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Short report

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Conditional disruption of AMP kinase in dopaminergic neurons promotes Parkinson's disease-associated phenotypes in mice

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Keywords: Parkinson's disease; AMPK; mitochondria

Abstract

Emerging studies implicate energy dysregulation as an underlying trigger for Parkinson's disease (PD), suggesting that a better understanding of the molecular pathways governing energy homeostasis could help elucidate therapeutic targets for the disease. A critical cellular energy regulator is AMP kinase (AMPK), which we have previously shown to be protective in PD. However, precisely how AMPK function impacts on dopaminergic neuronal survival and disease pathogenesis remains elusive. Here, we created a tissue-specific AMPK-knockout mouse model where the catalytic subunits of AMPK are ablated in nigral dopaminergic neurons. Using this model, we demonstrated that loss of AMPK function promotes dopaminergic neurodegeneration and associated locomotor aberrations. Accompanying this is a substantial reduction in the number of mitochondria in the surviving AMPK-deficient nigral dopaminergic neurons, suggesting that an impairment in mitochondrial biogenesis may underlie the observed PD-associated phenotypes. Importantly, the loss of AMPK function enhances the susceptibility of nigral dopaminergic neurons in these mice to 6-hydroxydopamine-induced toxicity. Taken together, these findings highlight the importance of neuronal energy homeostasis by AMPK in PD and position AMPK pathway as an attractive target for future therapeutic exploitation.

Background

Parkinson's disease (PD) is a prevalent neurodegenerative movement disorder that is characterized pathologically principally by the loss of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc), which normally innervate the striatum to form the nigrostriatal pathway that is important for movement coordination. Although the mechanism underlying the pathology in PD remains unclear, emerging evidence suggests that energy dysregulation may predispose DA neurons toward degeneration. Notably, SNpc DA neurons are known to exhibit high energy demand as a result of their unique characteristics, including possessing a complex neuronal architecture, poorly myelinated axons and pacemaker firing driven by bioenergetically-expensive L-type Ca^{2+} currents (1-3). Given this, one could appreciate the need for SNpc DA neurons to maintain exquisite energy homeostasis for optimal function and survival.

A critical energy regulator in eukaryotic cells is 5' adenosine monophosphate-activated protein kinase (AMPK). AMPK exists as a heterotrimer comprised of a catalytic α subunit ($\alpha 1$ or $\alpha 2$) and two regulatory subunits: β ($\beta 1$ or $\beta 2$) and γ subunits ($\gamma 1$, $\gamma 2$ or $\gamma 3$). The enzyme becomes activated upon phosphorylation of the α subunit on threonine-172 in response to falling energy supply, e.g. ATP depletion or glucose starvation. Using phosphorylated AMPK as a surrogate marker for energy demand, we have recently reported that its physiological level is disproportionately elevated in the ventral midbrain (containing SNpc) compared to various other regions in the mouse brain, which reflects its unique energy requirements (4). Conversely, AMPK activation is selectively disrupted in the midbrain of Parkin- and PTEN-induced kinase 1 (PINK1)-deficient mice; the loss of function of which in humans causes recessive Parkinsonism (4). Our results suggest that disruption of the AMPK pathway may precipitate PD whereas augmenting AMPK activity may mitigate the disease. Supporting this, we have demonstrated that AMPK activation ameliorates the disease phenotypes in *Drosophila* genetic

Short Report

models of PD (5), and in a manner that is dependent on its downstream target peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), a regulator of mitochondrial biogenesis (6). We further shown that pharmacological administration of AMPK activators restores the levels of midbrain pAMPK and PGC-1 α expression in Parkin-deficient mice (4).

To test our hypothesis that deficiency in AMPK activity predisposes DA neurons toward degeneration, we report here the generation and characterization of a AMPK mutant mouse model where the catalytic α 1 and α 2 subunits are conditionally ablated in DA neurons. The conditional AMPK knockout (AMPK-cKO) animal exhibits DA neuronal loss and associated locomotor deficits. We also recorded a significant reduction in the number of mitochondria in AMPK-deficient neurons, that is consistent with the role of AMPK in promoting mitochondrial biogenesis. Importantly, these mutant mice show enhanced susceptibility to 6-hydroxydopamine (6-OHDA)-induced DA neurotoxicity. Taken together, our findings highlight the importance of AMPK in DA neuronal homeostasis and its utility as a potential therapeutic target for PD.

