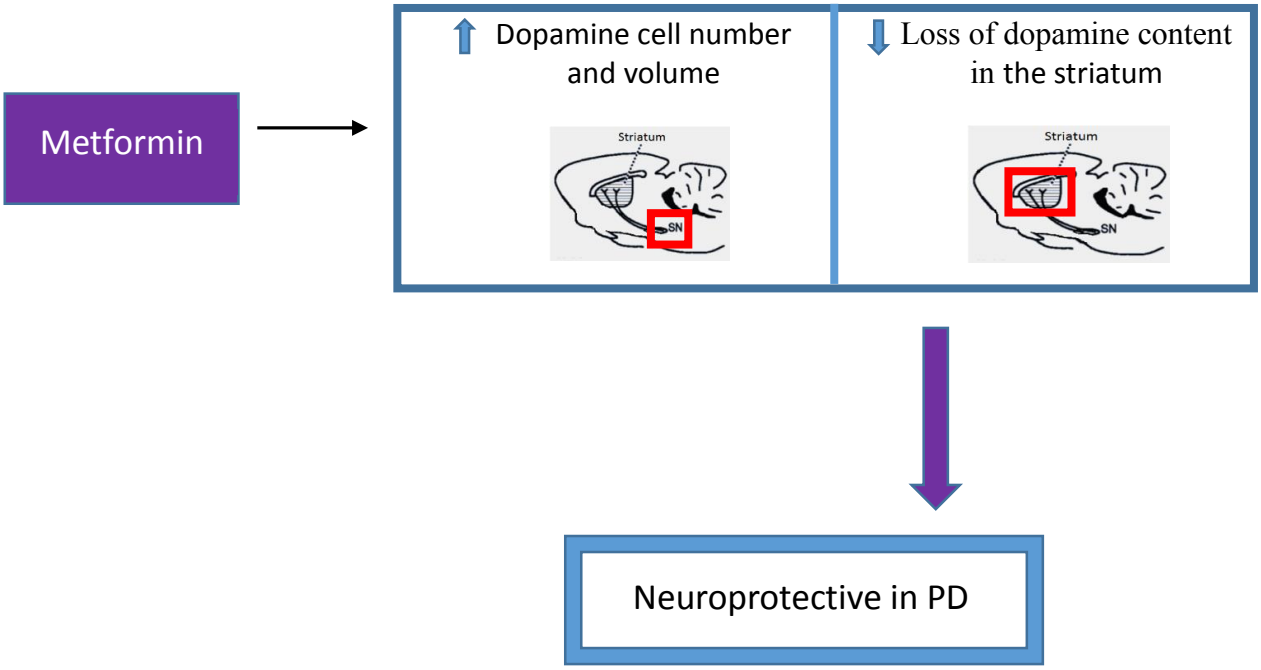


Figure 4.4: Summary for Chapter 4. Metformin, independent of AMPK activity in dopaminergic neurons, is neuroprotective in PD.

Chapter 5 - Discussion

Calorie restriction (CR) has numerous health benefits, which include preventing age-related neurodegeneration. However, the difficulty to adhere to such a diet makes it an impractical and potentially detrimental therapeutic option. Thus, we need to understand the key molecular mechanisms underlying the protective effects of CR in order to develop viable therapeutic strategies. The "hunger hormone" ghrelin, which is elevated during CR (Figure 3.1), has been extensively researched in terms of its metabolic actions to increase food intake. Recently, numerous non-metabolic actions have been discovered, including cardioprotective, hepatoprotective and neuroprotective effects. Importantly, ghrelin exists in two isoforms, acyl and des-acyl ghrelin. Although studies show the acyl ghrelin is protective in models of Parkinson's Disease (PD) (Andrews et al., 2009; Moon et al., 2009), des-acyl ghrelin's role in PD progression is unknown. Due to the broad-spanning effects of ghrelin we wanted to determine the relative contribution each isoform of ghrelin plays during PD progression. We then wanted to determine the role that ghrelin plays in CR and any downstream targets that can be manipulated to recreate the protective role of CR without the need to reduce caloric intake. This thesis has focused on understanding the neuroprotective mechanisms through which CR prevents degeneration associated with PD.

Chapter 2: Acyl but not des-acyl ghrelin is neuroprotective in Parkinson's Disease

Recently, ghrelin administration has been investigated to treat many diseases including stroke (Liu et al., 2009), epilepsy (Lucchi et al., 2013), encephalomyelitis (Theil et al., 2009) and Alzheimer's disease (Dhurandhar et al., 2013). However, these studies have focused solely on the beneficial effects of acyl ghrelin or did not consider des-acyl ghrelin as a potential protective agent. Hence, some of these reported actions of acyl ghrelin could be due to the actions of des-acyl ghrelin as it is readily deacetylated in the bloodstream (Tong et al., 2013). In our first study we examined the relative contributions of each isoform of ghrelin, since this is an important consideration for potential therapeutic applications of ghrelin treatment in human diseases. In order to do this we chronically injected acyl or des-acyl ghrelin into Ghrelin KO mice, in order to avoid the potential confounding variable of endogenous acyl

