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RESEARCH ARTICLE

Metformin Prevents Nigrostriatal Dopamine Degeneration Independent of AMPK Activation in Dopamine Neurons

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Abbreviations: DA, Dopamine; GFAP, Glial Fibrillary Acidic Protein; GLP1, Glucagon Like Peptide 1; IBA1,

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 then exposed to MPTP, the 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

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.

year 2030 [1]. 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 [2–4]. Intriguingly, there is a correlation between the incidence of diabetes preceding PD development in individuals [5], 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 Alzheimers [6, 7]. 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 [8].

Metformin is a biguanide 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 [9]. In mice Metformin produced approximately a 6% lifespan extension, which was also accompanied by improved locomotor performance [10]. Indeed, in a human study patients with T2D with Metformin monotherapy had a longer survival than matched non-diabetic controls [11]. 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 [12], stroke risk [13], enhancing neurogenesis [14] as well as the traditional lowering of blood glucose. As CR is beneficial for PD [15] and T2D [16], 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 [17]. In a *Drosophila* model of PD, Metformin treatment alleviated dopaminergic dysfunction and mitochondrial abnormalities [18]. Metformin chronically administered to mice reduces oxidative stress, dopaminergic degeneration and motor abnormalities associated with MPTP (a mouse model for PD) administration [19]. Hence, Metformin treatment has a protective effect in PD. 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 [20], it is an ideal therapeutic for the treatment of PD. Recently, a mechanism of action for how Metformin suppresses gluconeogenesis has been discovered [21], 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 [9]. Metformin has also been shown to inhibit mTOR to enhance mitochondrial function [22, 23]. Metformin can also activate AMPK by inhibiting complex I of the ETC [24]. This results in an increased AMP/ATP ratio and the subsequent activation of AMPK. AMPK acts to increase mitochondrial biogenesis [25] 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 [17]. In a *Drosophila* model of PD, loss of AMPK activity exacerbated neuronal loss and its associated phenotypes [18]. Resveratrol, an AMPK activator is neuroprotective in a rodent model of PD [26]. In

response to MPTP AMPK is phosphorylated and inhibition of AMPK by Compound C enhanced MPTP-induced cell death [27]. 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 [26, 28, 29] 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.

Material and Methods

Animals

All experiments were conducted in accordance with Monash University Animal Ethics Committee guidelines. The experiments in the study were specifically approved the Monash Animal Research Platform Animal Ethic Committee (MARF/2012/042). Male mice were maintained under standard laboratory conditions with free access to standard chow and water. Environmental enrichment consisting of tissues and cardboard boxes cut to fit the cages was provided in every cage. Mice were weighed and monitored daily. No mice died prior to the endpoint. Due to the use of MPTP early termination was designated if suffering was observed or if body weight fell below 70% of initial weight. All efforts were made to minimise suffering and isoflurane was used at end of experiment. 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 AMPK β 1 & β 2 only in DAT-expressing neurons, we crossed Dat-Cre knock-in mice from Jax Lab [Stock number 006660; B6.SJL-Slc6a3<tm1.1(cre)bkmn>/j] with AMPK β 1 (β 1) and β 2 (β 2) floxed mice [30]. 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 [31].

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 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 [32]. 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 deeply anaesthetised using isoflurane and 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 with isoflurane 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 (30 μ m) was collected and stored in cryoprotectant (30% Ethylene Glycol, 20% Glycerol in 0.1M PB) at -20°C. The sections were

washed thoroughly with 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 blocked with 4% normal horse serum and 0.3% Triton X-100 in 0.1M PB for one hour and then blocked again with Affi-niPure 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 anaesthetised 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; Indianapolis, USA), Corticosterone (Abnova; CA, USA) and Blood glucose (Sigma; Missouri, USA). Plasma insulin concentration was determined 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 blocked 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-AMPK α (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 \pm 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 [33]. Blood glucose was measured with ACCU-CHEK Active (Roche DiagnosticsGnH, Tokyo, Japan) and then a 25% D-glucose solution (1.5g/kg) was given by oral gavage 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 i.p 1U/kg insulin.

Statistical Analysis

All data are represented as Mean \pm 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 (raw data available; [S1–S11 Files](#)). There were no genotype differences in body weight ([Fig 1A, 1C & 1D](#)), blood glucose ([Fig 1E & 1F](#)) or insulin levels ([Fig 1G & 1H](#)). Because Metformin is known to promote insulin sensitivity [34] 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 ([Fig 1I](#)), Area under the curve ([Fig 1J](#)) and the ITT ([Fig 1M](#)). However, in AMPK KO mice Metformin significantly increased glucose clearance ([Fig 1K](#)) at the 15-minute time-point, however the area under the curve showed no significant differences between water and Metformin treatment ([Fig 1L](#)). There was no difference in the ITT ([Fig 1N](#)). This indicates that AMPK activation in dopamine neurons does not promote peripheral glucose clearance during an oGTT or ITT.

Plasma analysis showed a significant main effect of MPTP to increase Triglycerides ([Fig 2A & 2B](#)), Non-Esterified Fatty Acids (NEFA) ([Fig 2C & 2D](#)) and corticosterone ([Fig 2E & 2F](#)) levels, which occurred with a concurrent reduction in body weight ([Fig 1A](#)) indicating the stress placed on these mice. These hormones were measured to determine if metabolic feedback was playing a role in the neuroprotective actions of Metformin. To confirm the inability of AMPK

