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Metformin Protects Cells from Mutant Huntingtin Toxicity Through Activation of AMPK and Modulation of Mitochondrial Dynamics

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Abstract Huntington's disease (HD) is a devastating neurodegenerative disease caused by the pathological elongation of the CAG repeats in the huntingtin gene. Caloric restriction (CR) has been the most reproducible environmental intervention to improve health and prolong life span. We have demonstrated that CR delayed onset and slowed disease progression in a mouse model of HD. Metformin, an antidiabetic drug, mimics CR by acting on cell metabolism at multiple levels. Long-term administration of metformin improved health and life span in mice. In this study, we showed that metformin rescued cells from mutant huntingtin (HTT)-induced toxicity, as indicated by reduced lactate dehydrogenase (LDH) release from cells and preserved ATP levels in cells expressing mutant HTT.

Further mechanistic study indicated that metformin activated AMP-activated protein kinase (AMPK) and that inhibition of AMPK activation reduced its protective effects on mutant HTT toxicity, suggesting that AMPK mediates the protection of metformin in HD cells. Furthermore, metformin treatment prevented mitochondrial membrane depolarization and excess fission and modulated the disturbed mitochondrial dynamics in HD cells. We confirmed that metformin crossed the blood–brain barrier after oral administration and activated AMPK in the mouse brain. Our results urge further evaluation of the clinical potential for use of metformin in HD treatment.

Keywords Metformin · Huntington's disease · Mitochondria · AMPK

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Introduction

Huntington disease (HD) is a neurodegenerative disease that is caused by the pathological elongation of the CAG repeats in exon 1 of the huntingtin gene (MacDonald et al. 1993). To date, a number of palliative therapies have been shown to be effective in reducing chorea, but these treatments produce only slight symptomatic benefit. No treatment has been shown to delay onset and/or slow the progression of disease in humans. The pathogenesis of HD is manifested by dysfunction and loss of striatal neurons in the initial stages and in the later stages of the disease involves the cortex and other brain regions (Brandt et al. 1996). Although the function of normal huntingtin (HTT) is largely unknown and the reason for the increased sensitivity of striatal neurons to mutant HTT has not been identified, a role for mitochondrial/metabolic dysfunction in HD has been proposed.

Abnormalities in mitochondrial function have been observed in postmortem HD brains, including decreases in mitochondrial complex II/III activity as well as evidence of altered energy metabolism (Mann et al. 1990; Browne et al. 1997). Mitochondria from HD patients and mice show compromised ability to handle calcium (Giacomello et al. 2011). In summary, compelling evidence suggests that mitochondrial dysfunction is central to the pathogenesis of HD. In addition, energy depletion occurs in cells expressing mutant HTT, detected as reduction in adenosine triphosphate (ATP) (Milakovic and Johnson 2005). Given the evidence that bioenergetics dysfunction may contribute to the pathology of HD, treatment strategies designed to reduce these defects may be beneficial. Caloric restriction (CR) has been the most reproducible and environmental intervention to improve health and prolong life span. We have shown that CR delayed onset and slowed disease progression in a mouse model of HD and other neurodegenerative diseases (Duan et al. 2003).

Metformin, an antidiabetic drug, mimics CR, acting on cell metabolism at multiple levels (Ingram et al. 2006; Blagosklonny 2007; Ravn et al. 2013). A recent study has shown that long-term administration of metformin improved health and life span in mice (Martin-Montalvo et al. 2013). Metformin also promotes adult neurogenesis (Wang et al. 2012). At the molecular level, metformin increases AMP-activated protein kinase (AMPK) activity (Martin-Montalvo et al. 2013) and activates atypical protein kinase C (aPKC) and CREB binding protein (CBP) (Wang et al. 2012). The signaling pathways activated by metformin may counteract mutant HTT-induced toxicity and thereby protect neurons in HD. Indeed, administration of metformin in drinking water significantly prolongs survival in a R6/2 HD mouse model (Ma et al. 2007). However, in this study, the mechanism of protective effect of metformin was not further investigated. Metformin is a well-tolerated FDA-approved oral medication and rapidly crosses the blood–brain barrier in rats (Labuzek et al. 2010). The compelling evidence suggests that metformin may be an ideal repositioning drug for HD treatment.

On the basis of the information described above, we investigated the regulatory role of metformin in mutant HTT-induced energy depletion and cell toxicity. Our data suggest that metformin protects striatal cells against mutant HTT toxicity and, by using compound C as an AMPK inhibitor, further confirmed our hypothesis that AMPK is involved in the mechanism of protection by metformin in HD cells. Orally administered metformin crosses the blood–brain barrier and activates AMPK in the striatum in mice. The current study supports a speedy and relatively low-cost strategy for the development of new HD therapeutics.

Materials and Methods

Materials

Metformin was purchased from Cayman Chemical (Michigan, USA). Compound C was purchased from Sigma (USA.). A potentiometric fluorescent dye tetramethylrhodamine ethyl ester (TMRE) was purchased from Life Technology (USA.); $iTaq^{TM}$ universal SYBR® Green Supermix was purchased from Bio-Rad (USA). Antibodies were obtained from the following commercial sources: mouse actin (Sigma); total mouse AMPK (Cell Signaling); phospho-AMPK (Cell Signaling); and anti-Tomm-20 (Santa Cruz Biotechnology). Protein assay BCA kits were purchased from Thermo Scientific. Cell culture medium and supplements including Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin/streptomycin, G418, and GlutaMAX were purchased from Life Technology (USA). All other chemicals and reagents were commercially available and of the highest grade. Dominant-negative (DN) myc-tag labeled AMPKα cDNA construct in pcDNA3 (Invitrogen) plasmid was generously provided by Dr. David Carling MRC Clinical Sciences Centre, Imperial College, London, UK, the plasmid was amplified, and cDNAs were extracted and provided by Dr. Edward Gabrielson Laboratory at Johns Hopkins University School of Medicine.