and des-acyl ghrelin. Ghrelin KO mice cannot produce acyl or des-acyl ghrelin throughout their entire lifespan and as such we can determine exclusively the effect of each ghrelin peptide. As anticipated there was a protective effect of chronic acyl ghrelin administration in terms of attenuated TH (dopamine) neuronal loss, reduced gliosis and dopamine turnover (as determine by the DOPAC:DA ratio). Acyl ghrelin has been previously described to be neuroprotective in mouse models of PD. Intraperitoneal injection of acyl ghrelin diminishes the dopaminergic cell loss in the SN after MPTP administration (Andrews et al., 2009). Acyl ghrelin administration also prevented the inflammatory response which participates in the pathogenesis of PD in the SN in mice treated with MPTP (Jiang et al., 2008). However, we observed that acyl ghrelin injection also increased the concentration of des-acyl ghrelin due to endogenous deacylation. Hence, the protective effect may not be exclusively attributed to acyl ghrelin. Indeed des-acyl ghrelin has been shown to be protective in other neurological conditions. For example, des-acyl ghrelin administration prevents cell death in cultured neurons exposed to oxygen and glucose deprivation in the presence of a GHSR antagonist (Chung et al., 2008; Hwang et al., 2009). *In vivo*, des-acyl ghrelin administration exhibits a vasodilator response (Ku et al., 2015) and both acyl and des-acyl ghrelin are protective after transient focal ischemia reperfusion (Hwang et al., 2009). In microglia exposed to amyloid beta, des-acyl ghrelin counteracted the activation of the pro-inflammatory cytokine IL-6, whereas acyl ghrelin had no effect (Bulgarelli et al., 2009). Because of these studies we examined if exogenous des-acyl ghrelin was able to prevent nigrostriatal degeneration in a mouse model of PD.

To determine if des-acyl ghrelin is neuroprotective in a mouse model of PD we chronically injected des-acyl ghrelin into Ghrelin KO mice. Analysis of TH cell number and protein expression in the SN and Striatum showed no protective effect of chronic des-acyl ghrelin administration and no conversion to acyl-ghrelin in the plasma. To definitively show that des-acyl ghrelin is not neuroprotective we used a genetic approach using GOAT KO mice. By deleting the enzyme that acylates ghrelin these mice have been exposed to only elevated des-acyl ghrelin throughout their entire lifespan. This approach allows us to analyse the physiological consequences of a deficiency of ghrelin acylation. Indeed, there was no neuroprotective effect between genotypes confirming that elevated des-acyl ghrelin is not neuroprotective in PD. Recently des-acyl ghrelin, but not acyl ghrelin, has elicited vasodilatory actions in the cerebral endothelium in a model of stroke. This effect was

independent of GHSR1a, potentially acting through a novel des-acyl ghrelin receptor in cerebral arteries (Ku et al., 2015). In another study using Ghrelin receptor KO mice, des-acyl ghrelin increased the pilocarpine-evoked seizure threshold, thus acting as an anticonvulsant, in ghrelin receptor WT but not KO mice (Portelli et al., 2015). In a study examining the role of each isoform during myocardial injury, there was a protective therapeutic action with acyl ghrelin that was significantly weaker than des-acyl ghrelin (Li et al., 2006). This raises the possibility that des-acyl ghrelin maybe protective in some diseases/treatments but not in others. This might reflect differences in expression of the unidentified des-acyl ghrelin receptor in different brain regions or peripheral tissues, although this requires experimental proof.

Interestingly, chronic injections of des-acyl ghrelin but not acyl ghrelin, increased corticosterone in the plasma. Corticosterone is often referred to as the stress hormone and has detrimental effects on the nigrostriatal system by increasing oxidative stress products (Kim et al., 2005) as well as reducing striatal DA levels (Rasheed et al., 2010) making the system more susceptible to degeneration. As chronic acyl ghrelin injections alone did not elevate corticosterone levels we predict that the artificially elevated corticosterone by des-acyl ghrelin may contribute to the lack of neuroprotection.

Throughout this thesis rat acyl- and des-acyl ghrelin was used due to the homologous sequence between rat and mouse. We chose this species based on previous research showing neurochemical and protective actions in various brain regions of peripherally administered ghrelin. Numerous studies show that peripheral administration of acyl-ghrelin affects behaviour and physiological function including enhanced food intake (Tschop et al., 2000), learning and memory (Diano et al., 2006) and was neuroprotective in PD (Andrews et al., 2009; Moon et al., 2009). These studies imply either an amplification of the message from the plasma into the brain or a direct pathway to various brain regions thus mitigating the need for acyl-ghrelin to cross the Blood Brain Barrier. Recent studies indicate that acyl-ghrelin passes through fenestrated capillaries in the Arcuate nucleus and adjacent brain regions thus bypassing the need to enter the brain (Cabral et al., 2013; Schaeffer et al., 2013). These studies focused on the role of the Arcuate nucleus to rapidly control appetite and energy status, hence the pathway to circumvent the tightly sealed vasculature of the Blood Brain Barrier is essential for rapid changes. We believe that the more caudal neurons in the SN do

not need to respond rapidly for the neuroprotective actions of acyl-ghrelin and thus not reliant upon the extension of fenestrated capillaries into the SN. This theory is based on the observation that increased vulnerability to ghrelin receptor KO mice was rescued by the re-expression of the receptor on TH-positive cells (Andrews et al., 2009). Indeed, within this thesis we show that the neuroprotective actions of acyl-ghrelin are abolished in AMPK KO mice. These studies indicate that a physical presence of acyl-ghrelin is required to bind to the SN dopaminergic neurons. However, it is also important to note that TH positive cells exist both within and external to the SN, including within the hypothalamus, potentially playing the role of messenger to the SN. Future research is required to fully elucidate the mechanism acyl-ghrelin enters the brain and has access to SN dopaminergic neurons.

Overall, we show that acyl ghrelin is a potential therapeutic target to reduce PD progression by reducing gliosis and minimising neuronal cell loss in the nigrostriatal dopaminergic system. Des-acyl ghrelin elevated corticosterone levels potentially having a detrimental effect to the nigrostriatal system. This study importantly tells us that acyl ghrelin is responsible for the neuroprotective actions in a mouse model of PD. This information can now be used to inform future studies about the therapeutic potential of the acylated isoform of ghrelin. Furthermore, our studies suggest pharmacological approaches that prevent plasma conversion from acyl ghrelin to des-acyl ghrelin, such as Acyl Protein Thioesterase-1 (APT-1) (Satou et al., 2010) and Butyrylcholinesterase (BChE) (De Vriese et al., 2004), may have clinical efficacy to help slow or prevent the debilitating effects of PD. With this information from Chapter 2 we next wanted to determine if acyl ghrelin was responsible for the neuroprotective actions of CR.

