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Treadmill exercise rescues mitochondrial function and motor behavior in the CAG_{140} knock-in mouse model of Huntington's disease

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ABSTRACT

Background: Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by polyglutamine (CAG) expansion in the Huntingtin (*HTT*) gene. The CAG_{140} knock-in (KI) mouse model recapitulates the progression of motor symptoms emerging at 12 months of age.
Objective: This study was aimed at assessing the effects of exercise, in the form of treadmill running, and examining its impact on motor behavior and markers of metabolism in the CAG_{140} KI mouse model of HD after motor symptoms have emerged.
Methods: CAG_{140} KI mice at 13–15 months of age were subjected to treadmill exercise 3 days per week for 1 h per day or remained sedentary. After 12 weeks of exercise brain tissues were analyzed for enzymatic activity including mitochondria Complexes I, II/III, and IV, transglutaminase, aconitase, pyruvate dehydrogenase, and phosphofructokinase1/2. In addition, the concentration was determined for nitrate/nitrite, pyruvate carboxylase, NAD^+/NADH, and glutamate as well as the ratio of mitochondria and nuclear DNA. Motor behavior was tested using the rotarod.
Results: Exercise resulted in increased [nitrite + nitrate] levels (surmised as nitric oxide), reduced transglutaminase activity, increased aconitase activity with increased tricarboxylic acid-generated reducing equivalents and mitochondrial oxidative phosphorylation complexes activity. Mitochondrial function was strengthened by increases in glycolysis, pyruvate dehydrogenase activity, and anaplerosis component represented by pyruvate carboxylase.
Conclusions: These changes in mitochondrial function were associated with improved motor performance on the rotarod test. These findings suggest that exercise may have beneficial effects on motor behavior by reversing deficits in mitochondrial function in a rodent model of HD.

1. Introduction

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease that impacts 1 in 10,000 people. Patients phenotypically are characterized by progressive decline in cognitive and motor functions with neuropsychiatric disturbances leading ultimately to premature death 15–20 years after the onset of motor symptoms [1]. The disease is defined genetically by an excessive polyglutamine (CAG) expansion in the Huntingtin (*HTT*) gene on exon 1 of chromosome 4 resulting in a mutated form of the huntingtin (HTT) protein [2].

Therapeutic strategies for attenuating or reversing the progression of HD are still under development. Mounting evidence supports that cardiovascular exercise (physical activity) is associated with reduced risk of neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease [3,4]. The application of exercise and its impact on neurodegenerative disorders over a lifetime is speculated to act in a neuroprotective fashion blocking the onset of disease including its genetic and toxicant initiators. Factors of lifestyle may also provide resilience, where cognitive and motor reserves in some individuals may fend off disease over their lifetime. On the other hand, mechanisms of resilience may be different than those engaged in either the prodromal or early stages of disease and may impact disease by modifying

Abbreviations: HD, Huntington's disease; NAD, nicotinamide adenine dinucleotide; NO, nitric oxidase; PC, pyruvate carboxylase; PFK1/2, phosphofructokinase 1/2; PDH, pyruvate dehydrogenase complex; TCA, tricarboxylic acid cycle; TG, transglutaminase

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progression in terms of severity, time course, and symptomology. Animal models of human neurological disorders provide an opportunity to investigate the impact of interventions such as exercise and begin to identify underlying mechanisms, both within the central nervous system (CNS) and the periphery, that may serve as novel therapeutic targets to treat patients.