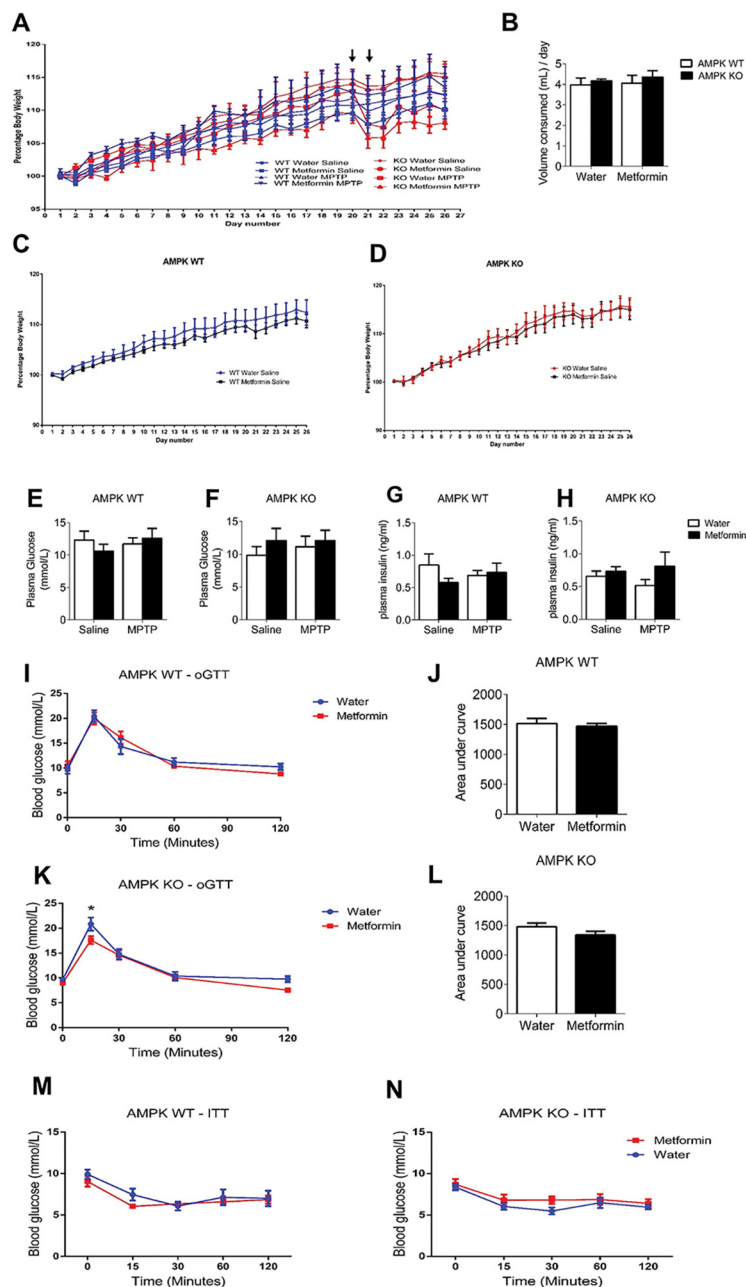


Fig 1. Body Weight and blood glucose measurements in AMPK WT and KO mice. A–D, throughout the experiment there was no difference in body weight or volume of water consumed comparing genotype or treatment. Arrows indicate MPTP injections. E–H, Plasma analysis of insulin and glucose levels in trunk blood show no differences between genotype and treatment. During an oGTT Metformin treatment does not alter glucose clearance in AMPK WT (I & J) or AMPK KO mice (K & L). Insulin sensitivity is not altered during an ITT in AMPK WT (M) or KO (N) mice treated with Metformin. * = $p < 0.05$. Data are represented as mean \pm SEM ($n = 8–10$, two-way ANOVA, $p < 0.05$).

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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 (Fig 2I & 2K). AMPK KO mice exhibited no significant change in the pAMPK/AMPK ratio in response to MPTP in either the SN (Fig 2J) or Striatum (Fig 2L)

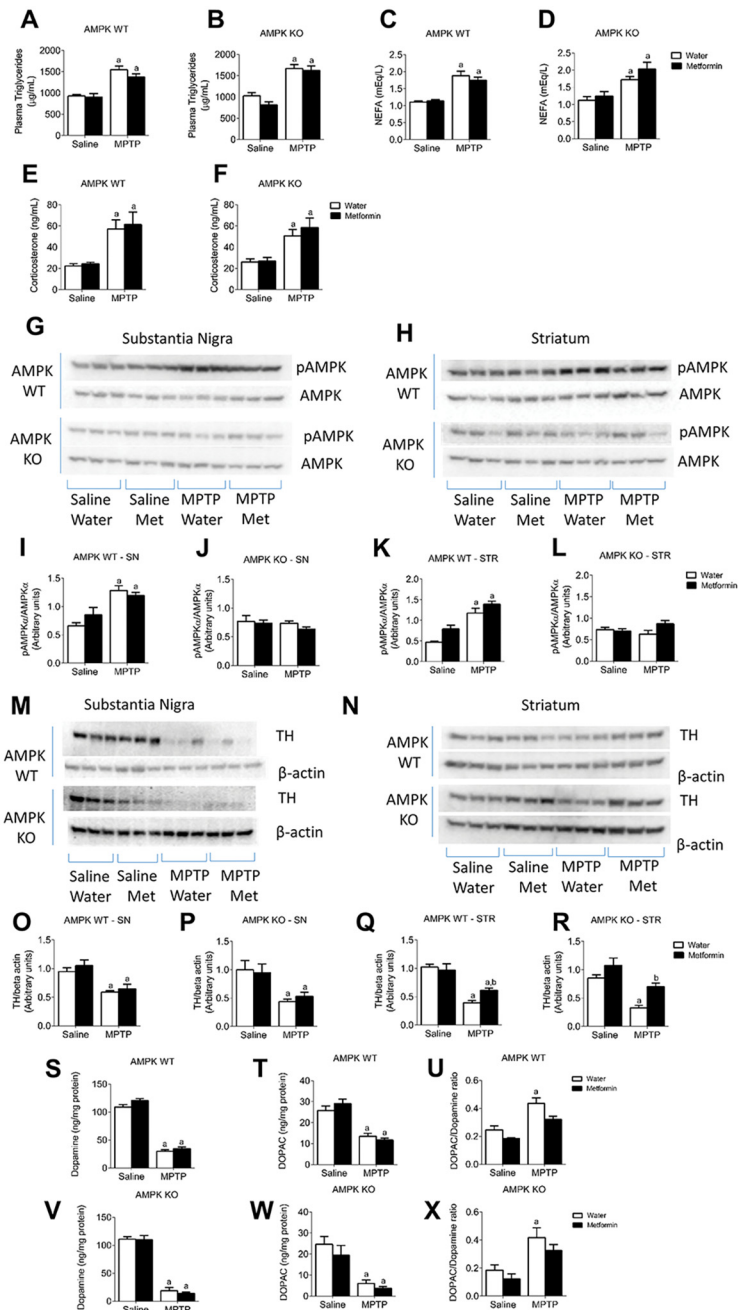


Fig 2. Metformin is neuroprotective in AMPK WT and KO mice. Plasma Triglycerides (**A & B**), NEFA (**C & D**) and corticosterone (**E & F**) are elevated in response to MPTP. Representative Western Blot images of pAMPK/AMPK in the SN (**G**) and Striatum (**H**). Protein analysis of the pAMPK/AMPK ratio 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 \pm SEM ($n = 6-9$, two-way ANOVA, $p < 0.05$).