Chapter 3: Ghrelin-AMPK signalling mediates the neuroprotective effects of Calorie Restriction in Parkinson's Disease

In Chapter 3 we show that CR increases acyl ghrelin to directly target DA neurons and prevent degeneration in a mouse model of PD. CR protects against a number of pathological conditions including diabetes, cancer, heart disease and neurodegeneration. In models of Parkinson's Disease (PD) an alternate-day feeding schedule, where mice consumed 30-40%

less calories than ad-libitum controls was neuroprotective post MPTP exposure (Duan and Mattson, 1999). Primates with a chronic overall 30% reduction in food intake were also resistant to MPTP induced neurotoxicity (Maswood et al., 2004). These studies show that CR is beneficial in models of PD, however the difficulty to adhere to CR means that an alternative method is required to recapitulate the neuroprotective benefits of CR whilst bypassing dietary constraints. Evidence from cells treated with serum from CR rats suggests a hormonal factor improves mitochondrial function and cell viability (Lopez-Lluch et al., 2006). We hypothesised that acyl ghrelin may be this hormonal factor, because CR increases plasma acyl ghrelin (Cummings et al., 2002)(Figure 3.1a) and acyl ghrelin restricts degeneration in models of PD (Figure 2.1). In Chapter 3 we wanted to show that the previously determined protective actions of acyl ghrelin are responsible for the protective actions of CR. To do this we used ad libitum or CR Ghrelin WT or Ghrelin KO mice exposed to MPTP or saline. We found that in the absence of ghrelin there is no neuroprotective effect of CR. Although Ghrelin KO mice lack both acyl and des-acyl ghrelin our results from Figure 2.1 show that the protective effect of CR is most likely due to acyl ghrelin. This protective effect was seen in the SN with an attenuated loss of dopamine (TH) neurons concomitant with an attenuated gliosis response. Striatal TH loss was attenuated following MPTP administration in CR Ghrelin WT but not KO mice, indicating the protective role of ghrelin. However, despite a protective effect of CR on TH neuronal number and protein expression in the striatum, TH protein levels in the SN were not affected by CR in Ghrelin WT mice, suggesting a site specific neuroprotective action of CR. We propose that despite the neuronal cell loss due to MPTP, under CR conditions these remaining neurons are able to compensate by functioning more efficiently to ultimately maintain adequate levels of DA in the striatum. Indeed, in human PD patients there is up to an 80% reduction in dopaminergic neurons before the reduction of dopamine in the striatum leads to clinical symptoms (Riederer and Wuketich, 1976).

We further show that AMPK in SN DA neurons is a molecular target of ghrelin during CR to maintain neuronal function. Firstly, metabolic stress (CR) and/or toxic stress (MPTP) promoted AMPK activity in striatal dopamine nerve terminals in Ghrelin WT but not Ghrelin KO mice. The ability of MPTP to increase AMPK activity is supported by previous studies in mice and cells (Choi et al., 2010). AMPK enhances mitochondrial function and biogenesis (Reznick and Shulman, 2006) as such, we suggest CR-induced AMPK phosphorylation at the nerve terminal promotes neuronal energy metabolism and supports ongoing dopaminergic

neuronal activity. This is supported by the reduced striatal DOPAC:DA ratio in CR Ghrelin WT but not KO mice. Also, AMPK activity diminishes with age (Reznick et al., 2007), an effect that is consistent with age-related neurodegeneration, which contributes to the onset of PD. Hence, we believe that CR activated AMPK activity in a ghrelin-dependent manner potentially attenuates any age-related decline in the nigrostriatal system. In further support of this argument, both plasma ghrelin and ghrelin's function diminish with age, an effect that can be reversed with CR (Englander et al., 2004; Smith et al., 2007; Sun et al., 2007; Yang et al., 2007; Takeda et al., 2010). Specifically, PD patients have a diminished ghrelin response as shown by reduced postprandial plasma ghrelin levels (Unger et al., 2011).

In Chapter 3 we link CR-induced elevated acyl ghrelin levels with AMPK activation in dopaminergic neurons. We show that in cultured dopaminergic neurons both acyl ghrelin and a ghrelin agonist elicited a robust increase in AMPK activation. Acute acyl ghrelin injection *in vivo* increased both AMPK and ACC phosphorylation in the SN but not the striatum. This is the first *in vivo* study that shows that acyl ghrelin activates AMPK activity in the midbrain, similar to numerous reports showing acyl ghrelin activates AMPK activity in the hypothalamus (Andersson et al., 2004; Kola et al., 2005; Andrews et al., 2008). As shown in Figure 3.3, CR drives ghrelin-induced AMPK phosphorylation in the striatum, but not the SN, yet acute acyl ghrelin injection *in vivo* increased AMPK phosphorylation in the SN but not the striatum (Figure 3.4). We consider this discrepancy may be due to chronically elevated acyl ghrelin vs. an acute ghrelin injection. Chronically high plasma acyl ghrelin, as seen in CR Ghrelin WT mice, activates SN dopamine neurons via the GHSR, which then facilitates and propagates AMPK phosphorylation in areas of metabolic need, in this case striatal nerve terminals, in order to prevent degeneration. Although acute injection of acyl ghrelin increases the pAMPK/AMPK ratio in the SN after 45 minutes, this narrow time frame presumably prevents propagation of AMPK phosphorylation in the striatum. It is important to note that the ghrelin receptor, GHSR, is abundantly expressed in the SN with little or no expression in the striatum (Zigman et al., 2006).