Immortalized Striatal Cells Culture and Treatment

Immortalized striatal precursor cells with normal HTT (SThdh$^{Q7/Q7}$) or mutant HTT (SThdh$^{Q111/Q111}$) were derived from HTT knock-in mice and were kindly provided by Dr. Marcy McDonald[25]. These cells were maintained at 33 °C in high-glucose DMEM medium (Invitrogen) with 10 % fetal bovine serum (FBS, GIBCO), 1 % penicillin–streptomycin (Invitrogen), 1 % L-glutamate (Invitrogen), and 400 μg/ml of G418 (Mediatech), in a humidified atmosphere of 95 % air: 5 % CO$_2$. Cells were cultured in 96-well or 24-well plates for 24 h to about 70 % confluence. Then cells were switched to serum free medium. Different concentrations of metformin were then added to the medium and incubated for another 24 h. For the compound C-treated group, 1 μM compound C was used. After 24 h, medium LDH and cellular ATP levels were assessed, respectively. For DN-AMPKα transfection, cells were transfected with plasmid DNA using Lipofectamine® 2000 (Invitrogen). After 24 h, medium LDH and cellular ATP levels were assessed, respectively.

Lactate Dehydrogenase (LDH) and ATP Assays

Cells were cultured in 96-well plates and treated with different chemicals for 24 h. Cell toxicity was determined

by measuring the release of LDH into the culture medium with use of a spectrophotometer and an LDH assay kit (Roche). LDH release was assessed from at least 3 cell culture wells for each experimental condition and was repeated at least three times. Culture medium was collected from 96-well plates and incubated with substrate mixture from the LDH assay kit for 30 min at room temperature. The plate was read at 492 nm in a colorimetric plate reader (Beckman).

Cell viability was measured by the CellTiter-Glo® Luminescent Cell Viability Assay, in which the amount of ATP was quantified by luminescence signal. Briefly, cells were cultured in opaque-walled 96-well plates with the same density at 10,000/100 µl per well. After the indicated treatment, the plate and its contents were equilibrated at room temperature for about 30 min and 100 µl of CellTiter-Glo® reagent (Promega) was added to each well and mixed thoroughly. The plate was then maintained at room temperature for 10 min. Luminescence signal was measured with a FluoroSkan AcsentFL luminescence reader (ThermoLabsystem).

Western Blotting

Cells were lysed or mouse brain tissues were homogenized with RIPA buffer containing protease inhibitors and phosphatase inhibitors. Protein concentrations were determined with the BAC kit (Pierce) according to the manufacturer's recommendations. Equal amounts of protein were separated by sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) by using 4–20 % polyacrylamide gels and then transferred onto nitrocellulose membranes (Millipore, Bedford, MA, USA) for Western blotting analyses. Anti-total AMPK (Cell Signaling, USA), anti-phospho-AMPK (Cell Signaling, USA), and anti-β-actin (Sigma, USA) were used with slight revision of the manufacturers' recommended dilutions. Horseradish peroxidase (HRP)-conjugated secondary antibodies and ECL luminescence reagent (both from GE Healthcare, UK) were used to detect the signals. The densitometry analysis was performed using Image J.

Mitochondrial Membrane Potential Measurement in Live Cells

Mitochondrial membrane potential was determined by using a fluorescent probe TMRE. Striatal cells were cultured in 24-well plates to 70 % confluence. Medium was changed to serum withdrawal medium, and cells were treated with metformin for a further 24 h. After incubation with TMRE for 1 h, the fluorescence signal was captured with an epifluorescence microscope (Zeiss Axio-imager M1) and Axiovision software (Zeiss). Cells were collected

and analyzed with a flow cytometer (FACSCalibur). The mean fluorescence intensity of 10,000 cells was assessed in each sample and corrected for autofluorescence from unlabeled cells.

Immunocytochemistry and Mitochondrial Morphology Determination

Cells were cultured in 24-well plates with glass coverslips for 24 h to about 70 % confluence. Then, the medium was changed to serum free medium with or without metformin, and cells were incubated for a further 24 h. Cells were then fixed with 4 % paraformaldehyde for 15 min and washed for 3 times with PBS. Then, cells were stained with anti-Tom 20 antibody to visualize the morphology of the mitochondria. Briefly, the sections were washed 3 times with PBS for 5 min each time, then permeabilized by incubating with 0.3 % Triton X-100 for 5 min, followed by incubation with blocking solution containing 3 % goat serum and 0.1 % Triton X-100 for 1 h, and incubated with primary antibody-anti-Tom 20 (1:1000) at 4 °C overnight. After three washings with PBS, the sections were incubated with Alexa Fluor-488-conjugated secondary antibody for 2 h at room temperature and washed 3 times with PBS, followed by incubation with DAPI for 10 min to counterstain the nucleus. Coverslips were mounted on the slides, and confocal fluorescence images were acquired a Zeiss LSM510 confocal microscope.

Totals of 200–300 cells were observed in each group to define the mitochondrial morphology. Cells were assigned to one of four groups based on the morphology of their mitochondria (tubular, mixed, fragmented, or swollen) as reported previously (Yu et al. 2005). The percentages of cells containing different types of mitochondria were calculated and represented as mean ± SEM.

RNA Isolation and Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated by using Trizol reagent (Invitrogen) as the manufacturer recommended. First-strand cDNA was synthesized by using MuLV reverse transcriptase with hexamer random primer. Quantitative RT-PCR was carried out with the iTaq™ universal SYBR® Green Supermix in ABI 7900HT Fast Real-Time PCR System. The sequences of primers were: DRP1forward primer 5′-AGCTGGTCCA TGAGGAGATG-3′, DRP1reverse primer 5′-GCACCAT TTCATTTGTCACG-3′, Fis1 forward primer 5′-AGACTG AGCCCCAGAACAAC-3′, Fis1 reverse primer 5′-ACAG CCAGTCCAATGAGTCC-3′, Mfn1forward primer 5′-GA GGTGGCAAACAAGGTTTC-3′, Mfn1 reverse primer 5′-TTTCTTCCCATGCCATCTTC-3′, Mfn2 forward primer 5′-TTACCACGGAAGAGCAGGTC-3′, Mfn2 reverse

primer 5′-GCAGAACTTTGTCCCAGAGC-3′, Bcl2 forward primer 5′-AGAGCGTCAACAGGGAGATG TTACC ACGGAAGAGCAGGTC-3′, Bcl2 reverse primer 5′-GC TGAGCAGGGTCTTCAGAG-3′, β-actin forward primer 5′-AGATCTGGCACCACACCTTC-3′, and β-actin reverse primer 5′-CCAGAGGCATACAGGGACAG-3′. After 40 cycles of amplification, the melt curve was analyzed and relative expression of each gene was calculated after normalizing to β-actin expression by applying the comparative quantification method.