A number of key molecules including nitric oxide (NO) play an important role in a variety of signal transduction pathways that are crucial for maintaining the physiologic functions of vascular, respiratory, immune, muscular, and nervous systems. Within the CNS, NO plays a major role in the regulation of cerebral blood flow and local brain metabolism, gene expression, memory function including receptor trafficking, neuroendocrine secretion, and synaptic plasticity [5]. While exercise has a wide spectrum of pleiotropic effects involving many biochemical targets, it is known to elevate levels of NO in the body especially muscle [6] as well as within the CNS [7,8]. Neuronal nitric oxide synthase (nNOS) activity and nNOS protein expression are reduced in HD possibly contributing to symptom progression in both animal models [9,10] and in patients [11]. Such findings suggest that the decrease in NO is a major contributing factor to HD-related cell death by a variety of mechanisms including excitotoxicity, the generation of reactive oxygen species (ROS), and activation of developmental cell death programs such as apoptosis [11]. Reversing the loss of NO activity may be neuroprotective as well as providing a means to restore or enhance functions compromised in disease. For example, several deficiencies of mitochondrial complex activities within the electron transport chain have been observed in preclinical and clinical HD [12–14]. Increased production of NO could be beneficial in mitochondrial enzymatic interactions. Specifically, NO donors at a linear rate could inactive tissue transglutaminase (TG), a mitochondrial protein that plays a critical role in mitochondrial homeostasis including the control of mitophagy and bioenergetics, shown to be elevated in HD [15]. The inactive TG can no longer use aconitase as a substrate and thus, preserve its function in the TCA cycle as a generator of reducing equivalents such as NADH. This improvement is seen downstream in the mitochondrial OXPHOS complex activity with increases in complexes I, II/III, and IV.

The purpose of this study was to explore a potential mechanisms by which chronic exercise impacts the metabolic and bioenergetic profile of aged HD mice focusing on mechanisms centered on the enhanced levels of brain [nitrite + nitrate] (surmised as NO) caused by exercise that results in changes in the metabolic profile elicited by initial modifications including TG activity. For these studies, we utilized the HD CAG_{140} Knock In (KI) mouse model which is characterized by 140 CAG repeats in exon 1 of the mouse huntingtin gene ortholog (hdh) and has been reported to demonstrate a protracted prodromal phase with motor features presenting after 12 months of age [16]. The CAG_{140} KI mouse is a model for investigating the mechanisms of exercise-induced NO production on disease modification and evaluating the functional outcomes linked to biochemical markers of brain bioenergetics. The impact of these metabolic changes was examined as a functional outcome that entails motor behavior in the rotarod test. Few studies have investigated the effects of exercise on HD models and even fewer have addressed the impact on aged HD animals [17,18].

2. Materials and Methods

2.1. Mice

For these studies we used knock-in mice that contained a chimeric mouse/human exon 1 with 140 CAG repeats inserted into the mouse gene by homologous targeting [16]. The CAG_{140} KI mice were maintained in-house using lines descended from heterozygous pairing. The founder mice for the colony were a gift of Drs. Michael Levine and Carlos Cepeda (UCLA) with permission from Dr. Scott Zeitlin (University of Virginia) through a Material Transfer Agreement. Mice were backcrossed onto the C57BL/6J background annually to maintain vigor. Mouse genotypes from tail biopsies were determined using PCR (Transnetyx, Inc., Cordova, TN). The protracted prodromal phase with motor features is present in the HD CAG_{140} KI mouse model after 12 months of age. The CAG140 KI mouse line has a life expectancy of about 2 years. Mice 13–15 months of age were used and represent moderate to advanced HD. Mice were randomly assigned to one of 2 groups (i) CAG_{140}KI, $n = 8$ (3 males and 5 females) and (ii) CAG_{140}KI + exercise, $n = 8$ (3 males and 5 females). No differences between males and females HD mice have been reported; hence groups were collapsed for analysis. Mice were group housed with a reverse light cycle (lights off from 7 a.m. to 7 p.m.) and were allowed access to food (rodent chow manufactured by Purina Inc.) and water *ad libitum*. Experimental procedures were approved by the University of Southern California's Institutional Animal Care and Use Committee (IACUC) and conducted in accordance with the National Research Council Guide for the Care and Use of Laboratory Animals (DHEW Publication 80–23, 2011, Office of Laboratory Animal Welfare, DRR/NIH, Bethesda, MD). All efforts were made to minimize animal suffering and to reduce the number of animals used to achieve statistical significance.