Materials and Methods

Antibodies. The following antibodies were used: monoclonal anti-phospho-AMPK α 1/ α 2 (Cell Signaling Technology Cat# 2535), anti-AMPK α 1/ α 2 (Abcam Cat# ab80039) and anti- β -actin (Sigma Cat# A5441); polyclonal anti-tyrosine hydroxylase (TH) (Pel-Freez Biologicals Cat# P40101-0) and anti-TOM-20 (Santa Cruz Biotechnology Cat# sc-11415); anti-mouse horseradish peroxidase and anti-rabbit horseradish peroxidase (GE Healthcare, UK).

Generation of TH-specific AMPK α 1/ α 2-knockout (AMPK-cKO) mice. All animal-related studies were approved by and conformed to the guidelines of the Institutional Animal Care and Use Committee (TTSH-NNI: TNI-15-13-021). Mice were maintained on a 12-h light/dark cycle with food and water available *ad libitum*. Transgenic TH-Cre mice (*TH-Cre*) were crossed with AMPK floxed mice that were homozygous for *Prkaa1* (AMPK α 1) and *Prkaa2* (AMPK α 2) floxed genes (*Ampk^{ff}*), generating offspring (50%) that were heterozygous for AMPK α 1/ α 2 floxed gene and TH-Cre (*TH-Cre; Ampk^{ff/+}*). *TH-Cre; Ampk^{ff/+}* mice were backcrossed to parental *Ampk^{ff}* mice to give *TH-Cre; Ampk^{ff}* mice (12.5%, AMPK-cKO mice) and *Ampk^{ff}* mice (12.5%, AMPK-WT mice). Subsequently, AMPK-cKO mice were crossed with AMPK-WT mice to generate offspring of the same genotypes in the ratio of 1:1 based on Mendelian inheritance. Genomic DNA was extracted from mouse tails and the presence of TH-Cre gene, AMPK α 1 and AMPK α 2 floxed genes were verified using PCR. A summary of the primers used and expected PCR products is provided in Table 1. The PCR products were separated on a 1% agarose gel for visualization.

Table 1. Primers used to genotype AMPK-WT and AMPK-cKO mice and their expected PCR products

Gene	Primer	Primer sequence (5'→3')	Expected PCR product (bp)
<i>Prkaa1</i> floxed	Forward	CCCACCATCACTCCATCTCT	450
	Reverse	AGCCTGCTTGGCACACTTAT	
<i>Prkaa2</i> floxed	Forward	GCAGGCGAATTTCTGAGTTC	450
	Reverse	TCCCCTTGAACAAGCATACC	
<i>TH-Cre</i>	Forward	GCGGTCTGGCAGTAAAACTATC	100
	Reverse	GTGAAACAGCATTGCTGTCACCT	
<i>TH-Cre</i> internal positive control	Forward	CTAGGCCACAGAATTGAAAGATCT	324
	Reverse	GTAGGTGGAAATTCTAGCATCATCC	

Mice behavioral assessment. All animals were transported to the animal behavioral room for habituation to the novel room at least 30 minutes prior to experiments. Locomotor activity recordings were carried out in an opaque open field arena (45 x 45 cm). The testing apparatus was enclosed in a ventilated, quiet and dim procedure room to minimize animals' stress levels while allowing normal exploratory behavior. Animals were introduced into the arena and allowed to explore freely for 10 minutes. Exploratory behavior was recorded using a monochrome industrial camera (DMK 22AUC03, The Imaging Source, Taiwan) and locomotor activity data was collected using the ANY-maze video tracking system. Motor performance assays were also carried out using a rotarod as previously described (7).

Short Report

Generation and characterization of 6-OHDA lesion PD model and apomorphine-induced rotation assay. 7 AMPK-WT or 13 AMPK-cKO mice were lesioned with 1.5 μ l of 5 μ g/ μ L 6-hydroxydopamine (6-OHDA) using a method previously described (7). For every week post-surgery, up to five weeks, the apomorphine-induced rotation assay was carried out and the number of rotations made in one minute was counted and recorded. 4 to 5-month-old wild-type C57B6 male mice injected with 6-OHDA were sacrificed via cervical dislocation. Mice brains were harvested and processed for western blot analysis as previously described (4).