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implying an inability to be phosphorylated in response to a toxic insult as a protective mechanism, as observed previously [31].

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 (Fig 2M–2R) an effect that was attenuated by metformin in both AMPK WT and KO mice in the Striatum (Fig 2Q & 2R) but not in the SN (Fig 2O & 2P). In support of this data, HPLC analysis of dopamine (Fig 2S & 2V) and DOPAC (Fig 2T & 2W) 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 (Fig 2U & 2X). These results indicate that Metformin has site-specific protective effects in the Striatum.

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 (Fig 3A) and KO mice (Fig 3B) and also prevented a reduction in cell volume in both genotypes (Fig 3C & 3D), with the majority of the beneficial effect occurring in smaller cells between 1000–2000 μm^3 (Fig 3E & 3F). 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 (Fig 3G–3I) and GFAP (Fig 3J–3L) 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 (Fig 3H–3K).

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.

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 [35], hepatic [36], cardiac [37, 38] and neurological conditions [39]. Indeed, Alzheimers Disease (AD) is closely associated with impaired insulin signalling and glucose metabolism and is often referred to as “type 3 diabetes” [40]. There is a correlation between insulin resistance and an elevated risk for AD development [41] and studies conducted in obese leptin-resistant mice showed that Metformin attenuated AD-like neuropathology and biological markers [39]. 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 [42].

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 [8]. 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

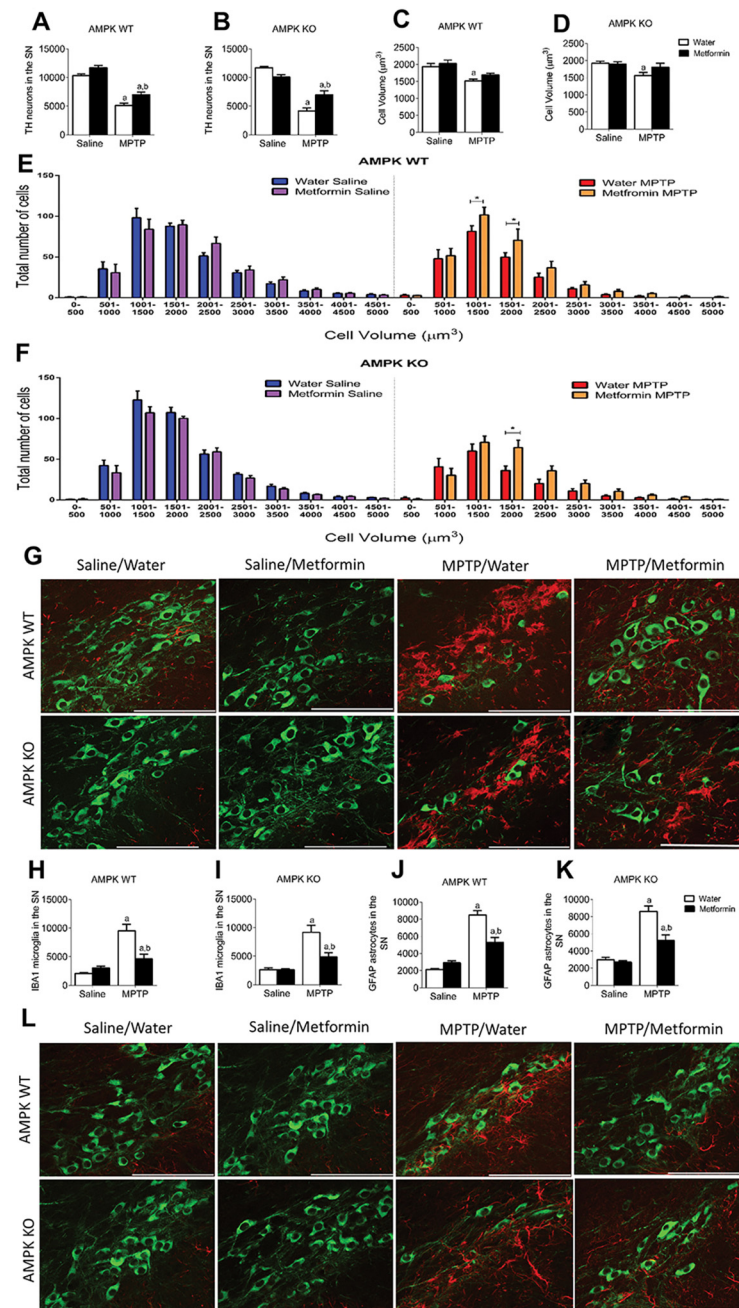


Fig 3. Metformin preserves cell number and volume after MPTP exposure, independent of genotype. Stereological quantification of TH levels in the SN shows a protective effect of Metformin after MPTP exposure in both AMPK WT (A) and KO (B) mice. Overall cell volume shows a significant reduction after MPTP 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 (1000–2000 μm^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 \pm SEM ($n = 7-10$, two-way ANOVA, $p < 0.05$).

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Metformin to reduce the risk for developing PD and although the evidence for neuroprotection is clear in animal and cell-based models [19, 43, 44], 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 [24] and tissues such as the liver [24] and muscle [45]. In our studies we clearly demonstrate 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. In this study we used a mouse model where AMPK β 1 and AMPK β 2 were successfully deleted in DAT expressing neurons. AMPK is composed of three subunits: alpha, beta and gamma. The alpha subunit plays a catalytic role whereas beta and gamma are regulatory. We chose the beta subunit as deletion of both AMPK β 1 and AMPK β 2 in muscle ablated AMPK phosphorylation [30]. A potential limitation in this model is the use of transgenic mice. There is potential that these controls (CRE negative, floxed positive) react differently to Metformin and/or MPTP as the floxed allele has been carried from conception. 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 [31]. 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 [18]. Other activators of AMPK including Resveratrol [26] and ghrelin [31, 46] are neuroprotective in vivo. Overexpression of alpha synuclein in cells (as a model of PD) activates AMPK in order to restrict cell death [17]. Although AMPK activation is neuroprotective in PD and Metformin induces direct protective effects through AMPK in other disease states such stroke [47], our studies show that Metformin doesn't activate AMPK in dopamine neurons to prevent degeneration in a mouse model of PD. However, this model was using mice which selectively had AMPK activity removed in dopaminergic neurons hence AMPK could elicit neuroprotective actions within cells external to the neurons. As there was a significant reduction in gliosis with Metformin treatment in both AMPK WT and KO mice after MPTP treatment there is potential for AMPK activity in microglia / astrocytes to elicit neuroprotective actions. Indeed, in vitro studies indicate that AMPK activation within microglia suppresses pro-inflammatory responses [48]. As inflammation is a key hallmark in PD [49], AMPK activation within microglia may be responsible for the neuroprotective actions of Metformin, although this theory requires experimental proof.