2.2. Exercise regimen

Treadmill running was initiated in CAG_{140} KI mice at 13–15 months of age, performed on a Model EXER-6M Motorized Treadmill (Columbus Instruments, Columbus, Ohio). The treadmill exercise protocol was conducted based on previous publications with modifications [19,20]. Briefly, on day 1 of week 1, mice in the exercise group ran at a speed of 8.0 ± 0.5 m/min for 40 min and closely monitored for any adverse reaction to the treadmill, inability to run, or failure to learn the task. Exercise continued at a velocity of 10.0 ± 1.5 m/min 3 times per week for a 12-week period. Treadmill speed was gradually increased to 20 ± 1.5 m/min by the final month. A non-noxious stimulus (metal beaded curtain) was used as a tactile incentive to prevent animals from drifting back on the treadmill. All mice were weighed at the end of each week and closely assessed for adverse reactions including stress; there were no abnormalities in weight. Treadmill running is not stressful based on the evaluation of anxiety, depression, and corticosterone levels [21]. All mice tolerated the exercise regimen, with no dropouts.

2.3. Rotarod test for motor behavior

Behavioral testing was carried out after completion of the exercise regimen and consisted of motor performance using the accelerating rotarod. Outcome was measured by latency to fall (in seconds). Mice were given 10 min to acclimate to the behavior room environment on the day of the test. The rotarod was set to its base speed as mice were assigned to their respective lanes. At t = 0s, the rotarod began the acceleration phase reaching peak velocity at t = 24 s before decelerating. At t = 48 s, the rotarod switched directions and repeated the cycle. Endpoint criteria for the rotarod test were: (i) mouse falling off the cylinder onto the platform; (ii) mouse completing three revolutions via clutching onto the cylinder pad; or (iii) mouse showing any signs of pain or distress. For criteria 1 and 2, the time was recorded, and the mouse was given a rest period of 5 min with access to food and water. This was repeated for a total of 6 trials. For criteria 3, the mouse would be removed from the study completely. No mice were removed from the rotarod test. For each mouse, times for the first two trials were removed so rotarod data would accurately reflect the ability to perform the test. The average time across trials was calculated for each mouse. A two-factor ANOVA with replication was performed to determine significance.

2.4. Mitochondrial isolation and mitochondrial complex activities

For all subsequent analyses whole brain was utilized. Briefly, mice

were quickly decapitated, and brain removed fresh while kept cold on wet ice. The forebrain, excluding the cerebellum, was isolated and utilized for subsequent enzymatic and mitochondrial analyses. Brain mitochondria was isolated by a Percoll gradient as previously described [22]. The resulting mitochondrial samples were used immediately or stored at −80 °C for later protein and enzymatic assays. During mitochondrial purification, aliquots were collected and evaluated for confirmation of mitochondrial purity and integrity. Isolated brain mitochondria were plated in Abcam Mitoscience complex activity assays. Complex I activity was expressed as $OD\lambda_{450nm}$ as NADH oxidation (ab109721), Complex II/III was expressed as $OD\lambda_{550nm}$ as cytochrome C reduction (ab109905), and complex IV as $OD\lambda_{550nm}$ as cytochrome *c* oxidation (ab109911).

2.5. Enzyme activities

Transglutaminase activity was measured using the Abcam Transglutaminase Activity Assay Kit (ab204700) measured colorimetrically at 525 nm. The limit of quantification of this assay is ~10 μU in cell/tissue lysates. Aconitase activity was measured using the Cayman Aconitase Assay Kit (cat. N° 705502); the reactions were monitored by measuring the increase in absorbance at 340 nm associated with the formation of NADH proportional to aconitase activity. Pyruvate Dehydrogenase (PDH) activity was measured in whole brain homogenate by the Abcam PDH enzyme activity microplate assay (ab109902), which captures fully functional PDH. Activity is determined by the rate of reduction of NAD^+ to NADH linked to a reporter dye that can be detected at 450 nm. The Abcam phosphofructokinase Activity (PFK) assay kit (ab155898) measured PFK1 and PFK2 and activities were measured in whole brain homogenates and the color product detected at 450 nm.

Nitrate and nitrite concentration was measured in brain homogenates with the Cayman Chemical Nitrate/Nitrite Assay Kit (cat. N° 780001) and photometric measurement of the absorbance due to the azochromophore corresponding to nitrate concentration. Pyruvate carboxylase (PC) concentration was measured using the Cloud-Clone Corp. PC immunosorbent assay kit (Product serial# 6ECC39CD3D). Color product was measured at 450 nm. NAD^+ and NADH concentration was measured using NAD/NADH Assay Kit (ab65348) which measures intracellular nucleotides NAD^+, NADH, and their ratio. Glutamate concentration was measured in whole brain and isolated brain mitochondria using the Abcam Glutamate Assay ELISA Kit (ab83389) that recognizes glutamate as a specific substrate forming a color product at 450 nm.