AMPK activation from various other sources including AICAR, Resveratrol, and GPA as well as molecular activation have shown to result in neuroprotection in PD (Jin et al., 2008; Horvath et al., 2011; Ng et al., 2012). We observed that after acyl ghrelin administration there was increased AMPK activity in the nigrostriatal system and hypothesised this as a key

mechanism preventing degeneration. To determine conclusively that AMPK activation is essential for the neuroprotective actions of acyl ghrelin, we generated a novel AMPK KO mouse line. AMPK KO mice lack the ability of AMPK to become phosphorylated selectively in dopaminergic neurons. We show that there was a neuroprotective effect of chronic acyl ghrelin only in AMPK WT mice. This result indicates that acyl ghrelin acts via AMPK activation in DA neurons to prevent neurodegeneration. However, in the AMPK KO mice the mice have lacked functional activity from conception. To prevent any compensatory effects during development which potentially could have altered metabolism a conditional KO could be used. A time dependent removal during adulthood by using mice with inducible expression of Cre recombinase could reduce any up-regulation of other subunits as well as any compensatory effects observed. Collectively from Chapter 2 and 3 we show that acyl ghrelin is the isoform responsible for neuroprotection in PD and that acyl ghrelin is responsible for the neuroprotective actions of CR in PD. We further show that acyl ghrelin acts on SN dopaminergic neurons to elevate AMPK activity and that this elevation is ultimately responsible for this protective action (see Figure 5.1).

Glia become activated to remove neuronal damage by phagocytosis (Neumann et al., 2009), however during chronic over-activation such as during PD glia cause neuronal damage due to the release of pro-inflammatory cytokines and ROS (Luo and Chen, 2012). Studies in vitro indicate ghrelin's ability to directly inhibit glial activation to diminish the inflammatory response (Lee and Yune, 2014). Also, in response to the bacterial endotoxin lipopolysaccharide, ghrelin inhibits the release of the pro-inflammatory cytokine interleukin-6 in dopaminergic cells (Beynon et al., 2013). Collectively, these studies imply ghrelin's role to elicit anti-inflammatory actions. In this study mice chronically administered ghrelin then treated with MPTP show reduced gliosis compared to saline treated controls. We believe this is due to an overall enhanced neuronal profile in AMPK WT mice to ultimately reduce dopaminergic cell death in response to MPTP and minimise the need for additional glia. However, in mice lacking AMPK selectively in dopaminergic neurons (AMPK KO) there was no attenuation of gliosis in response to ghrelin due to the elevated loss of dopamine cell number, content and TH expression in AMPK KO mice. Consequently there was an increased need for the removal of the damaged cells, hence an elevated gliosis response.

Although we have determined that AMPK is a therapeutic target for the protective actions of CR, we wanted an agent that would artificially elevate dopaminergic AMPK activation. One such example of a therapeutic that is well tolerated and has evidence of neuroprotection in PD is Metformin. The mechanism of action is unknown, although thought to act through AMPK. In Chapter 4 we wanted to determine if Metformin acts through AMPK in dopaminergic neurons to elicit a neuroprotective effect.

Chapter 4: Metformin is neuroprotective in a mouse model of Parkinson's Disease, independent of AMPK activation in dopamine neurons.

Metformin is a drug widely used in the treatment of type 2 Diabetes Mellitus (T2DM). It is well documented that Metformin treatment activates AMPK both *in vitro* and *in vivo*, in peripheral tissues (Zhou et al., 2001; Musi et al., 2002; Zou et al., 2004). However, in the brain, Metformin's actions are less clear. AMPK activation has been attributed to many of Metformin's actions including a reduction in glucose production, increased glucose uptake into muscles and even plays a role in cardioprotection. As T2DM is known to be detrimental to PD symptoms (Sandyk, 1993), there is a potential overlap whereby therapeutics can benefit both disease states. In Chapter 4 we determined if AMPK activation in dopaminergic neurons was responsible for the neuroprotective effect of Metformin. To do this we used AMPK WT/KO mice and chronically exposed them to Metformin in their drinking water. Mice receiving Metformin water had an attenuated response to MPTP. This protective effect was apparent in terms of reduced TH cell loss and gliosis in the SN, reduced TH loss in the striatum and enhanced DA turnover, as determined by the DOPAC:DA ratio. AMPK activation was observed in AMPK WT mice in response to MPTP treatment as an attempt to minimise cell loss, in accordance with its well-known protective effects mentioned previously in Chapter 3. However, there was no increase in AMPK activation in the SN or striatum in AMPK KO mice in response to MPTP. These results suggest that functional AMPK is required to increase the pAMPK/AMPK ratio in response to MPTP, similar to Figure 3.3 I-L. Despite the lack of an increase in the pAMPK/AMPK ratio in AMPK KO mice after MPTP, there were no genotype differences in TH protein expression, TH cell number, TH cell volume and DA content measured by HPLC before or after MPTP. These results indicate that the inability of AMPK to be phosphorylated in response to a toxic insult did not preclude the

neuroprotective actions of Metformin treatment. It is important to note that throughout this thesis a single model of PD has been used for the purpose of continuity. It is important to mention that many models of PD exist with each having its own merits and limitations. Future research should focus on the use of other models including transgenic mice such as alpha synuclein overexpression which have the additional benefit of having the model of the disease since birth.