There are many other potential mechanisms through which Metformin can act. For example Metformin inhibits apoptosis in neuronal cortical cells [43], prevents oxidative stress-related cellular death [50] and plays an inhibitory role on inflammatory transcription factor NF- κ B [51]. In mice exposed to Metformin there was reduced superoxide leakage in the mitochondria, indicating greater efficiency of mitochondrial complexes [10]. As complex I activity is diminished in PD patients [52] this enhanced mitochondrial efficiency coupled with reduced oxidative stress could be responsible for the neuroprotective actions of Metformin. Metformin also activates Sirtuins (SIRT's) and PGC-1 α . SIRT's are responsible for a variety of cellular processes including enhancing mitochondrial function, cellular metabolism, gluconeogenesis as well as aging [53]. There are increased levels and activity of SIRT's in the livers of Metformin treated mice [54]. SIRT activation improves mitochondrial function and extends lifespan [55]. Indeed, lifespan is increased in mice overexpressing SIRT1 [56] and decreased in SIRT1 KO [57]. Another potential target that Metformin could act through is PGC-1 α , a transcriptional regulator involved in mitochondrial biogenesis. Metformin has been shown to increase PGC-1 α protein expression in the liver [58] and skeletal muscle [59]. PGC-1 α deficient mice are

more susceptible to MPTP-induced dopaminergic neuronal loss [60]. In mice, overexpression of PARIS, which represses the expression of PGC-1 α , results in selective degeneration of substantia nigra dopaminergic neurons [61]. In humans polymorphisms of PGC-1 α are associated with early onset PD [62]. Collectively, these studies show that Metformin's neuroprotective actions could be due to many other agents that enhance mitochondrial function independent of AMPK activation. It is important to note that AMPK interacts with SIRT1 and PGC-1 α . AMPK enhances SIRT1 activity, enhances the downstream target PGC-1 α and increases mitochondrial biogenesis [25]. Also, AMPK can both activate and be activated by SIRT1 [63], creating a complex interplay between AMPK, SIRT1 and PGC-1 α . Future research should determine if SIRT1 and PGC-1 α play a role in the neuroprotective actions of Metformin.

Metformin can increase circulating levels of the gut hormone GLP-1 to help control blood glucose levels [64]. GLP1 also has receptors on SN dopaminergic neurons and prevents neurodegeneration in a mouse model of PD [65, 66]. 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 and dose 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-1 α in SN dopamine neurons.

Supporting Information

S1 File. Data for Corticosterone assay.

(XLSX)

S2 File. Data for oral glucose tolerance tests.

(XLSX)

S3 File. Data for HPLC analysis.

(XLSX)

S4 File. Body weight data for 1 cohort of metformin treated mice.

(XLSX)

S5 File. Data for blood glucose.

(XLSX)

S6 File. Data for body weight for another cohort of mice.

(XLSX)

S7 File. Data for plasma insulin assay.

(XLS)

S8 File. Data for stereology.

(XLSX)

S9 File. Data for western blot analysis.

(XLSX)

S10 File. Data for plasma NEFA analysis.
(XLSX)

S11 File. Data for plasma TG analysis.
(XLSX)

Author Contributions

Conceived and designed the experiments: JAB ZBA. Performed the experiments: JAB VVS MD ML JE. Analyzed the data: JAB JE. Contributed reagents/materials/analysis tools: BEK JSD. Wrote the paper: JAB ZBA.