Mitochondrial biogenesis was expressed as the ratio of COXII/gamma-Globin corresponding to mitochondrial (mtDNA) and nuclear DNA (nDNA), respectively. Primers were developed by Integrated DNA Technologies. Primer sequences (5′-3′) COXII F:GCCGACTAAATCAAG CAACA R:CAATGGGCATAAA GCTATGG gamma-Globin; F:GAAG CGA TTCTAGGGAGCAG R:GGAG CAGCGATTCTGAGTAGA.

2.6. Activities of c-JUN N-Terminal kinase (JNK) and serine/threonine kinase Akt

JNK activity was measured in whole brain homogenates using the Abcam JNK1/2 (pT183/Y185) +Total JNK1/2 ELISA kit (abcam176662) following the manufacturer's instructions. The kit employs an affinity tag labeled capture antibody coated in the wells of the plate. A reporter conjugated detector antibody immunocaptures the analytes within the sample and a stop solution generates a signal detected at 450 nm. Activity was determined by the ratio of Phospho~JNK/JNK. AKT activity was measured in whole brain homogenate using the Abcam AKT1/2/3 (pS473) + AKT Total ELISA kit (abcam176657) following the manufacturer's instructions. The kit employs an affinity tag labeled capture antibody coated in the wells of the plate and a reporter conjugated detector antibody that immunocaptures the analyte within the sample and stop solution generating a signal detected at 450 nm. Activity was determined by the ratio of Phospho~AKT/AKT.

2.7. Data analysis

Data are reported as means ± SEM of at least three experimental replicates. Group size (*n*) is eight unless otherwise stated for specific method. Statistical significance between means was determined by Student's two-tailed *t*-test of paired data. The level of statistical significance is indicated in the respective figures ($*p < 0.05$, $**p < 0.01$). Two-way ANOVA between the sedentary group and exercised group of HD mice was used for the rotarod test. Post-hoc analysis using Student-Neuman Keuls with statistical significance considered if $p < 0.05$.

3. Results

3.1. Exercise generated markers of nitric oxide

We found that exercise increased [NO_2^- + NO_3^-] levels (surmised as NO) in the brain by 48% when compared to the sedentary group (Fig. 1A). Total nitrite and nitrate [NO_2^- + NO_3^-] were measured in brain homogenates of the HD mouse model in both the sedentary group (483 ± 110 μM) and the exercise group (714 ± 32 μM) ($p < 0.05$).

3.2. Exercise decreases transglutaminase (TG) activity

Since NO inhibits TG cross-linking activity by S-nitrosylation of Cys^{277} in the catalytic core of the enzyme, we examined TG expression in HD mice with and without exercise [23]. We found that TG activity in isolated brain mitochondria was decreased 52% in the exercise group (0.399 ± 0.087 μmol/min/mg protein) compared to the sedentary group (0.800 ± 0.141 μmol/min/mg protein) ($p < 0.05$) (Fig. 1B). TG activity in whole brain homogenates of the sedentary (0.240 ± 0.024 μmol/min/mg protein) and exercise (0.184 ± 0.011 μmol/min/mg protein) groups, showed a decrease of approximately 24%, which did not reach statistical significance, probably because the effect was diluted by other cellular proteins since the majority of TG activity resides within mitochondria [24]. Since TG protein level and activity are highly expressed in liver [25] we examined the systemic effects of exercise. We found that TG activity was also decreased with exercise (1.241 ± 0.091 μmol/min/mg protein) compared to the sedentary (2.622 ± 0.442 μmol/min/mg protein) group ($p < 0.05$).

3.3. Aconitase activity and TCA reducing equivalents

Since aconitase is a substrate for TG and has been shown to be altered in HD, we examined if changes in TG expression and activity also resulted in changes in aconitase activity [24]. We found that exercise increased brain mitochondrial aconitase activity by 22% comparing the sedentary group (13.92 ± 0.78 μmol/min/mg protein) with the exercise group (17.02 ± 1.05 μmol/min/mg protein) ($p < 0.05$) (Fig. 1C). This suggests that the decrease in TG activity observed in brain and liver has minimized the crosslinking with aconitase thereby rescuing aconitase activity.