Although we show that Metformin is not acting through AMPK in dopaminergic neurons, there are many other potential mechanisms through which Metformin could be acting. For example Metformin inhibits apoptosis in neuronal cortical cells (El-Mir et al., 2008), prevents oxidative stress-related cellular death (Chakraborty et al., 2011) and plays an inhibitory role on inflammatory transcription factor NF-kB (Hirsch et al., 2013). In mice exposed to Metformin there was reduced superoxide leakage in the mitochondria, indicating greater efficiency of mitochondrial complexes (Martin-Montalvo et al., 2013). As complex I activity is diminished in PD patients (Schapira et al., 1990) this enhanced mitochondrial efficiency coupled with reduced oxidative stress could be responsible for the neuroprotective actions of Metformin. Metformin also activates Sirtuins (SIRTs). SIRTs are responsible for a variety of processes including enhanced mitochondrial function, cellular metabolism, gluconeogenesis and also regulation of lifespan (Finkel et al., 2009). There are increased levels and activity of SIRTs in the livers of Metformin treated mice (Caton et al., 2010). SIRT activation improves mitochondrial function and extends lifespan (Mitchell et al., 2014). Indeed, lifespan is increased in mice overexpressing SIRT1 (Bordone et al., 2007) and decreased in SIRT1 KO (Boily et al., 2008). Metformin has also been shown to influence other metabolic gut hormones such as glucagon like peptide 1 (GLP1). Metformin increases circulating levels of GLP1 to help control blood glucose levels (Mulherin et al., 2011). GLP1 also has receptors on SN dopaminergic neurons and prevents neurodegeneration in a mouse model of PD (Bertilsson et al., 2008; Li et al., 2009). This elevation could be responsible for the protective actions of Metformin and raises the possibility that the neuroprotective actions of Metformin are secondary to changes in peripheral metabolism. Collectively these studies give precedence for the neuroprotective actions of Metformin independent of AMPK activation.

Although Metformin has recently been shown to be neuroprotective in PD (Wahlqvist et al., 2012), our results show it is through a mechanism not dependent upon AMPK activation in

dopaminergic neurons (see Figure 5.1). Future studies should focus on the neuroprotective actions of Metformin in combination with a drug that activates AMPK in dopaminergic neurons. This polypharmacy approach could potentially enhance the neuroprotective actions of Metformin.

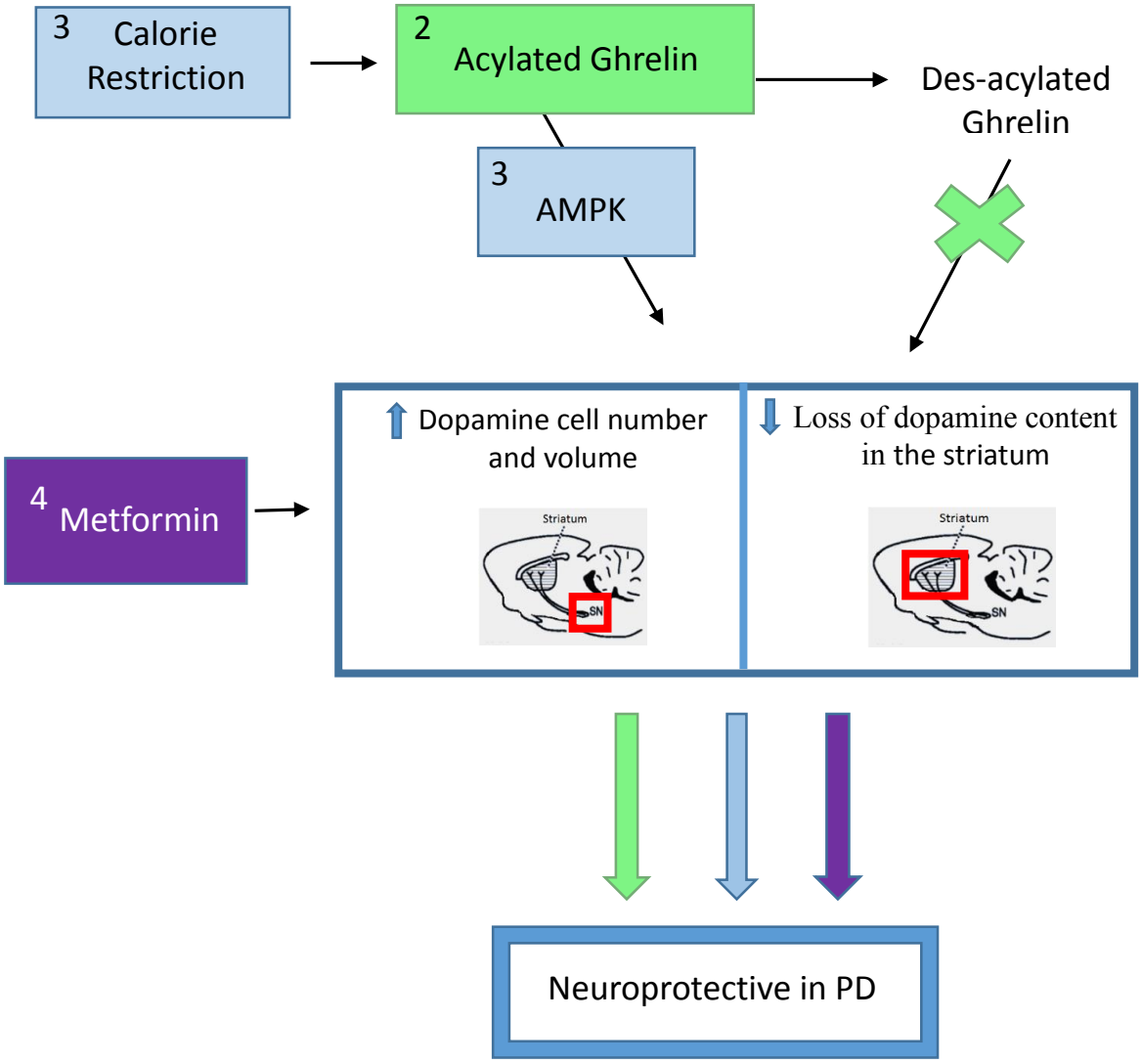


Figure 5.1: Neuroprotective actions of CR, Acyl ghrelin and Metformin. Acylated, not des-acylated ghrelin is responsible for the neuroprotective actions of 'ghrelin' (Chapter 2 – green). Calorie restriction elicits neuroprotective actions via elevated acylated ghrelin levels in the plasma, which consequently activates AMPK to increase dopamine cell number and volume and elevate dopamine content in the striatum (Chapter 3 – blue). Metformin, independent of AMPK activity in dopaminergic neurons is neuroprotective in PD (Chapter 4 – purple).