Animals, Metformin Administration, and Measurement of Metformin Levels In Vivo

Two-month-old male C57BL/6 mice were obtained from Jackson Laboratory and housed under specific pathogen-free conditions with a 12-h light/dark cycle at 23 °C. Mice were provided with food and water ad libitum. All procedures were conducted in strict compliance with the guide for the Animal Care and Use Committee of Johns Hopkins University. Mice received a single dose of metformin orally at 100 or 200 mg/kg. Mice were humanely killed, and plasma and brain tissues were harvested at 4 and 6 h after drug administration to determine metformin concentrations. Metformin was extracted from 20 μL of mouse EDTA plasma with 100 μL of extraction solution with internal standard (50 ng/mL of metformin-d6 in acetonitrile (ACN)). Brain tissue homogenates were prepared at a concentration of 200 mg/mL in mouse EDTA plasma before extraction using ACN. Samples were mixed well and centrifuged. The supernatant was removed, and 10 μL was injected onto a HPLC tandem mass spectrometric detection (LC–MS/MS) for analysis. Chromatographic separation was performed on a Waters BEH HILIC 2.1 × 50 mm, 1.7 μm HPLC column running ACN/water (80:20, v/v) mobile phase containing 0.2 % formic acid using an isocratic flow rate of 0.4 mL/min. Metformin and its internal standard were detected by positive mode electrospray mass spectrometry to obtain the following MRM (multiple reaction monitoring) transitions: $130.0 \rightarrow 71.0$ for metformin and $136.1 \rightarrow 77.2$ for metformin-d6. The calibration range was from 5 to 1000 ng/mL. Brain samples were then quantified in ng/g wet weight as: nominal concentration (ng/mL) × standardized dilution. The brain-to-plasma ratio at each time point was calculated as $C_{\text{brain}}/C_{\text{plasma}}$.

Statistical Analyses

Data are expressed as mean ± SEM. Standard Student's t test was used for two group comparison with significance level set as $p < 0.05$. A Wilcoxon signed-rank test was used for two group comparisons of the pharmacokinetic data with significance level set as $p < 0.05$.

Results

Metformin Protects Striatal Cells from Mutant Huntingtin Toxicity by Activating AMPK

To determine whether metformin protects mutant HTT-induced cell toxicity, we used the striatal cell line expressing full-length mutant HTT (STHdh^Q111/Q111) or wild-type HTT (STHdh^Q7/Q7). The mutant HTT caused toxicity in the cells, as indicated by increased LDH release and reduced ATP levels in STHdh^Q111/Q111 compared to those in STHdh^Q7/Q7 cells under serum withdrawal conditions. Pretreatment with metformin at indicated concentrations significantly reduced the LDH release and preserved ATP levels in the cells expressing mutant HTT (Fig. 1a, b), indicating that metformin protects these cells from mutant HTT toxicity.

To further explore the signaling pathway activated by metformin in the striatal cells, we measured levels of p-AMPK which indicates activated AMPK and found that p-AMPK levels were significantly decreased in the STHdh^Q111/Q111 cells and that metformin activated AMPK in striatal cells, indicated by increased p-AMPK levels (Fig. 1c, d).

In order to determine whether activation of AMPK mediated the protective effects of metformin in HD cells, we pretreated cells with AMPK inhibitor compound C or transfected cells with DN-AMPKα before exposure to metformin. We found that both compound C and DN-AMPK significantly reduced the protection of metformin in HD cells, evidenced by the levels of ATP and LDH release (Fig. 1 e–h), suggesting that activation of AMPK mediated the protection of metformin in mutant HTT-induced toxicity. Our results also suggest that other signaling pathway(s) in addition to AMPK are involved in the protection of metformin, as blocking AMPK activation did not completely abolish this protection.

Metformin Prevents Mutant Huntingtin-Induced Mitochondrial Depolarization

Mutant HTT impaired mitochondrial function, as indicated by loss of mitochondrial membrane potential in STHdh^Q111/Q111 cells compared to STHdh^Q7/Q7 cells (Fig. 2). To determine whether metformin prevents mitochondrial depolarization, cells were treated with metformin for 24 h and then loaded with the mitochondrial dye TRME, and changes in mitochondrial membrane potential were measured by FACS. Cells expressing mutant HTT
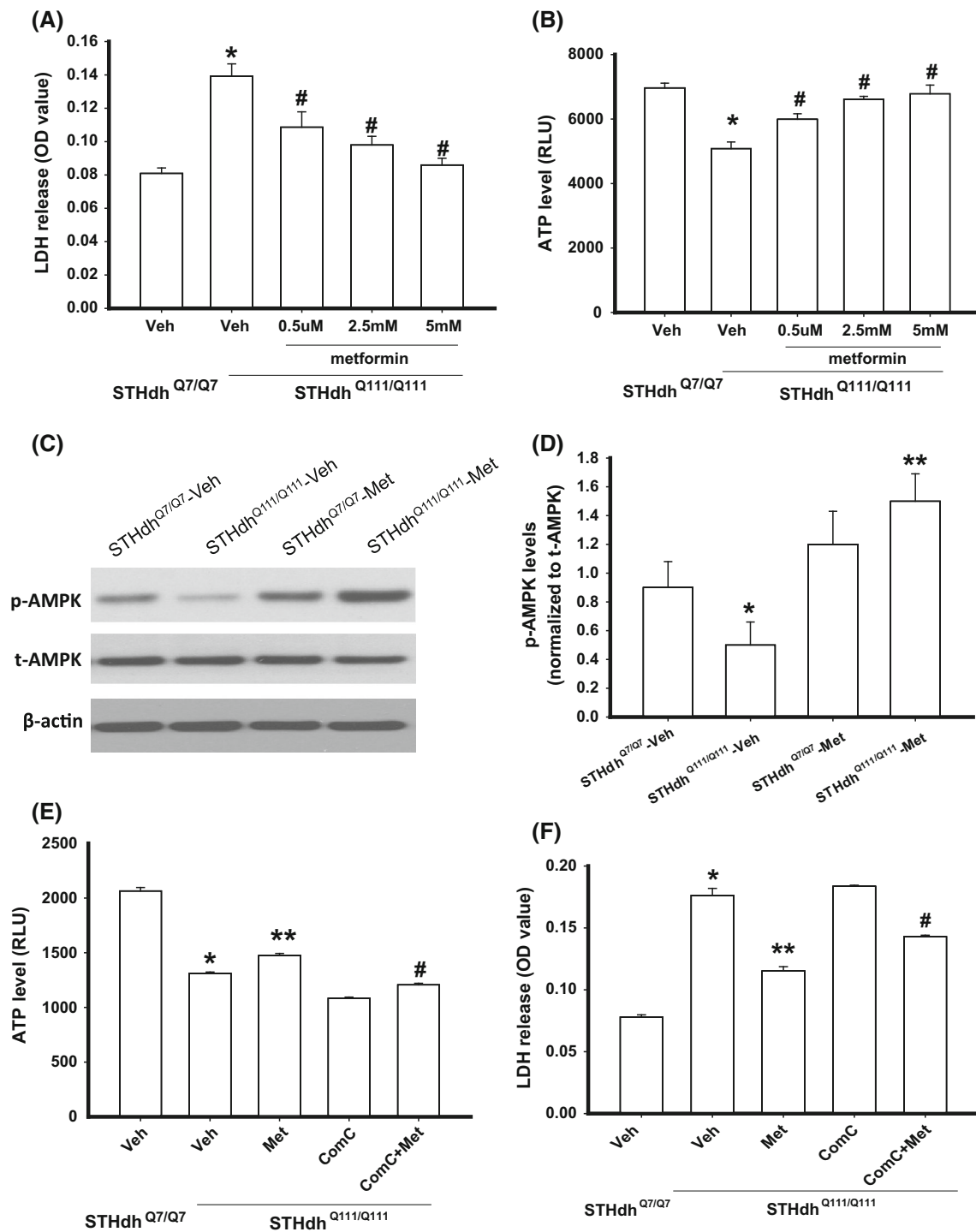
Fig. 1 Metformin activates AMPK and protects cells from mutant huntingtin (HTT) neurotoxicity. **a, b** Mutant HTT-induced toxicity in striatal cells indicated by lower ATP levels and increased LDH release. Metformin treatment restored ATP levels and reduced LDH release. **c, d** Striatal cells expressing normal HTT (STHdh^Q7/Q7) or mutant HTT (STHdhQ^111/Q111) are treated with metformin (500 µM) for 48 h, and activation of AMPK (p-AMPK) is detected by Western blots. Representative blots (**c**) and quantification data (**d**) are shown. **e, f** Cells are pretreated with AMPK inhibitor compound C (1 µM) before exposure to metformin; ATP levels and LDH release are measured. All data are from three independent experiments,