References

1. Dorsey ER, Constantinescu R, Thompson JP, Biglan KM, Holloway RG, Kieburtz K, et al. Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030. *Neurology*. 2007; 68(5):384–6. Epub 2006/11/04. doi: [10.1212/01.wnl.0000247740.47667.03](https://doi.org/10.1212/01.wnl.0000247740.47667.03) PMID: [17082464](https://pubmed.ncbi.nlm.nih.gov/17082464/).
2. Driver JA, Smith A, Buring JE, Gaziano JM, Kurth T, Logroscino G. Prospective cohort study of type 2 diabetes and the risk of Parkinson's disease. *Diabetes care*. 2008; 31(10):2003–5. Epub 2008/07/05. doi: [10.2337/dc08-0688](https://doi.org/10.2337/dc08-0688) PMID: [18599528](https://pubmed.ncbi.nlm.nih.gov/18599528/); PubMed Central PMCID: PMC2551644.
3. Hu G, Jousilahti P, Bidel S, Antikainen R, Tuomilehto J. Type 2 diabetes and the risk of Parkinson's disease. *Diabetes Care*. 2007; 30(4):842–7. PMID: [17251276](https://pubmed.ncbi.nlm.nih.gov/17251276/).
4. Sandyk R. The relationship between diabetes mellitus and Parkinson's disease. *The International journal of neuroscience*. 1993; 69(1–4):125–30. Epub 1993/03/01. PMID: [8082998](https://pubmed.ncbi.nlm.nih.gov/8082998/).
5. D'Amelio M, Ragonese P, Callari G, Di Benedetto N, Palmeri B, Terruso V, et al. Diabetes preceding Parkinson's disease onset. A case-control study. *Parkinsonism Relat Disord*. 2009; 15(9):660–4. Epub 2009/04/10. doi: [10.1016/j.parkreldis.2009.02.013](https://doi.org/10.1016/j.parkreldis.2009.02.013) PMID: [19356970](https://pubmed.ncbi.nlm.nih.gov/19356970/).
6. Pasquier F, Boulogne A, Leys D, Fontaine P. Diabetes mellitus and dementia. *Diabetes & metabolism*. 2006; 32(5 Pt 1):403–14. Epub 2006/11/18. PMID: [17110895](https://pubmed.ncbi.nlm.nih.gov/17110895/).
7. Peila R, Rodriguez BL, Launer LJ. Type 2 diabetes, APOE gene, and the risk for dementia and related pathologies: The Honolulu-Asia Aging Study. *Diabetes*. 2002; 51(4):1256–62. Epub 2002/03/28. PMID: [11916953](https://pubmed.ncbi.nlm.nih.gov/11916953/).
8. Wahlqvist ML, Lee MS, Hsu CC, Chuang SY, Lee JT, Tsai HN. Metformin-inclusive sulfonylurea therapy reduces the risk of Parkinson's disease occurring with Type 2 diabetes in a Taiwanese population cohort. *Parkinsonism Relat Disord*. 2012; 18(6):753–8. Epub 2012/04/14. doi: [10.1016/j.parkreldis.2012.03.010](https://doi.org/10.1016/j.parkreldis.2012.03.010) PMID: [22498320](https://pubmed.ncbi.nlm.nih.gov/22498320/).
9. Onken B, Driscoll M. Metformin induces a dietary restriction-like state and the oxidative stress response to extend *C. elegans* Healthspan via AMPK, LKB1, and SKN-1. *PLoS One*. 2010; 5(1):e8758. Epub 2010/01/22. doi: [10.1371/journal.pone.0008758](https://doi.org/10.1371/journal.pone.0008758) PMID: [20090912](https://pubmed.ncbi.nlm.nih.gov/20090912/); PubMed Central PMCID: PMC2807458.
10. Martin-Montalvo A, Mercken EM, Mitchell SJ, Palacios HH, Mote PL, Scheibye-Knudsen M, et al. Metformin improves healthspan and lifespan in mice. *Nature communications*. 2013; 4:2192. Epub 2013/08/01. doi: [10.1038/ncomms3192](https://doi.org/10.1038/ncomms3192) PMID: [23900241](https://pubmed.ncbi.nlm.nih.gov/23900241/); PubMed Central PMCID: PMC3736576.
11. Bannister CA, Holden SE, Jenkins-Jones S, Morgan CL, Halcox JP, Scherthaner G, et al. Can people with type 2 diabetes live longer than those without? A comparison of mortality in people initiated with metformin or sulphonylurea monotherapy and matched, non-diabetic controls. *Diabetes, obesity & metabolism*. 2014; 16(11):1165–73. Epub 2014/07/22. doi: [10.1111/dom.12354](https://doi.org/10.1111/dom.12354) PMID: [25041462](https://pubmed.ncbi.nlm.nih.gov/25041462/).
12. Evans JM, Donnelly LA, Emslie-Smith AM, Alessi DR, Morris AD. Metformin and reduced risk of cancer in diabetic patients. *BMJ (Clinical research ed)*. 2005; 330(7503):1304–5. Epub 2005/04/26. doi: [10.1136/bmj.38415.708634.F7](https://doi.org/10.1136/bmj.38415.708634.F7) PMID: [15849206](https://pubmed.ncbi.nlm.nih.gov/15849206/); PubMed Central PMCID: PMC2558205.
13. Cheng YY, Leu HB, Chen TJ, Chen CL, Kuo CH, Lee SD, et al. Metformin-inclusive therapy reduces the risk of stroke in patients with diabetes: a 4-year follow-up study. *Journal of stroke and cerebrovascular diseases: the official journal of National Stroke Association*. 2014; 23(2):e99–105. Epub 2013/10/15. doi: [10.1016/j.jstrokecerebrovasdis.2013.09.001](https://doi.org/10.1016/j.jstrokecerebrovasdis.2013.09.001) PMID: [24119365](https://pubmed.ncbi.nlm.nih.gov/24119365/).
14. Wang J, Gallagher D, DeVito LM, Cancino GI, Tsui D, He L, et al. Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. *Cell stem cell*. 2012; 11(1):23–35. Epub 2012/07/10. doi: [10.1016/j.stem.2012.03.016](https://doi.org/10.1016/j.stem.2012.03.016) PMID: [22770240](https://pubmed.ncbi.nlm.nih.gov/22770240/).