Immunohistochemistry, Stereological analysis of TH-positive neurons and mitochondrial analysis. Mice brains were processed for immunohistochemistry and stereological analysis as previously described (7). For mitochondrial analysis, mice brains were sectioned in the coronal plane at 10 μ m thickness. Brain sections containing the SNpc were washed with washing buffer (0.1% Triton X-100/PBS) before they were blocked with blocking buffer (5% NGS in 0.1% Triton X-100/PBS) for one hour at room temperature. Brain sections were then incubated in anti-TH and anti-TOM20 antibodies in blocking buffer overnight at 4°C and washed with washing buffer the next day. Following which, brains sections were incubated in secondary fluorescent antibodies at room temperature for four hours and washed with washing buffer before being mounted on glass slides with Vectorshield mounting media containing DAPI. Slides were viewed under the Olympus Fluoview FV1000 Laser Scanning Confocal Microscope. Confocal images of 20 stained mice brain sections (~1000 TH-positive cells) per group were analysed using a MATLAB algorithm that was modified from (8).

Short Report

Statistical analysis. Statistical analyses were performed using the student's two-tailed unpaired t-test (* $p < 0.05$, ** $p < 0.001$ or unless otherwise stated). All data was expressed as mean (S.D.) generated from at least three independent experiments unless otherwise stated.

Results

AMPK α expression is abolished in TH-expressing cells of AMPK-cKO mice brains

We used the Cre/loxP-mediated recombination strategy to conditionally target the *Prkaa1/2* loci in the mouse brain. AMPK floxed mice (*Prkaa1/2^{ff}*) in which the sequence encoding the catalytic site of both of the α subunits was flanked by loxP sequences has previously been described (9). To obtain DA neuronal-specific disruption of AMPK activity, *Prkaa1/2^{ff}* mice were crossed with *TH-Cre* transgenic mice expressing Cre recombinase under the control of the tyrosine hydroxylase promoter, to create *TH-Cre; Prkaa1/2^{ff}* (*Prkaa1/2^{ff}-TH-cKO*) mice (herein refers to as AMPK cKO mice). Using PCR, we verified the presence of *Prkaa1/2^{ff}* in AMPK-WT mice and presence of both *TH-Cre* and *Prkaa1/2^{ff}* in AMPK-cKO mice (Fig. 1A). We further ascertained the deletion of AMPK α subunits in TH-expressing cells via immunofluorescence staining of AMPK-WT and AMPK-cKO mice brain sections using antibodies specific to AMPK- α 1 and AMPK- α 2 subunits. In both the nigral and striatal regions of AMPK-cKO mice, the expression of AMPK- α 1 is significantly reduced ($p=0.0000222$) (Fig. 1B-C and E). Unlike the predominantly cytoplasmic AMPK- α 1 species, AMPK- α 2 is normally localized to the nucleus (10). Thus, we examined AMPK- α 2 expression mainly in the SNpc DA neurons where the cell bodies reside. We similarly recorded a significant reduction in AMPK- α 2 expression in AMPK-cKO compared to AMPK-WT mice ($p=0.00161$) (Fig. 1D-E). Whereas dual knockout of the genes encoding AMPK- α 1 and AMPK- α 2 in the germline is embryonic lethal (11), AMPK-cKO mice do not exhibit developmental defects, gross abnormalities, or accelerated mortality (not shown).

AMPK-cKO mice have reduced dopaminergic neuronal count and exhibit locomotor deficits compared to age-matched AMPK-WT mice

Working on the premise that AMPK is critical for DA neuronal survival, we anticipate that AMPK deficiency predisposes DA neurons toward degeneration. As early as three-months-old, AMPK-cKO mice exhibit reduced TH-positive staining of the neurites in the striatum and the cell bodies in the SNpc (Fig. 2A). This difference is significant when quantified using an unbiased stereology-based approach ($p=0.00369$) and persists even after aging to 22 months old ($p=0.00641$) (Fig. 2B). As SNpc DA neurons are predominantly responsible for modulating locomotion, we anticipate that the loss of these neurons in the AMPK-cKO mice will result in locomotor deficits, which are characteristic of mice with PD (12). The locomotor function of the AMPK-cKO mice and their wild-type counterparts were assessed using the open field and rotarod tests at various experimental time points (3, 8, 12 and 22 months old) in order to account for age-associated changes. In general, AMPK-cKO mice travel much less than AMPK-WT mice in the open field, as evident from the representative track plots (Fig. 2C). At three months old, the total distance travelled horizontally by the AMPK-cKO mice within the arena is significantly reduced when compared to age-matched AMPK-WT mice ($p=0.00129$) (Fig. 2C). Likewise, vertical rearing activity in the arena is also markedly reduced in the AMPK-cKO mice when compared to AMPK-WT mice and this difference persists through age ($p=0.0253$ at 3 months; $p=0.00108$ at 8 months; $p=0.0101$ at 12 months; $p=0.0229$ at 22 months) (Fig. 2D). This is further supported by observations from the rotarod assay, a commonly used behavioural assessment for motor coordination, where AMPK-cKO mice falls off the accelerating rod more readily than AMPK-WT mice from eight to 22 months old ($p=0.0000422$ at 8 months; $p=0.0120$ at 12 months; $p=0.0408$ at 22 months) (Fig. 2E), suggesting that motor coordination is impaired in the AMPK-cKO mice, especially as they age. Taken together, these findings indicate that loss of AMPK function predisposes