$*p < 0.05$ versus values in vehicle-treated STHdh^Q7/Q7 cells, $**p < 0.05$ versus vehicle-treated STHdhQ^111/Q111 cells; #$p < 0.05$ versus metformin-treated STHdhQ^111/Q111 cells by standard Student's t tests. **g, h** Cells transfected with dominant-negative (DN) AMPK cDNAs before exposure to metformin. ATP levels and LDH release are measured. All data are from three independent experiments, $*p < 0.05$ versus values in vehicle-treated STHdh^Q7/Q7 cells, $**p < 0.05$ versus vehicle-treated STHdhQ^111/Q111 cells; #$p < 0.05$ versus metformin-treated STHdhQ^111/Q111 cells by standard Student's t tests
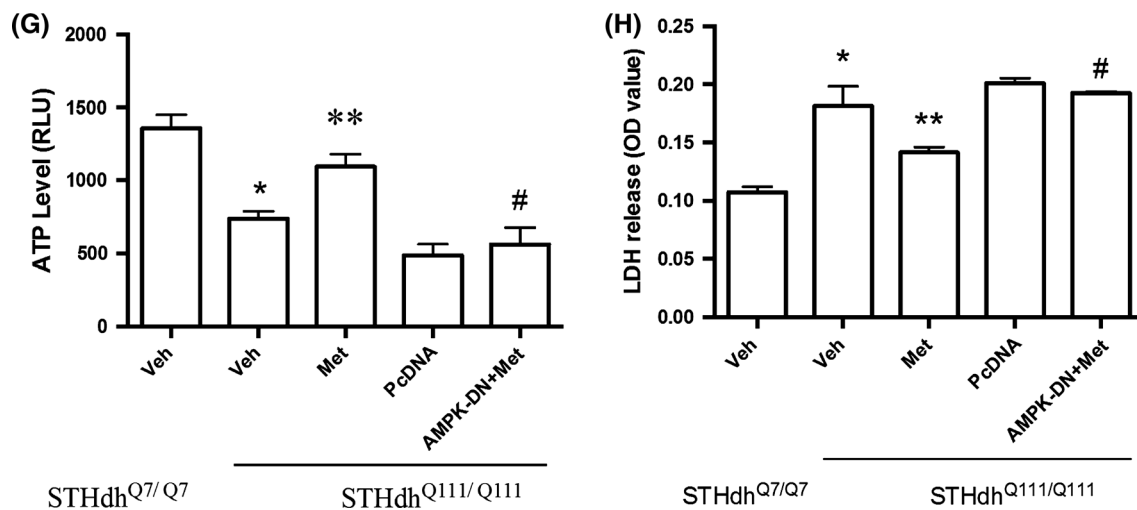
Fig. 1 continued

exhibited lower mitochondrial membrane potential after serum withdrawal than did those cells expressing wild-type HTT (Fig. 2a–c) and that metformin treatment significantly reduced the loss of mitochondrial membrane potential that occurred in response to serum withdrawal in cells expressing mutant huntingtin (Fig. 2a–c).

Metformin Maintains Mitochondrial Morphology and Prevents Mitochondrial Fragmentation Induced by Mutant Huntingtin

Mitochondria continually change shape through the combined actions of fission and fusion. Under normal conditions, mitochondrial fusion prevails and, as a result, the organelles show tubular structure revealed by Tom 20 staining (Fig. 3a). In cells expressing mutant HTT, however, the dynamic balance is shifted to fission, leading to mitochondrial fragmentation or swollen mitochondria. STHdh$^{Q111/111}$ cells contained more fragmented mitochondria compared to STHdh$^{Q7/7}$ cells (Jin et al. 2013), and we found that STHdh$^{Q111/Q111}$ cells also contained more swollen mitochondria than did those in STHdh$^{Q7/7}$ cells (Fig. 3). Emerging evidence suggests that mitochondrial fragmentation contributes to loss of mitochondrial membrane potential and consequent release of apoptotic factors (Heath-Engel and Shore 2006). By staining the mitochondria with Tom 20, a mitochondrial outer membrane protein, we found that metformin significantly reduced the number of fragmented and swollen mitochondria in STHdh$^{Q111/Q111}$ cells, as indicated by more cells in metformin-treated group containing normal tubular structure mitochondria and fewer fragmented and swollen mitochondria (Fig. 3b).