Several steps in the TCA cycle generate the reduced form of nicotinamide adenine dinucleotide (NADH) that serves as a reducing agent to donate electrons in oxidative phosphorylation. We found that NAD^+ was increased 6-fold in the exercise group (0.710 ± 0.187 μM) compared to the sedentary group (0.117 ± 0.0729 μM) ($p < 0.05$) (Fig. 1D). NADH levels also increased by 22% in the exercise group but this did not reach statistical significance: sedentary (1.918 ± 0.297 μM) versus exercise (2.330 ± 0.051 μM) (Fig. 1D). The significant increase in NAD^+ suggests a higher rate of NADH

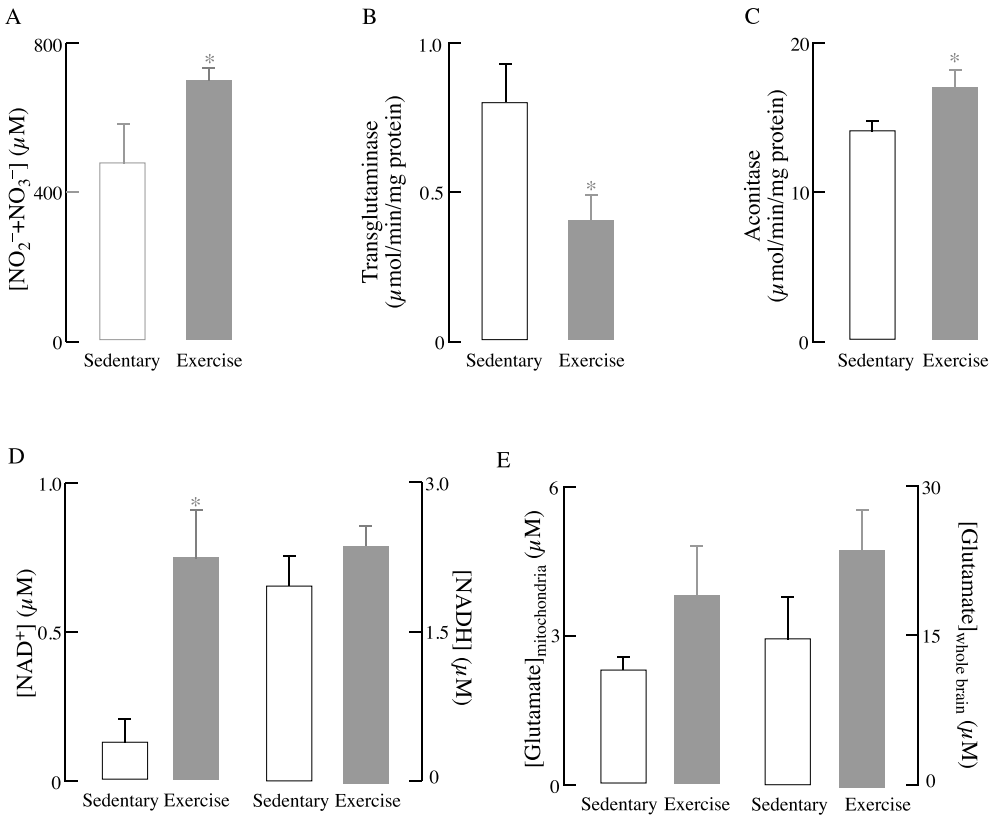

Fig. 1. *Effect of Treadmill Exercise on Metabolic Parameters*. (A) [NO_2^- + NO_3^-] levels were measured in the whole brain homogenate of the HD mouse model. (B) Transglutaminase activity was measured in isolated brain mitochondria. (C) Aconitase activity was measured in isolated brain mitochondria. (D) NAD^+ and NADH concentrations. (E) Glutamate levels in mitochondria and whole brain homogenate. Experimental conditions as described in the Materials and Methods section. $n = 8$; *$p < 0.05$.

utilization due to enhanced mitochondrial complex activities (see below and Fig. 2A–C).