Short Report

SNpc DA neurons to earlier degeneration, which manifests phenotypically in the form of locomotor deficits.

AMPK-cKO mice display aberrations in mitochondrial homeostasis

To better understand the molecular underpinnings of our observations, we examined for any changes in terms of mitochondrial homeostasis, considering that the protective effects of AMPK in ameliorating PD-related phenotypes is mediated by its downstream effector and mitochondrial biogenesis regulator PGC-1 α (6). From the representative confocal images, there are fewer mitochondria (reduction in TOM20 staining) in the TH-expressing nigral DA neurons of AMPK-cKO mice compared to AMPK-WT mice (Fig. 3A). When quantified using a MATLAB algorithm, this reduction in the number of mitochondria is significant ($p=0.0169$) (Fig. 3B), indicating the possibility of impaired mitochondrial biogenesis or enhanced mitophagy (clearance of damaged mitochondria). Since mitophagy is typically preceded by mitochondrial fission, we examined whether the mitochondria in AMPK-deficient nigral DA neurons tended toward fragmentation. However, the average mitochondrial sizes and aspect ratios were comparable between the AMPK-WT and AMPK-cKO groups ($p=0.174$) (Fig. 3B), suggesting that impaired mitophagy is an unlikely explanation for the reduction in the number of mitochondria in AMPK-deficient nigral DA neurons. Collectively, these findings indicate that AMPK deficiency affects mitochondrial homeostasis, in particular mitochondrial biogenesis.

AMPK-cKO mice are more susceptible to 6-hydroxydopamine (6-OHDA)-mediated toxicity

Next, we wondered if these mice would be more susceptible to mitochondrial toxins given the changes in mitochondrial homeostasis. To study this, we subjected them to a PD-related toxin 6-hydroxydopamine (6-OHDA) that is known to form free radicals and potentially inhibit mitochondrial complexes I and IV. In wild-type mice injected with 6-OHDA, levels of the phosphorylated and active form of AMPK (pAMPK) is upregulated in the ventral midbrain region (containing SNpc) of the injected hemisphere (Fig. 4A), possibly a result of mitochondrial dysfunction that triggers a consequent reduction in ATP production (13). With the knowledge that functional AMPK signalling is required for prompt cellular response to metabolic changes and stresses (14), we speculate that this upregulation of AMPK activity may mediate a protective response against 6-OHDA-mediated toxicity and accordingly, loss of AMPK function may enhance susceptibility towards this toxin. Indeed, there is a significant reduction in the number of TH-positive nigral DA neurons on the injected (ipsilateral) side of AMPK-cKO mice compared to that of AMPK-WT mice ($p=0.0117$) (Fig. 4C). This can be visualized rather apparently from the representative images of mice brain sections stained with TH (Fig. 4D). Unlike the AMPK-WT mice, AMPK-cKO mice have complete lesioning of the striatum at five weeks post-lesion accompanying the dramatic reduction in SNpc TH-staining (Fig. 4C-D). Loss of DA neurons unilaterally gives rise to a rotational phenotype in the contralateral direction when these mice are injected with apomorphine, a DA agonist (15), which can be quantified to reflect the severity of the lesion. From two to five weeks post-surgery, AMPK-cKO mice exhibit more pronounced rotational behaviour compared to AMPK-WT mice ($p=0.0225$ at week 2; $p=0.00126$ at week 3; $p=0.00621$ at week 4; $p=0.0141$ at week 5) (Fig. 4B). Collectively, these results show that the ablation of AMPK catalytic subunits aggravates 6-OHDA-induced DA neuronal loss.