15. Duan W, Mattson MP. Dietary restriction and 2-deoxyglucose administration improve behavioral outcome and reduce degeneration of dopaminergic neurons in models of Parkinson's disease. *J Neurosci Res*. 1999; 57(2):195–206. PMID: [10398297](#).
16. Pi-Sunyer X, Blackburn G, Brancati FL, Bray GA, Bright R, Clark JM, et al. Reduction in weight and cardiovascular disease risk factors in individuals with type 2 diabetes: one-year results of the look AHEAD trial. *Diabetes care*. 2007; 30(6):1374–83. Epub 2007/03/17. doi: [10.2337/dc07-0048](#) PMID: [17363746](#); PubMed Central PMCID: PMCPMC2665929.
17. Dulovic M, Jovanovic M, Xilouri M, Stefanis L, Harhaji-Trajkovic L, Kravic-Stevovic T, et al. The protective role of AMP-activated protein kinase in alpha-synuclein neurotoxicity in vitro. *Neurobiol Dis*. 2014; 63:1–11. Epub 2013/11/26. doi: [10.1016/j.nbd.2013.11.002](#) PMID: [24269733](#).
18. Ng CH, Guan MS, Koh C, Ouyang X, Yu F, Tan EK, et al. AMP kinase activation mitigates dopaminergic dysfunction and mitochondrial abnormalities in *Drosophila* models of Parkinson's disease. *J Neurosci*. 2012; 32(41):14311–7. Epub 2012/10/12. doi: [10.1523/JNEUROSCI.0499-12.2012](#) PMID: [23055502](#).
19. Patil SP, Jain PD, Ghumatkar PJ, Tambe R, Sathaye S. Neuroprotective effect of metformin in MPTP-induced Parkinson's disease in mice. *Neuroscience*. 2014; 277:747–54. Epub 2014/08/12. PMID: [25108167](#).
20. Labuzek K, Suchy D, Gabryel B, Bielecka A, Liber S, Okopien B. Quantification of metformin by the HPLC method in brain regions, cerebrospinal fluid and plasma of rats treated with lipopolysaccharide. *Pharmacological reports: PR*. 2010; 62(5):956–65. Epub 2010/11/26. PMID: [21098880](#).
21. Madiraju AK, Erion DM, Rahimi Y, Zhang XM, Braddock DT, Albright RA, et al. Metformin suppresses gluconeogenesis by inhibiting mitochondrial glycerophosphate dehydrogenase. *Nature*. 2014; 510(7506):542–6. Epub 2014/05/23. doi: [10.1038/nature13270](#) PMID: [24847880](#); PubMed Central PMCID: PMCPMC4074244.
22. Johnson SC, Rabinovitch PS, Kaeberlein M. mTOR is a key modulator of ageing and age-related disease. *Nature*. 2013; 493(7432):338–45. Epub 2013/01/18. doi: [10.1038/nature11861](#) PMID: [23325216](#); PubMed Central PMCID: PMCPMC3687363.
23. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. *Cancer research*. 2007; 67(22):10804–12. Epub 2007/11/17. doi: [10.1158/0008-5472.can-07-2310](#) PMID: [18006825](#).
24. Zhou G, Myers R, Li Y, Chen Y, Shen X, Fenyk-Melody J, et al. Role of AMP-activated protein kinase in mechanism of metformin action. *J Clin Invest*. 2001; 108(8):1167–74. Epub 2001/10/17. doi: [10.1172/JCI13505](#) PMID: [11602624](#); PubMed Central PMCID: PMC209533.
25. Canto C, Gerhart-Hines Z, Feige JN, Lagouge M, Noriega L, Milne JC, et al. AMPK regulates energy expenditure by modulating NAD⁺ metabolism and SIRT1 activity. *Nature*. 2009; 458(7241):1056–60. Epub 2009/03/06. nature07813 [pii] doi: [10.1038/nature07813](#) PMID: [19262508](#).
26. Jin F, Wu Q, Lu YF, Gong QH, Shi JS. Neuroprotective effect of resveratrol on 6-OHDA-induced Parkinson's disease in rats. *Eur J Pharmacol*. 2008; 600(1–3):78–82. Epub 2008/10/23. S0014-2999(08)01024-8 [pii] doi: [10.1016/j.ejphar.2008.10.005](#) PMID: [18940189](#).
27. Choi JS, Park C, Jeong JW. AMP-activated protein kinase is activated in Parkinson's disease models mediated by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *Biochem Biophys Res Commun*. 2010; 391(1):147–51. Epub 2009/11/12. doi: [10.1016/j.bbrc.2009.11.022](#) PMID: [19903456](#).
28. Horvath TL, Erion DM, Elsworth JD, Roth RH, Shulman GI, Andrews ZB. GPA protects the nigrostriatal dopamine system by enhancing mitochondrial function. *Neurobiol Dis*. 2011; 43(1):152–62. doi: [10.1016/j.nbd.2011.03.005](#) PMID: [21406233](#); PubMed Central PMCID: PMC3623269.
29. Bayliss JA, Andrews ZB. Ghrelin is neuroprotective in Parkinson's disease: molecular mechanisms of metabolic neuroprotection. *Ther Adv Endocrinol Metab*. 2013; 4(1):25–36. Epub 2013/03/22. doi: [10.1177/2042018813479645](#) PMID: [23515333](#); PubMed Central PMCID: PMC3593299.
30. O'Neill HM, Maarbjer SJ, Crane JD, Jeppesen J, Jorgensen SB, Schertzer JD, et al. AMP-activated protein kinase (AMPK) beta1beta2 muscle null mice reveal an essential role for AMPK in maintaining mitochondrial content and glucose uptake during exercise. *Proc Natl Acad Sci U S A*. 2011; 108(38):16092–7. Epub 2011/09/08. doi: [10.1073/pnas.1105062108](#) PMID: [21896769](#); PubMed Central PMCID: PMC3179037.
31. Bayliss JA, Lemus MB, Stark R, Santos VV, Thompson A, Rees DJ, et al. Ghrelin-AMPK Signaling Mediates the Neuroprotective Effects of Calorie Restriction in Parkinson's Disease. *J Neurosci*. 2016; 36(10):3049–63. doi: [10.1523/JNEUROSCI.4373-15.2016](#) PMID: [26961958](#); PubMed Central PMCID: PMC4783502.
32. Li J, Benashski SE, Venna VR, McCullough LD. Effects of metformin in experimental stroke. *Stroke; a journal of cerebral circulation*. 2010; 41(11):2645–52. Epub 2010/09/18. doi: [10.1161/strokeaha.110.589697](#) PMID: [20847317](#); PubMed Central PMCID: PMCPMC2994809.