Metformin Balances Mitochondrial Fission and Fusion in STHdh$^{Q111/Q111}$ Cells

The mitochondria are a class of dynamic organelles, constantly undergoing fission and fusion, which are governed by distinct proteins. Whereas fission depends on Fis-1 and Drp1, fusion requires coordinated action of mitofusins (Mfn1 and Mfn2). The mRNA levels of these fission and fusion factors were altered in STHdh$^{Q111/Q111}$ cells (Fig. 4a–d). Interestingly, Fis1, Mfn1, and Mfn2 mRNA levels were increased in the STHdh$^{Q111/Q111}$ cells compared to STHdh$^{Q7/Q7}$ cells, while Drp1 mRNA levels did not differ between STHdh$^{Q111/Q111}$ cells and STHdh$^{Q7/Q7}$ cells (Fig. 4a–d). These results suggested that mutant HTT induced detrimental as well as compensatory responses in the cells. Metformin plays a role in modulating the balance between mitochondrial fission and fusion genes. Bcl-2 is located in the mitochondria and counteracts cell death, the levels of Bcl-2 mRNA declined significantly in cells expressing mutant HTT compared to cells expressing wild-type HTT, and metformin treatment attenuated the decline of this anti-apoptotic factor in STHdh$^{Q111/Q111}$ cells (Fig. 4e).

Metformin Crosses the Blood–Brain Barrier and Activates AMPK in the Mouse Brain

It has been suggested that metformin crosses the blood–brain barrier in the model of systemic inflammation in rats (Labuzek et al. 2010). By using a LC–MS/MS method, we measured the metformin concentrations in mouse brain and plasma. Metformin was detected in the brain with a statistically significant increase with dose at each time point
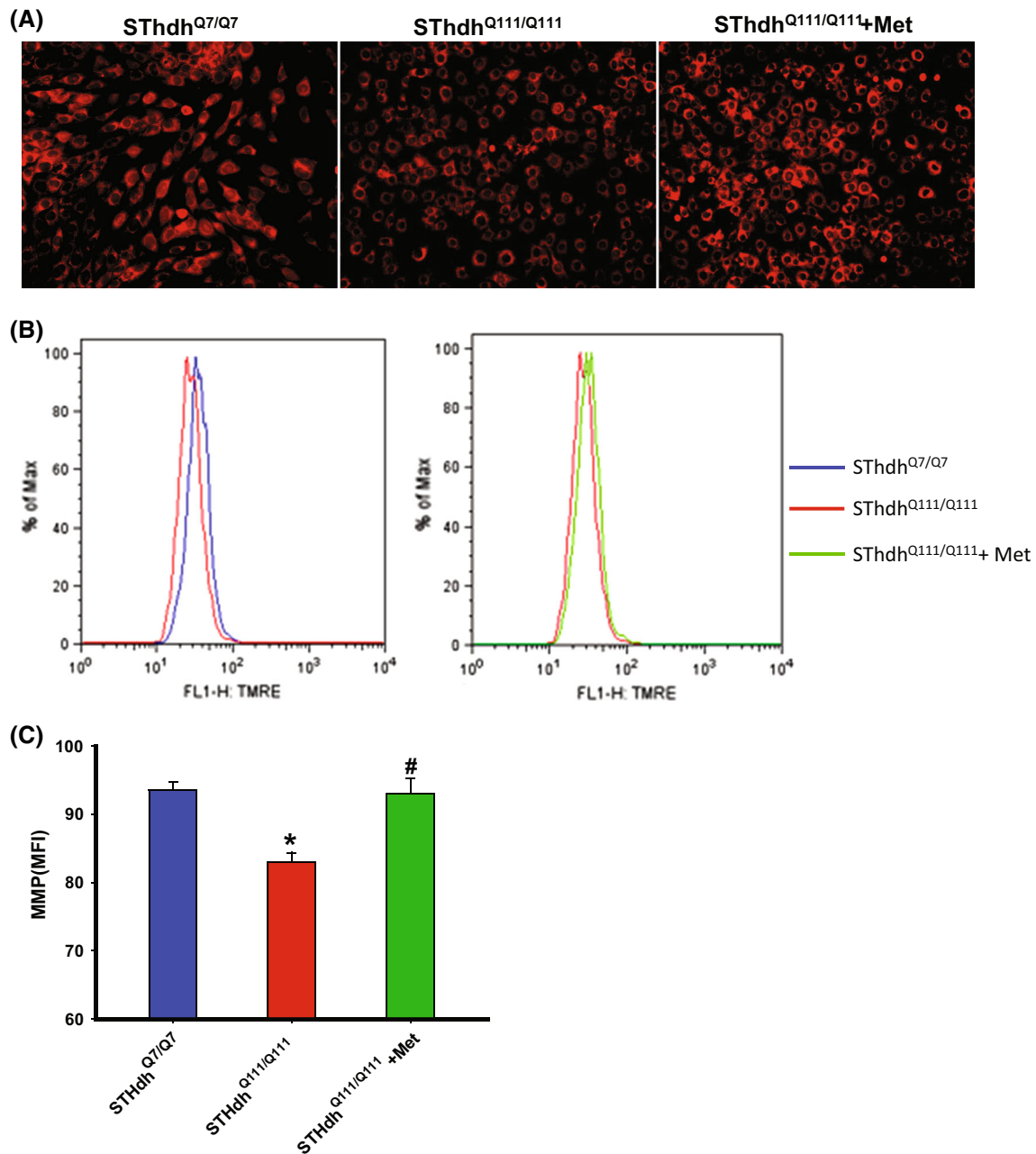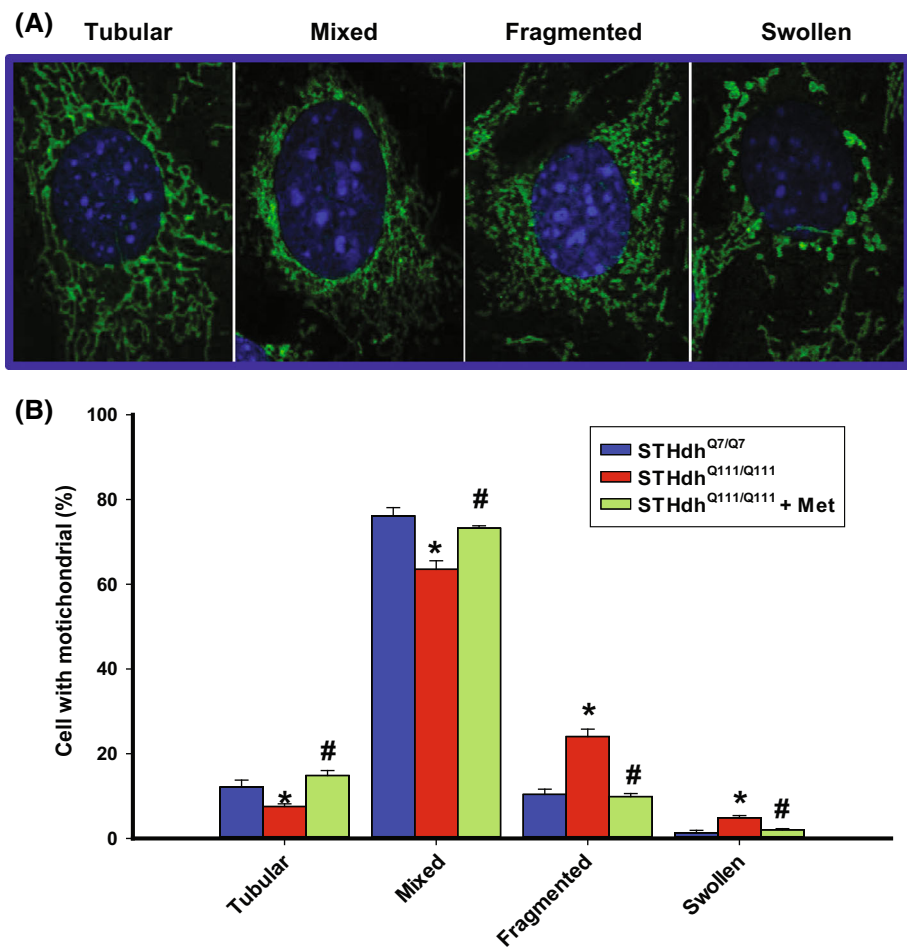
Fig. 2 Metformin preserves mitochondrial membrane potential in cells expressing mutant HTT. **a** Representative confocal microscope images of striatal cells loaded with TMRE in indicated groups. Note that mutant HTT expressing cells exhibited lower TMRE fluorescent intensity, indicating decreased mitochondrial membrane potential. **b** *Histograms* show that the peak of excitation of TMRE fluorescent dye shifted from the *right* in SThdh$^{Q7/Q7}$ cells (*blue curve*) to the *left* in STHdh$^{Q111/Q111}$ cells (*Red curve*); metformin treatment (*green curve*) shifted the peak to the *right*, indicating increased fluorescence intensity and mitochondrial membrane potential by metformin. **c** Quantification of fluorescence intensity by flow cytometry. *$p < 0.01$ versus SThdh$^{Q7/Q7}$ cells; #$p < 0.05$ versus vehicle-treated STHdh$^{Q111/Q111}$ cells by standard Student's *t* tests. *MMP* mitochondrial membrane potential; *MFI* mean fluorescence intensity (Color figure online)