Discussion

To study the role of AMPK in DA neurons and PD pathogenesis, we generated a conditional AMPK-KO mouse model where the catalytic alpha subunits of AMPK are ablated in TH-expressing DA neurons using the Cre/loxP-mediated recombination strategy. AMPK-cKO mice exhibit accelerated DA neuronal loss and associated locomotor deficits. Accompanying this is a reduction in the number of mitochondria in the surviving AMPK-deficient nigral DA neurons, suggesting that aberrant mitochondrial biogenesis may underlie the PD-associated phenotypes observed during AMPK deficiency. Importantly, the AMPK-cKO mice are more susceptible to DA neurotoxicity mediated by a mitochondrial and PD-related toxin, 6-OHDA. Taken together, our findings highlight the importance of AMPK in DA neuronal homeostasis and thereby its therapeutic potential for PD.

To date, studies that support a neuroprotective role of AMPK in PD mostly employed an overexpression or activation strategy, including our previous study in the *Drosophila* (5). To confirm these findings, it is necessary to evaluate the effects of AMPK ablation, which is anticipated to promote DA neurodegeneration. However, *in vivo* AMPK-KO models designed to study AMPK's function in the brain are few and far between, much less in neurodegenerative diseases like PD (Viollet et al., 2009). One such study by Bayliss et al. created a similar tissue-specific knockout model where AMPK β 1 and β 2 regulatory subunits (rather than the catalytic AMPK α subunits) are specifically deleted in dopamine transporter (DAT)-expressing DA neurons (16). Using this model, they demonstrated that the protective effects of calorie-restriction against MPTP toxicity occurs through the ghrelin-AMPK partnership. However, the baseline phenotypes of these conditional AMPK-KO mice were not reported, and it remains unclear what the effects of dysfunctional AMPK on DA neurons are. Addressing this gap, we showed here that loss of AMPK function in mice promotes nigral DA neurodegeneration and gives rise to locomotor deficits. Supporting this, several studies in *Drosophila* showed that

Short Report

genetic loss-of-function of AMPK regulatory subunits (β or γ), or the expression of a dominant-negative form of AMPK results in overt neurodegeneration, reduction in locomotor activity and overall survival (17-19). In mice, ablating the catalytic subunits of AMPK in photoreceptors leads to early retinal defects including axonal retraction and synaptic alterations that is normally associated with aging (20). Although these findings are not specific to the context of nigral DA neurons, they collectively highlight the importance of an intact AMPK heterotrimer in neuronal survival. Our study concurs with these by further demonstrating that AMPK's catalytic function is critical to nigral DA neuronal homeostasis.

At the molecular level, we showed that the PD-associated phenotypes in AMPK-cKO mice occur concurrently with a reduction in the number of mitochondria, suggesting that aberrant mitochondrial biogenesis likely underlies the degeneration of AMPK-deficient DA neurons. As the energy powerhouse, mitochondria play an important role in energy metabolism in neurons, which are especially dependent on oxidative phosphorylation. Our previous work demonstrated that AMPK-mediated rescue of mitochondrial abnormalities in PD mutant flies is dependent on the mitochondrial biogenesis regulator PGC-1 α (6). Conversely, when AMPK activity is blocked in rat cortical neurons, the regulation of downstream PGC-1 α -NRF-1 pathway is interrupted and neuronal activity becomes uncoupled from mitochondrial energy metabolism (21). In line with this, we found that the mitochondria numbers in nigral DA neurons of AMPK-deficient mice are reduced, reflecting a dysfunctional AMPK-PGC-1 α axis. Since the ability of the cell to ensure a steady supply of healthy mitochondria is impaired, mitochondrial reactive oxygen species (mROS) may be elevated, as reported in AMPK-KO mouse embryonic fibroblasts that undergo premature senescence due to elevated mROS (22). Given this, it seems logical that the AMPK-cKO mice are more vulnerable to 6-OHDA, whose toxicity is largely accepted to be mediated through oxidative stress (23). We (i.e. current observations) and others have shown that 6-OHDA enhances AMPK phosphorylation, perhaps

Short Report

as an attempt to cope with 6-OHDA-mediated toxicity (13). When mROS increases, AMPK activates downstream mitochondria-related pathways, including PGC-1 α -dependent mitochondrial biogenesis and antioxidant response (22). When this protective mechanism is impeded in AMPK-cKO mice subjected to a PD toxin that increases ROS, the nigral DA neurons become more susceptible to degeneration. A similar observation has been reported with yet another mitochondrial and PD-related toxin MPTP, where pharmacological or genetic inhibition of AMPK further reduces cell viability (24). Taken together, these findings demonstrate that mitochondrial dyshomeostasis occurs during AMPK deficiency and this compromises the ability of nigral DA neurons to elicit a protective response in face of mitochondria-related stress.