Overall conclusion

In humans, CR maintains cognitive function and prevents degeneration in a number of different disorders. However the underlying mechanisms of this protection are unknown. Despite the many known beneficial effects of CR including increased lifespan, reduced incidence of cancer and cardiovascular disease and protection in PD, adhering to CR is difficult. By understanding and exploring mechanisms of CR we attempted to create an alternative option that can mimic CR without having to reduce the amount of calories we consume. We focused on ghrelin due to its well-established elevation during CR and known neuroprotective activity during PD. We found that of the two isoforms of ghrelin, acyl and des-acyl, the acylated form plays a critical role in the protective effects of CR in PD. We also show that acyl ghrelin activates AMPK to attenuate the loss of dopaminergic neurons during PD progression. Further, we went on to show that Metformin's neuroprotective actions are independent from AMPK activation in dopaminergic neurons. Future research is required to find a drug that can be orally administered, which activates AMPK in dopaminergic neurons. Combination therapy of this drug with Metformin could be implemented as these two therapeutics would be acting on separate targets to elicit neuroprotection. Further implications of this study can be applied not only to PD, but also other diseases such as Alzheimer's, cardiovascular disease, cancer and even help to slow the aging process.

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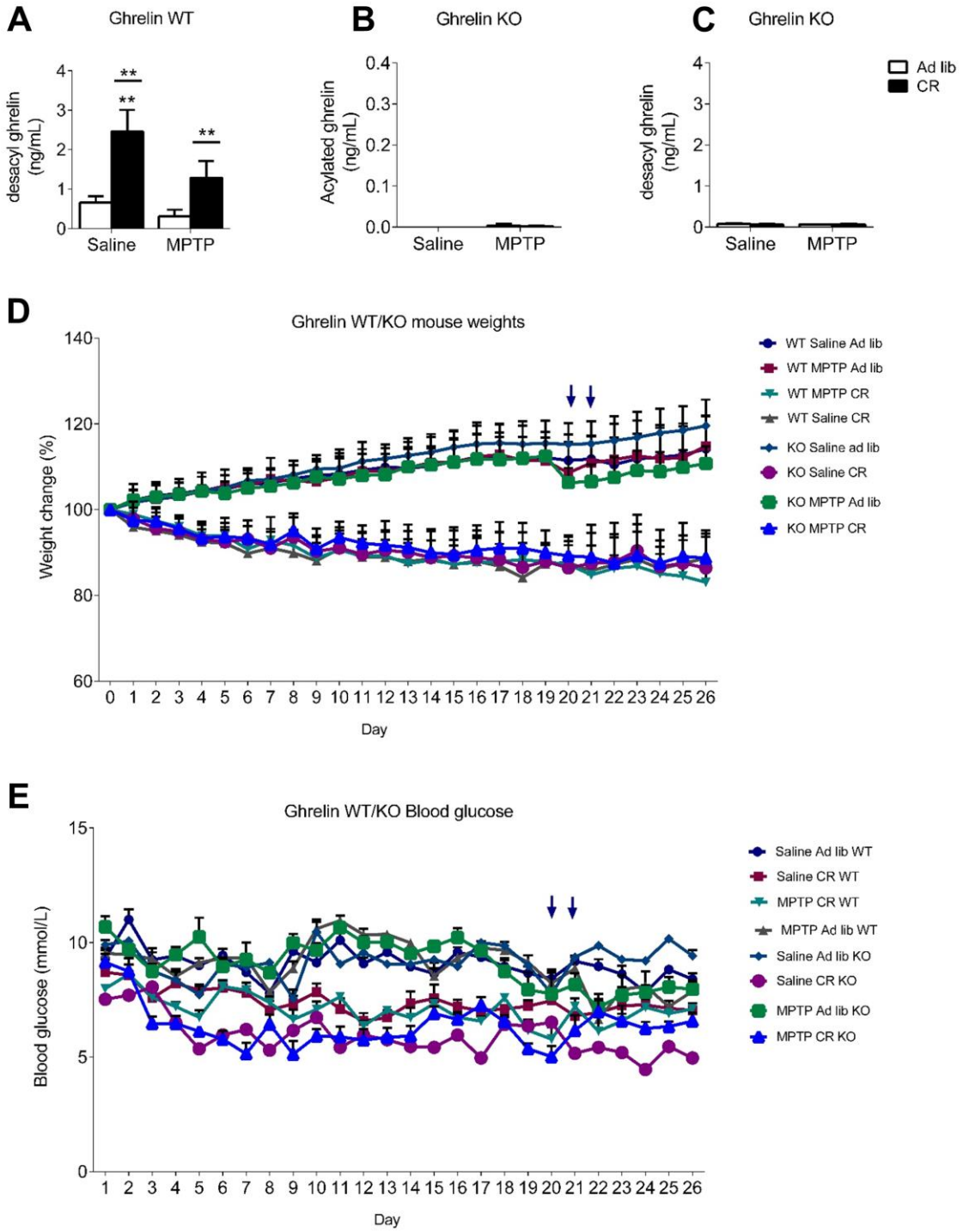
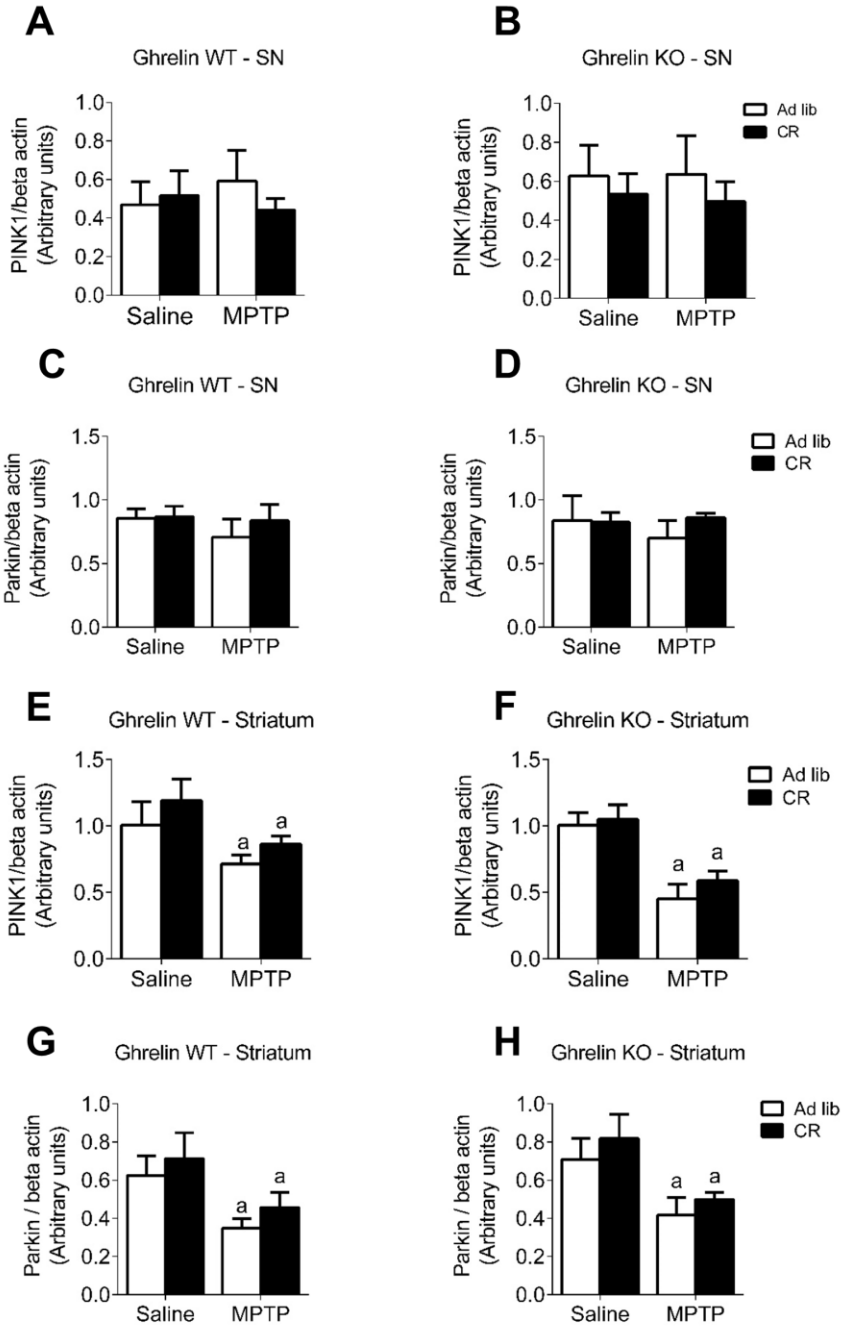
Appendix