33. Ayala JE, Samuel VT, Morton GJ, Obici S, Croniger CM, Shulman GI, et al. Standard operating procedures for describing and performing metabolic tests of glucose homeostasis in mice. *Disease models & mechanisms*. 2010; 3(9–10):525–34. Epub 2010/08/18. doi: [10.1242/dmm.006239](https://doi.org/10.1242/dmm.006239) PMID: [20713647](https://pubmed.ncbi.nlm.nih.gov/20713647/); PubMed Central PMCID: PMC2938392.
34. McIntyre HD, Ma A, Bird DM, Paterson CA, Ravenscroft PJ, Cameron DP. Metformin increases insulin sensitivity and basal glucose clearance in type 2 (non-insulin dependent) diabetes mellitus. *Australian and New Zealand journal of medicine*. 1991; 21(5):714–9. Epub 1991/10/01. PMID: [1759920](https://pubmed.ncbi.nlm.nih.gov/1759920/).
35. Pilmore HL. Review: metformin: potential benefits and use in chronic kidney disease. *Nephrology (Carlton, Vic)*. 2010; 15(4):412–8. Epub 2010/07/09. doi: [10.1111/j.1440-1797.2010.01328.x](https://doi.org/10.1111/j.1440-1797.2010.01328.x) PMID: [20609092](https://pubmed.ncbi.nlm.nih.gov/20609092/).
36. Mazza A, Fruci B, Garinis GA, Giuliano S, Malaguarnera R, Belfiore A. The role of metformin in the management of NAFLD. *Experimental diabetes research*. 2012; 2012:716404. Epub 2011/12/24. doi: [10.1155/2012/716404](https://doi.org/10.1155/2012/716404) PMID: [22194737](https://pubmed.ncbi.nlm.nih.gov/22194737/); PubMed Central PMCID: PMCPmc3238361.
37. El Messaoudi S, Rongen GA, de Boer RA, Riksen NP. The cardioprotective effects of metformin. *Current opinion in lipidology*. 2011; 22(6):445–53. Epub 2011/09/08. doi: [10.1097/MOL.0b013e32834ae1a7](https://doi.org/10.1097/MOL.0b013e32834ae1a7) PMID: [21897229](https://pubmed.ncbi.nlm.nih.gov/21897229/).
38. Bhamra GS, Hausenloy DJ, Davidson SM, Carr RD, Paiva M, Wynne AM, et al. Metformin protects the ischemic heart by the Akt-mediated inhibition of mitochondrial permeability transition pore opening. *Basic research in cardiology*. 2008; 103(3):274–84. Epub 2007/12/15. doi: [10.1007/s00395-007-0691-y](https://doi.org/10.1007/s00395-007-0691-y) PMID: [18080084](https://pubmed.ncbi.nlm.nih.gov/18080084/).
39. Li J, Deng J, Sheng W, Zuo Z. Metformin attenuates Alzheimer's disease-like neuropathology in obese, leptin-resistant mice. *Pharmacology, biochemistry, and behavior*. 2012; 101(4):564–74. Epub 2012/03/20. doi: [10.1016/j.pbb.2012.03.002](https://doi.org/10.1016/j.pbb.2012.03.002) PMID: [22425595](https://pubmed.ncbi.nlm.nih.gov/22425595/); PubMed Central PMCID: PMCPmc3327803.
40. de la Monte SM, Wands JR. Alzheimer's disease is type 3 diabetes-evidence reviewed. *Journal of diabetes science and technology*. 2008; 2(6):1101–13. Epub 2009/11/04. PMID: [19885299](https://pubmed.ncbi.nlm.nih.gov/19885299/); PubMed Central PMCID: PMCPmc2769828.
41. Matsuzaki T, Sasaki K, Tanizaki Y, Hata J, Fujimi K, Matsui Y, et al. Insulin resistance is associated with the pathology of Alzheimer disease: the Hisayama study. *Neurology*. 2010; 75(9):764–70. Epub 2010/08/27. doi: [10.1212/WNL.0b013e3281eee25f](https://doi.org/10.1212/WNL.0b013e3281eee25f) PMID: [20739649](https://pubmed.ncbi.nlm.nih.gov/20739649/).
42. Imfeld P, Bodmer M, Jick SS, Meier CR. Metformin, other antidiabetic drugs, and risk of Alzheimer's disease: a population-based case-control study. *Journal of the American Geriatrics Society*. 2012; 60(5):916–21. Epub 2012/03/31. doi: [10.1111/j.1532-5415.2012.03916.x](https://doi.org/10.1111/j.1532-5415.2012.03916.x) PMID: [22458300](https://pubmed.ncbi.nlm.nih.gov/22458300/).
43. El-Mir MY, Demaille D, G RV, Delgado-Esteban M, Guigas B, Attia S, et al. Neuroprotective role of antidiabetic drug metformin against apoptotic cell death in primary cortical neurons. *J Mol Neurosci*. 2008; 34(1):77–87. Epub 2007/11/28. doi: [10.1007/s12031-007-9002-1](https://doi.org/10.1007/s12031-007-9002-1) PMID: [18040888](https://pubmed.ncbi.nlm.nih.gov/18040888/).
44. Adeyemi OO, Ishola IO, Adedeji HA. Novel action of metformin in the prevention of haloperidol-induced catalepsy in mice: Potential in the treatment of Parkinson's disease? *Progress in neuro-psychopharmacology & biological psychiatry*. 2013. Epub 2014/02/12. doi: [10.1016/j.pnpbp.2013.10.014](https://doi.org/10.1016/j.pnpbp.2013.10.014) PMID: [24513020](https://pubmed.ncbi.nlm.nih.gov/24513020/).
45. Musi N, Hirshman MF, Nygren J, Svanfeldt M, Bavenholm P, Rooyackers O, et al. Metformin increases AMP-activated protein kinase activity in skeletal muscle of subjects with type 2 diabetes. *Diabetes*. 2002; 51(7):2074–81. Epub 2002/06/28. PMID: [12086935](https://pubmed.ncbi.nlm.nih.gov/12086935/).
46. Bayliss JA, Lemus M, Santos VV, Deo M, Elsworth J, Andrews ZB. Acylated but not des-acyl ghrelin is neuroprotective in an MPTP mouse model of Parkinson's Disease. *J Neurochem*. 2016. doi: [10.1111/jnc.13576](https://doi.org/10.1111/jnc.13576) PMID: [26872221](https://pubmed.ncbi.nlm.nih.gov/26872221/).
47. Jiang T, Yu JT, Zhu XC, Wang HF, Tan MS, Cao L, et al. Acute metformin preconditioning confers neuroprotection against focal cerebral ischaemia by pre-activation of AMPK-dependent autophagy. *British journal of pharmacology*. 2014; 171(13):3146–57. Epub 2014/03/13. doi: [10.1111/bph.12655](https://doi.org/10.1111/bph.12655) PMID: [24611741](https://pubmed.ncbi.nlm.nih.gov/24611741/); PubMed Central PMCID: PMCPmc4080970.
48. Lu DY, Tang CH, Chen YH, Wei IH. Berberine suppresses neuroinflammatory responses through AMP-activated protein kinase activation in BV-2 microglia. *Journal of cellular biochemistry*. 2010; 110(3):697–705. Epub 2010/06/01. doi: [10.1002/jcb.22580](https://doi.org/10.1002/jcb.22580) PMID: [20512929](https://pubmed.ncbi.nlm.nih.gov/20512929/).
49. Kim YS, Joh TH. Microglia, major player in the brain inflammation: their roles in the pathogenesis of Parkinson's disease. *Experimental & molecular medicine*. 2006; 38(4):333–47. Epub 2006/09/06. doi: [10.1038/emm.2006.40](https://doi.org/10.1038/emm.2006.40) PMID: [16953112](https://pubmed.ncbi.nlm.nih.gov/16953112/).
50. Chakraborty A, Chowdhury S, Bhattacharyya M. Effect of metformin on oxidative stress, nitrosative stress and inflammatory biomarkers in type 2 diabetes patients. *Diabetes research and clinical practice*. 2011; 93(1):56–62. Epub 2010/12/15. doi: [10.1016/j.diabres.2010.11.030](https://doi.org/10.1016/j.diabres.2010.11.030) PMID: [21146883](https://pubmed.ncbi.nlm.nih.gov/21146883/).
51. Hirsch HA, Iliopoulos D, Struhl K. Metformin inhibits the inflammatory response associated with cellular transformation and cancer stem cell growth. *Proc Natl Acad Sci U S A*. 2013; 110(3):972–7. Epub