Conclusion

In summary, our findings that AMPK deficiency in nigral DA neurons promotes their degeneration and results in locomotor aberrations in mice, supporting the protective role of AMPK in PD. We further demonstrated that changes in mitochondrial homeostasis are involved when AMPK activity is abolished in nigral DA neurons, and this correspondingly enhances vulnerability against the mitochondrial and PD toxin 6-OHDA. Our study highlights the importance of AMPK in DA neuronal homeostasis, implicating it as a potential molecular and therapeutic target for disease intervention.

Short Report

List of abbreviations

6-OHDA: 6-hydroxydopamine

AMPK: 5' adenosine monophosphate-activated protein kinase

cKO: Conditional knockout

DA: Dopaminergic

mROS: Mitochondrial reactive oxygen species

PD: Parkinson's disease

PGC-1 α : Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

PINK1: PTEN-induced kinase 1

SNpc: Substantia nigra pars compacta

TH: Tyrosine hydroxylase

WT: Wild-type

Declarations

Ethics approval and consent to participate

All animal-related studies were approved by and conformed to the guidelines of the Institutional Animal Care and Use Committee (TTSH-NNI: TNI-15-13-021) at National Neuroscience Institute, Singapore. This article does not contain any studies with human participants that were performed by any of the authors.

Consent for publication

Not applicable

Availability of supporting data

The datasets used and/or analysed during the current study are available from the

Short Report

corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HL, GG, WZ, CHY, JT performed the experiments and analysed the data. XS, LKP provided critical reagents and intellectual contributions. HL, LKL conceptualized the study, interpreted the results and wrote the manuscript. All authors read and approved the final manuscript.

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Short Report

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Figure legend

Fig. 1. AMPK α expression is abolished in TH-positive cells of AMPK-cKO mice brains.

(A) *Top*, PCR results indicating the presence of *TH-Cre* transgene (100bp product) in AMPK-cKO mice and the internal positive control (324bp product) in AMPK-WT and AMPK-cKO mice. *Bottom*, PCR results indicating the presence of *Prkaa1/2^{fl}* (450bp product) in both the AMPK-WT and AMPK-cKO mice. (B) Immunofluorescence labelling of striatal sections (Str) of AMPK-WT and AMPK-cKO mice brains with DAPI (blue, nucleus), TH (green) and AMPK α 1 (red). Merged image shows colocalization of DAPI, TH and AMPK α 1. (C) Same as (B) but in sections containing substantia nigra pars compacta (SNpc). (D) Same as (C) but red channel is AMPK α 2. (E) *Left*, representative confocal images of the respective enlarged views from (C) and (D). *Right*, quantification of the percentage of TH-positive cells expressing AMPK α 1/ α 2. Statistical analysis: student's two-tailed unpaired t-test (* p <0.05 and ** p <0.001 compared to AMPK-WT mice).

Fig. 2. AMPK-cKO mice have reduced dopaminergic neuronal count and locomotor deficits compared to age-matched AMPK-WT mice.

(A) *Left*, representative striatal sections and *right*, midbrain sections containing the SNpc of 3 months and 22 months old AMPK-WT and AMPK-cKO mice. The SNpc region is highlighted within the white dotted lines. Positive DAB staining of TH is indicated in brown. (B) *Left*, enlarged view of the SNpc regions highlighted within the white dotted lines from (A). *Right*, bar graph showing the stereological counts of nigral TH-positive DA neurons from 3 months and 22 months old AMPK-WT and AMPK-cKO mice. (C) Representative track plots, total distance travelled and (D) rearing activity of AMPK-WT ($n=19$, $n=12$, $n=20$, $n=11$) and AMPK-cKO mice ($n=19$, $n=12$, $n=25$, $n=12$) from the respective age groups (3, 8, 12 and 22 months old) during the open field test. (E) AMPK-WT and AMPK-cKO mice latency to fall from the rotarod at the same age groups.