Appendix 1



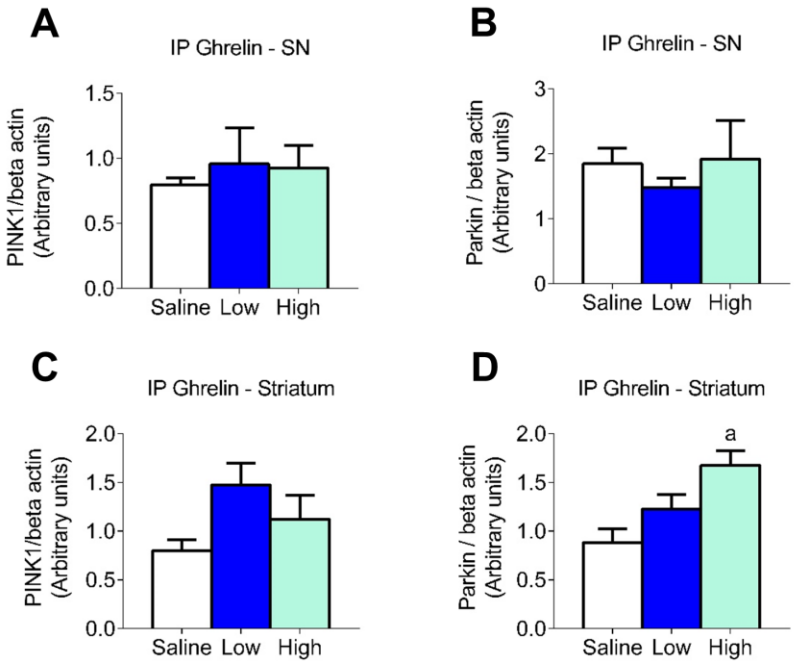
Appendix 1: Characterisation of the Ghrelin WT/KO mice. **A**, Calorie restriction significantly elevates plasma des-acyl ghrelin. **B & C**, Negligible levels of plasma acyl or des-acyl in Ghrelin KO mice. **D & E**, Daily body weight and blood glucose measurements for the duration of the study. * $p < 0.05$, ** $p < 0.01$. Data are represented as mean ± SEM (n=7-10, two-way ANOVA).