- 2013/01/02. doi: [10.1073/pnas.1221055110](https://doi.org/10.1073/pnas.1221055110) PMID: [23277563](https://pubmed.ncbi.nlm.nih.gov/23277563/); PubMed Central PMCID: PMCPmc3549132.
52. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. *J Neurochem*. 1990; 54(3):823–7. Epub 1990/03/01. PMID: [2154550](https://pubmed.ncbi.nlm.nih.gov/2154550/).
53. Finkel T, Deng CX, Mostoslavsky R. Recent progress in the biology and physiology of sirtuins. *Nature*. 2009; 460(7255):587–91. Epub 2009/07/31. doi: [10.1038/nature08197](https://doi.org/10.1038/nature08197) PMID: [19641587](https://pubmed.ncbi.nlm.nih.gov/19641587/); PubMed Central PMCID: PMCPmc3727385.
54. Caton PW, Nayuni NK, Kieswich J, Khan NQ, Yaqoob MM, Corder R. Metformin suppresses hepatic gluconeogenesis through induction of SIRT1 and GCN5. *J Endocrinol*. 2010; 205(1):97–106. Epub 2010/01/23. doi: [10.1677/joe-09-0345](https://doi.org/10.1677/joe-09-0345) PMID: [20093281](https://pubmed.ncbi.nlm.nih.gov/20093281/).
55. Mitchell SJ, Martin-Montalvo A, Mercken EM, Palacios HH, Ward TM, Abulwerdi G, et al. The SIRT1 activator SRT1720 extends lifespan and improves health of mice fed a standard diet. *Cell reports*. 2014; 6(5):836–43. Epub 2014/03/04. doi: [10.1016/j.celrep.2014.01.031](https://doi.org/10.1016/j.celrep.2014.01.031) PMID: [24582957](https://pubmed.ncbi.nlm.nih.gov/24582957/); PubMed Central PMCID: PMCPmc4010117.
56. Bordone L, Cohen D, Robinson A, Motta MC, van Veen E, Czopik A, et al. SIRT1 transgenic mice show phenotypes resembling calorie restriction. *Aging cell*. 2007; 6(6):759–67. Epub 2007/09/20. doi: [10.1111/j.1474-9726.2007.00335.x](https://doi.org/10.1111/j.1474-9726.2007.00335.x) PMID: [17877786](https://pubmed.ncbi.nlm.nih.gov/17877786/).
57. Boily G, Seifert EL, Bevilacqua L, He XH, Sabourin G, Estey C, et al. SirT1 regulates energy metabolism and response to caloric restriction in mice. *PLoS One*. 2008; 3(3):e1759. Epub 2008/03/13. doi: [10.1371/journal.pone.0001759](https://doi.org/10.1371/journal.pone.0001759) PMID: [18335035](https://pubmed.ncbi.nlm.nih.gov/18335035/); PubMed Central PMCID: PMCPmc2258149.
58. Aatsinki SM, Buler M, Salomaki H, Koulu M, Pavek P, Hakkola J. Metformin induces PGC-1alpha expression and selectively affects hepatic PGC-1alpha functions. *British journal of pharmacology*. 2014; 171(9):2351–63. Epub 2014/01/17. doi: [10.1111/bph.12585](https://doi.org/10.1111/bph.12585) PMID: [24428821](https://pubmed.ncbi.nlm.nih.gov/24428821/); PubMed Central PMCID: PMCPmc3997275.
59. Suwa M, Egashira T, Nakano H, Sasaki H, Kumagai S. Metformin increases the PGC-1alpha protein and oxidative enzyme activities possibly via AMPK phosphorylation in skeletal muscle in vivo. *Journal of applied physiology (Bethesda, Md: 1985)*. 2006; 101(6):1685–92. Epub 2006/08/12. doi: [10.1152/japplphysiol.00255.2006](https://doi.org/10.1152/japplphysiol.00255.2006) PMID: [16902066](https://pubmed.ncbi.nlm.nih.gov/16902066/).
60. St-Pierre J, Drori S, Uldry M, Silvaggi JM, Rhee J, Jager S, et al. Suppression of reactive oxygen species and neurodegeneration by the PGC-1 transcriptional coactivators. *Cell*. 2006; 127(2):397–408. PMID: [17055439](https://pubmed.ncbi.nlm.nih.gov/17055439/).
61. Shin JH, Ko HS, Kang H, Lee Y, Lee YI, Pletinkova O, et al. PARIS (ZNF746) repression of PGC-1alpha contributes to neurodegeneration in Parkinson's disease. *Cell*. 2011; 144(5):689–702. Epub 2011/03/08. doi: [10.1016/j.cell.2011.02.010](https://doi.org/10.1016/j.cell.2011.02.010) PMID: [21376232](https://pubmed.ncbi.nlm.nih.gov/21376232/); PubMed Central PMCID: PMC3063894.
62. Clark J, Reddy S, Zheng K, Betensky RA, Simon DK. Association of PGC-1alpha polymorphisms with age of onset and risk of Parkinson's disease. *BMC medical genetics*. 2011; 12:69. Epub 2011/05/21. doi: [10.1186/1471-2350-12-69](https://doi.org/10.1186/1471-2350-12-69) PMID: [21595954](https://pubmed.ncbi.nlm.nih.gov/21595954/); PubMed Central PMCID: PMCPmc3112073.
63. Price NL, Gomes AP, Ling AJ, Duarte FV, Martin-Montalvo A, North BJ, et al. SIRT1 is required for AMPK activation and the beneficial effects of resveratrol on mitochondrial function. *Cell Metab*. 2012; 15(5):675–90. Epub 2012/05/09. doi: [10.1016/j.cmet.2012.04.003](https://doi.org/10.1016/j.cmet.2012.04.003) PMID: [22560220](https://pubmed.ncbi.nlm.nih.gov/22560220/); PubMed Central PMCID: PMCPmc3545644.
64. Mulherin AJ, Oh AH, Kim H, Grieco A, Lauffer LM, Brubaker PL. Mechanisms underlying metformin-induced secretion of glucagon-like peptide-1 from the intestinal L cell. *Endocrinology*. 2011; 152(12):4610–9. Epub 2011/10/06. doi: [10.1210/en.2011-1485](https://doi.org/10.1210/en.2011-1485) PMID: [21971158](https://pubmed.ncbi.nlm.nih.gov/21971158/).
65. Bertilsson G, Patrone C, Zachrisson O, Andersson A, Dannaeus K, Heidrich J, et al. Peptide hormone exendin-4 stimulates subventricular zone neurogenesis in the adult rodent brain and induces recovery in an animal model of Parkinson's disease. *Journal of neuroscience research*. 2008; 86(2):326–38. Epub 2007/09/07. doi: [10.1002/jnr.21483](https://doi.org/10.1002/jnr.21483) PMID: [17803225](https://pubmed.ncbi.nlm.nih.gov/17803225/).
66. Li Y, Perry T, Kindy MS, Harvey BK, Tweedie D, Holloway HW, et al. GLP-1 receptor stimulation preserves primary cortical and dopaminergic neurons in cellular and rodent models of stroke and Parkinsonism. *Proc Natl Acad Sci U S A*. 2009; 106(4):1285–90. Epub 2009/01/24. doi: [10.1073/pnas.0806720106](https://doi.org/10.1073/pnas.0806720106) PMID: [19164583](https://pubmed.ncbi.nlm.nih.gov/19164583/); PubMed Central PMCID: PMCPmc2633544.