Short Report

Statistical analysis: student's two-tailed unpaired t-test ($*p<0.05$ and $**p<0.001$ compared to AMPK-WT mice at respective time points, $^ap<0.05$ and $^{aa}p<0.001$ compared to previous time point within the same group).

Fig. 3. AMPK-cKO mice display aberrations in mitochondrial homeostasis. (A) Representative images showing immunofluorescence labelling of SNpc-containing sections of AMPK-WT and AMPK-cKO mice brains with DAPI (blue, nucleus), TH (green) and TOM20 (red, mitochondria). TOM20 staining of the cell located in the white dotted lined box is shown in enlarged view. Merged image shows colocalization of DAPI, TH and TOM20. Scale bar: 20um. (B) *Left to right*, bar graphs showing the number of mitochondria per cell, average area of mitochondria and line graph showing the distribution of the mitochondria aspect ratios (mitochondria length to width ratios) between nigral TH-positive DA neurons of AMPK-WT and AMPK-cKO mice. Statistical analysis: student's two-tailed unpaired t-test ($*p<0.05$ compared to AMPK-WT).

Fig. 4. AMPK-cKO mice are more susceptible to 6-hydroxydopamine (6-OHDA)-mediated toxicity. (A) Representative immunoblots showing the expression of pAMPK, AMPK and actin across selected brain regions (cortex, striatum and ventral midbrain) of wild-type mice in the absence or presence of 6-OHDA. (B) Number of apomorphine-induced contralateral rotations of 6-OHDA-lesioned AMPK-WT mice ($n=7$), 6-OHDA-lesioned AMPK-cKO mice ($n=13$) and sham controls ($n=6$) from week 1 to week 5 post-surgery. (C) Stereological counts of TH-positive cells in the contralateral (non-lesioned) and ipsilateral (lesioned) sides of brain sections containing SNpc from AMPK-WT or AMPK-cKO mice. (D) Representative immunohistochemical images of the SNpc and striatal (Str) sections of AMPK-WT or AMPK-cKO mice five weeks post-surgery, arranged from anterior to posterior position.

Short Report

584 Positive brown stains indicate presence of TH-positive cells. Statistical analysis: student's two-
585 tailed unpaired t-test ($*p<0.05$ compared to AMPK-WT mice, $^{\#}p<0.05$ compared to
586 contralateral side).

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Figure 1

Figure 2

A

B

C

D

E

Figure 3

Figure 4

Figures

Figure 1

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Figure 2

AMPK-cKO mice have reduced dopaminergic neuronal count and locomotor deficits compared to age-matched AMPK-WT mice. (A) Left, representative striatal sections and right, midbrain sections containing the SNpc of 3 months and 22 months old AMPK-WT and AMPK-cKO mice. The SNpc region is highlighted within the white dotted lines. Positive DAB staining of TH is indicated in brown. (B) Left, enlarged view of the SNpc regions highlighted within the white dotted lines from (A). Right, bar graph showing the stereological counts of nigral TH-positive DA neurons from 3 months and 22 months old AMPK-WT and AMPK-cKO mice. (C) Representative track plots, total distance travelled and (D) rearing activity of AMPK-WT (n=19, n=12, n=20, n=11) and AMPK-cKO mice (n=19, n=12, n=25, n=12) from the respective age groups (3, 8, 12 and 22 months old) during the open field test. (E) AMPK-WT and AMPK-cKO mice latency to fall from the rotarod at the same age groups. Statistical analysis: student's two-tailed unpaired t-test (*p<0.05 and **p<0.001 compared to AMPK-WT mice at respective time points, ap<0.05 and aap<0.001 compared to previous time point within the same group).

Figure 3

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AMPK-cKO mice display aberrations in mitochondrial homeostasis. (A) Representative images showing immunofluorescence labelling of SNpc-containing sections of AMPK-WT and AMPK-cKO mice brains with

DAPI (blue, nucleus), TH (green) and TOM20 (red, mitochondria). TOM20 staining of the cell located in the white dotted lined box is shown in enlarged view. Merged image shows colocalization of DAPI, TH and TOM20. Scale bar: 20um. (B) Left to right, bar graphs showing the number of mitochondria per cell, average area of mitochondria and line graph showing the distribution of the mitochondria aspect ratios (mitochondria length to width ratios) between nigral TH-positive DA neurons of AMPK-WT and AMPK-cKO mice. Statistical analysis: student's two-tailed unpaired t-test (* $p < 0.05$ compared to AMPK-WT).

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