($p = 0.03$ for both 4 and 6 h) (Fig. 5a). However, the brain/plasma ratio was constant at each time point despite increased doses ($p = 0.47$ for both 4 and 6 h) (Fig. 5b). In order to determine whether metformin at the doses utilized in our experiment activates AMPK in the mouse brain, the brain samples from the same mice for the drug measurement were subjected to Western blotting analysis. As shown in Fig. 5d, e, metformin activated AMPK in the mouse brain, suggesting that AMPK activation could serve as a pharmacodynamic marker for metformin treatment in vivo.

Discussion

The present study provides strong evidence for a direct neuroprotective effect of metformin in HD cells. By using striatal cells expressing full-length mutant HTT under serum withdrawal conditions, we found that metformin preserved ATP levels and inhibited LDH release induced by mutant HTT, maintained mitochondrial morphology, and prevented mitochondrial depolarization. Moreover, this sequence of protective events is associated with activation of AMPK and balances mitochondrial fission and fusion. In addition, we demonstrated that metformin administered orally crosses the blood–brain barrier and activated AMPK in the mouse brain. It is worth mentioning that brain-to-plasma ratio detected in mice in our study is lower than that reported in rats (Labuzek et al. 2010). The difference in drug clearance and metabolic rate between species may contribute to the discrepancy partially; meanwhile, an additional contributing factor may be that Labuzek and colleagues measured metformin levels in rats treated with lipopolysaccharide, which cause inflammation and may alter the blood–brain barrier integrity. The differences in

pharmacokinetics (PK) observed between species suggest that PK in mice or rats cannot be directly extrapolated to the human. Metformin is a clinical drug which is commonly prescribed for diabetic patients; these findings support consideration of treatment with metformin in HD patients.