Appendix 2



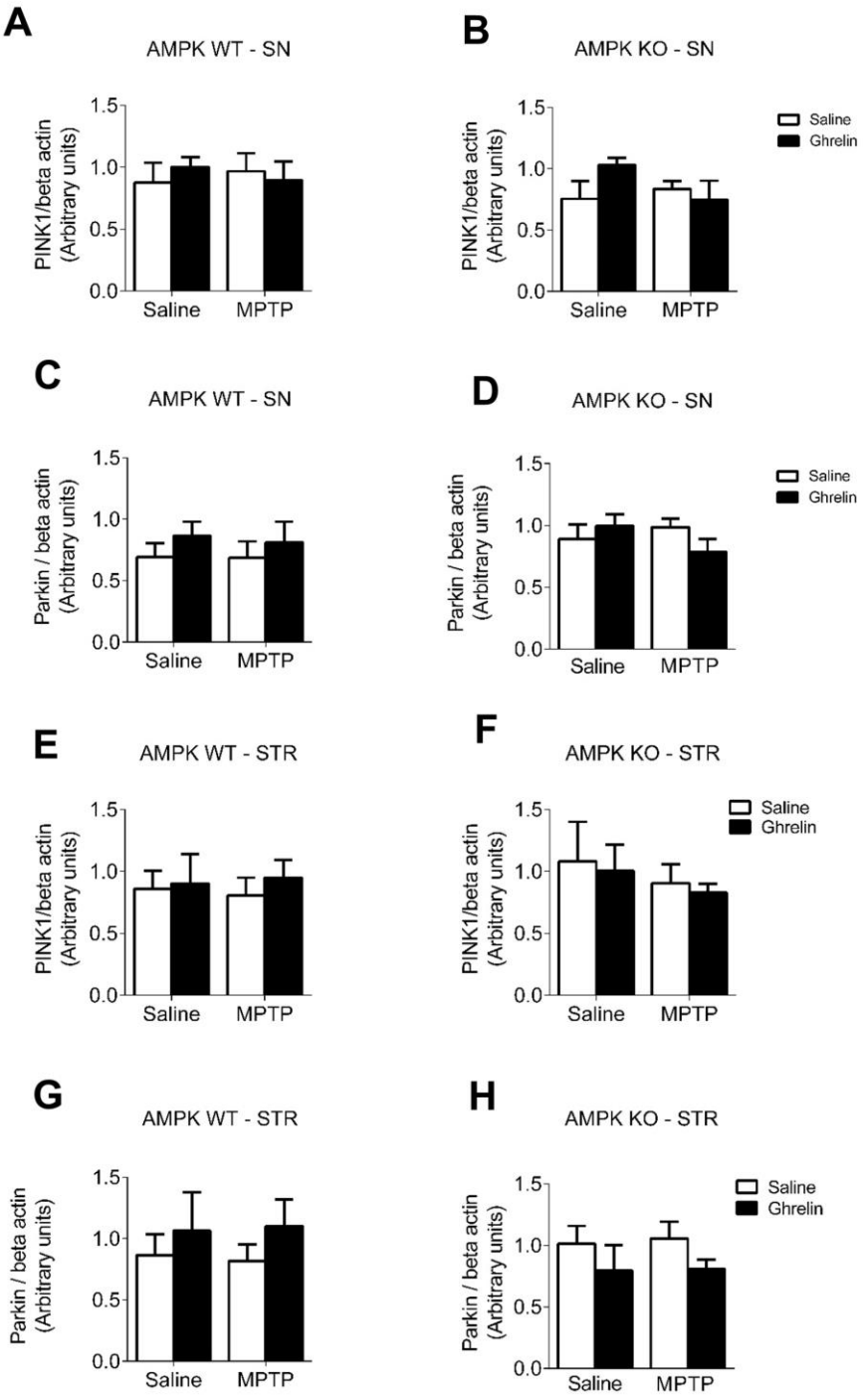
Appendix 2: Reduced mitophagy markers in the striatum in response to MPTP with no effect of genotype. **A - D**, Quantification of PINK1 and Parkin levels in the SN of Ghrelin WT and KO mice reveals no overall changes in response to treatment or diet. **E-H**, Quantification of PINK1 and Parkin levels in the Striatum of Ghrelin WT and KO mice shows a significant reduction in response to MPTP with no overall effect of diet. a, significant compared to saline ad-lib controls, b, significant compared to MPTP ad-lib controls. Data are represented as mean ± SEM (n=6-8, two-way ANOVA, $p<0.05$).

Appendix 3



Appendix 3: Acylated ghrelin significantly elevates Parkin levels in the Striatum. **A & B**, Quantification of PINK1 and Parkin levels in the SN shows no significant effect of low or high doses of acylated ghrelin. **C & D**, Quantification of PINK1 and Parkin in the striatum shows no change in PINK1 levels but a significant elevation in Parkin levels with a high dose of acylated ghrelin. a, significant compared to saline ad-lib controls, b, significant compared to MPTP ad-lib controls. Data are represented as mean ± SEM (n=5-7, two-way ANOVA, $p<0.05$).

Appendix 4



Appendix 4: No changes in mitophagy markers in AMPK WT/KO mice. **A - D**, Quantification of PINK1 and Parkin levels in the SN of AMPK WT and KO mice reveals no overall changes in response to ghrelin or MPTP. **E-H**, Quantification of PINK1 and Parkin levels in the striatum of AMPK WT and KO mice reveals no overall changes in response to ghrelin or MPTP. a, significant compared to saline ad-lib controls, b, significant compared to MPTP ad-lib controls. Data are represented as mean ± SEM (n=6-8, two-way ANOVA, $p<0.05$).