Previous reports have shown that metformin activates AMPK, and AMPK then triggers intracellular responses and cellular signaling pathways (Ishizuka et al. 2013; Dulovic et al. 2014), such as the pro-survival signaling pathway (Lieberthal et al. 2011; Pantovic et al. 2013). An additional target of metformin is the respiratory chain complex I of the mitochondria (Zou et al. 2004). However, we provide here a potential new mechanism that supports a protective role of metformin in mutant HTT neurotoxicity that is likely due to its maintenance of mitochondrial integrity and balancing mitochondrial dynamics. Although much is known about AMPK as a cellular energy sensor, until recently possible functions in neurons have remained unexplored. Neurons primarily use glucose as an energy source. While neurons have high energy demands, they are unable to store nutrient efficiently. Thus, with a narrow margin between energetic
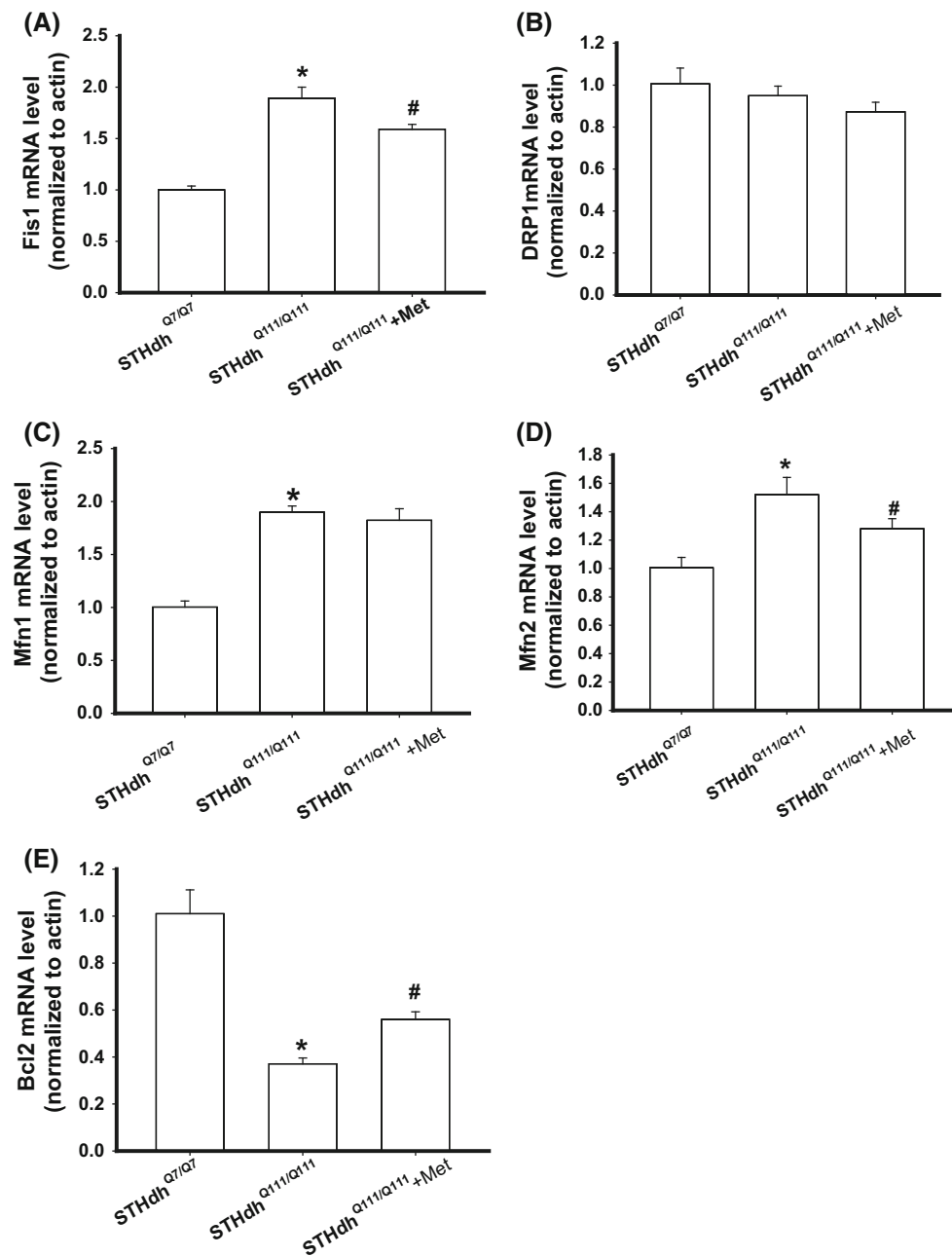
Fig. 4 Metformin modulates the mitochondrial fission and fusion factors and preserves Bcl-2 in cells expressing mutant HTT. The mRNA levels of Fis1 (**a**), Drp1 (**b**), Mfn1 (**c**), Mfn2 (**d**), and Bcl-2 (**e**) are determined by quantitative RT-PCR in indicated cells. $N = 3$, *$p < 0.01$ versus SThdh$^{Q7/Q7}$ group; # $p < 0.05$ versus untreated STHdh$^{Q111/Q111}$ group by standard Student's t tests

needs and capacity to store and generate energy, neurons are very sensitive to fluctuations in cellular energy. Consequently, it is not surprising that AMPK, which is expressed throughout the central nervous system, plays an important role in the functional maintenance and even survival of neurons. A continuous supply of energy is crucial for neurons because their survival requires large amounts of energy that they are unable to store. Therefore, neurons are extremely susceptible to insults that lead to energy depletion.

Metformin treatment prevented a marked decrease in ATP levels in mutant HTT expressing cells and this protective effect was dramatically reduced by AMPK inhibitor compound C, suggesting that metformin stimulates AMPK activities and preserves ATP levels and therefore could have a pivotal role in protecting neurons from mutant HTT, which results in bioenergetic failure.

The Bcl-2 protein family plays an important role in cell survival signal transduction by regulating mitochondrial
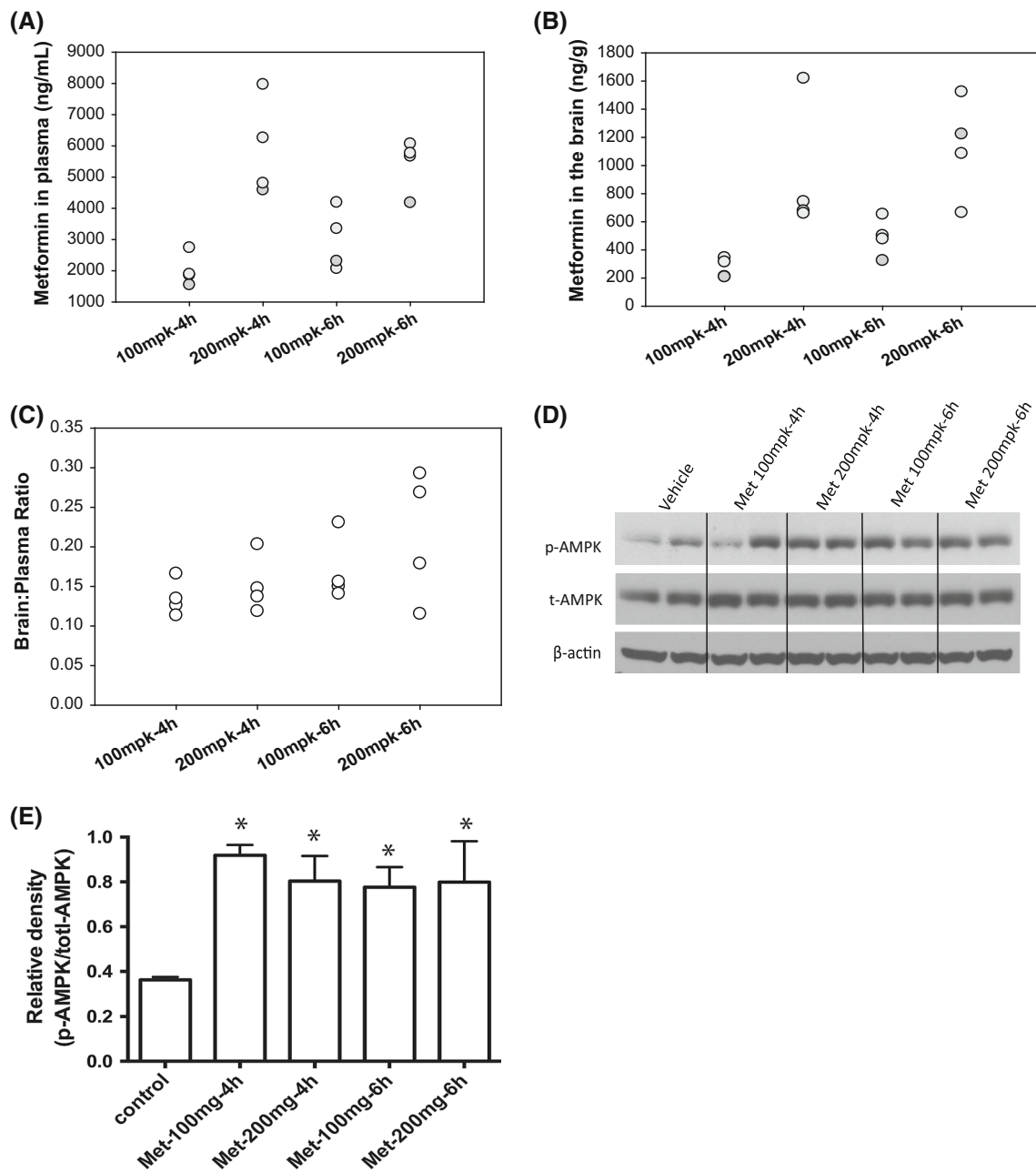
Fig. 5 Metformin crosses the blood–brain barrier and dose dependently activates AMPK in mouse brain. Mice are administered a single dose of metformin orally (100 or 200 mg/kg). Plasma and brain samples are harvested at 4 or 6 h after metformin administration. **a**, **b** The concentrations of metformin are measured by LC–MS/MS in plasma (**a**) and the mouse brain (**b**). **c** Brain/plasma ratio is calculated. **d**, **e** Metformin activated AMPK in vivo. Representative Western blotting imaging (**d**) and quantification data by densitometry ($n = 3$) using β-actin as loading control (**e**) are shown. $*p < 0.05$ versus control values by standard Student's t tests

function (Shi 2001). It has been reported that mitochondrial cytochrome C is released into the cytosol through the permeability transition pore and that Bcl-2 regulates the permeability of the mitochondrial outer membrane (Shi 2001). We found that metformin preserved Bcl-2 levels in the cells expressing mutant HTT, implying that metformin potentially boosts cell survival by regulating the mitochondrial pathway.

Changes in mitochondrial dynamics, resulting in increased fragmentation of the organelles, have been documented in HD (Reddy 2014). Mounting evidence suggests that structural and functional abnormalities in mitochondria are involved in HD pathogenesis (Wang et al. 2009; Kim et al. 2010; Song et al. 2011; Shirendeb et al. 2012). In neurons that express mutant HTT, an imbalance between fission and fusion leads to abnormalities in mitochondrial

structure and function and to cell death (Kim et al. 2010). Our current study suggests a complex response of cells to mutant HTT. The mRNA levels of Drp1 did not differ between cells expressing mutant HTT and those expressing wild-type HTT, whereas mRNA levels of Fis1, Mfn1, and Mfn2 were increased in mutant HTT cells. These results might be explained by induction of mitochondrial fission by mutant HTT and that cells showed a compensatory response by increasing mitochondrial fusion factors. Metformin modulates both fission and fusion factors to balance the mitochondrial dynamics. However, our current study could not determine whether mitochondrial fragmentation is a cause or a consequence of increased mitochondrial membrane depolarization in HD.

Although it has been demonstrated that metformin activated AMPK in the striatum and significantly prolonged survival in R6/2 transgenic mouse model of HD (Ma et al. 2007), the authors concluded that the site of metformin's action remains unclear and the improvement of peripheral system might contribute to the metformin-induced beneficial effects in R6/2 mice, as these mice have hyperglycemia (Ma et al. 2007). Our study provided the molecular mechanisms underlying the neuroprotection of metformin in HD models. However, another study suggested that AMPK may be activated in the striatum of HD mice at a late stage of the disease, and chronic exposure to high-dose regiments of the AMPK activator AICAR may worsen neuropathological and behavioral phenotypes (Ju et al. 2011). We speculate that AMPK activation may represent a compensatory response and useful approach for protecting neurons that, in the caudate nucleus, are targeted by the early phases of the cellular stress induced by mutant huntingtin in HD. Interestingly, our data suggest that AMPK activator metformin may protect from the early phases of the pathogenic process in HD. It has been reported that metformin improves health span and life span in mice (Martin-Montalvo et al. 2013). Hence, it may be possible that metformin may be rescuing neuronal toxicity of mutant huntingtin in human HD. To access the potential neuroprotection in vivo, we further verified permeation of metformin across the blood–brain barrier. Provided that metformin can be made to pass this frontier, it becomes a candidate of FDA-approved drug which can be considered in the treatment of HD. As mitochondrial dysfunction is a key event in HD pathogenesis, our results represent a potential mechanism by which metformin may be protective in HD.

In summary, this study demonstrated that metformin exerts a neuroprotective effect by preserving mitochondrial function, sustaining bioenergetics, and preventing excess fission. Although additional studies are needed to determine the molecular mechanisms underlying its actions in HD, metformin is a readily available and safe FDA-approved drug; it could eventually be used as a neuroprotectant against HD neurodegeneration. These data provide strong support for the rapid development of new therapy for HD.

Compliance with Ethical Standards

Conflict of interest All authors have declared the sources of research funding for this manuscript and have no financial or other contractual agreements that might cause (or be perceived as causes of) conflicts of interest.

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