The neurotransmitter glutamate is generated from α-ketoglutarate in the TCA cycle, and its impaired production and transport have been documented in HD [26]. We found that glutamate levels measured in whole brain homogenates increased 60% (sedentary (17.7 ± 4.9 μM) versus exercise (28.4 ± 4.9 μM)) and in isolated brain mitochondria increased 68% (sedentary (2.25 ± 0.25 μM) versus exercise (3.78 ± 1.07 μM)) (Fig. 1E), but did not reach statistical significance.

3.4. Mitochondrial complex activities

Since an increase in NAD + levels can be linked to increased mitochondrial complex activity, we determined the effect of exercise on the activity of Complexes I, II/III and IV (Fig. 1D) relative to sedentary mice: Complex I increased 41% (Fig. 2A) (sedentary (1.93 ± 0.14 mOD/min/mg protein) versus exercise (2.725 ± 0.20 mOD/min/mg protein) ($p < 0.01$); Complexes II/III increased 31% (Fig. 2B) (sedentary (1.24 ± 0.25 mOD/min/mg protein) versus exercise (1.62 ± 0.24 mOD/min/mg protein) ($p < 0.05$), and Complex IV increased 58% (Fig. 2C) (sedentary (1.11 ± 0.07 mOD/min/mg protein) versus exercise (1.77 ± 0.23 mOD/min/mg protein) ($p < 0.01$). The increased flow of reducing equivalents across the complexes suggests a higher rate of energy production in the form of proton gradient generated ATP.

The potential contribution of mitochondrial biogenesis was assessed by examining the mtDNA/nDNA ratio. While there was a trend for increased ratio with exercise (Fig. 2D): sedentary ($2.20 \pm 0.573 \times 10^{-4}$) versus exercise ($3.03 \pm 0.815 \times 10^{-4}$), this did not reach statistical significance.

3.5. Glycolysis regulation and upstream fuel production for TCA

Phosphofructokinase (PFK) is the key regulator of glycolysis leading to pyruvate formation in the cytosol and its further mitochondrial metabolism upon oxidative decarboxylation by the pyruvate dehydrogenase complex. This is consistent with previous reports on NO augmenting glycolysis in astrocytes through activation of PFK2 [27]. Total PFK activity (combination of PFK1 and PFK2), converting fructose-6-phosphate to fructose-(1/2),6-diphosphate was increased by 17% in the exercise group (3.486 ± 0.131 μmol/min/mg protein) as compared to sedentary (2.98 ± 0.135 μmol/min/mg protein) ($p < 0.05$) (Fig. 3A).

The pyruvate dehydrogenase (PDH) complex converts pyruvate to acetyl-CoA through oxidative decarboxylation allowing it to react with oxaloacetate to form citrate in the TCA cycle. We found that PDH complex activity was increased by 18% in the exercise (3.68 ± 0.104 μmol/min/mg protein) group versus sedentary (3.12 ± 0.227 μmol/min/mg protein) group ($p < 0.01$) (Fig. 3B).

Pyruvate carboxylase (PC) converts pyruvate to oxaloacetate, the acceptor of acetyl-CoA in the TCA cycle. Pyruvate carboxylase is expressed in astrocytes, and astrocytes are the sole *de novo* synthesizers of glutamate from glucose in the CNS [28]. Exercise increased pyruvate carboxylase concentration by 64% (4.16 ± 0.23 ng/mg protein) compared to sedentary (2.53 ± 0.19 ng/mg protein) ($p < 0.05$) (Fig. 3C).

3.6. JNK and Akt activity

Insulin signaling plays a pivotal role in peripheral tissues and the CNS where it participates in neuronal survival, synaptic plasticity, memory, and learning [29,30]. Insulin signaling is regulated by the stress-responsive Jun-N-terminal kinase (JNK) and both signaling processes interact to regulate metabolic homeostasis. The Akt pathway mediates some of the effects of insulin signaling in brain. Akt activity was increased by 22% in the exercise group expressed as the ratio of phospho~Akt/Akt; (sedentary 0.045 ± 0.0032, exercise 0.055 ± 0.0016), p < 0.05 (Fig. 4A), whereas JNK activity was decreased by 27% in the exercise group measured as a ratio phospho~JNK/JNK; (sedentary 2.93 ± 0.24, exercise 2.13 ± 0.19),

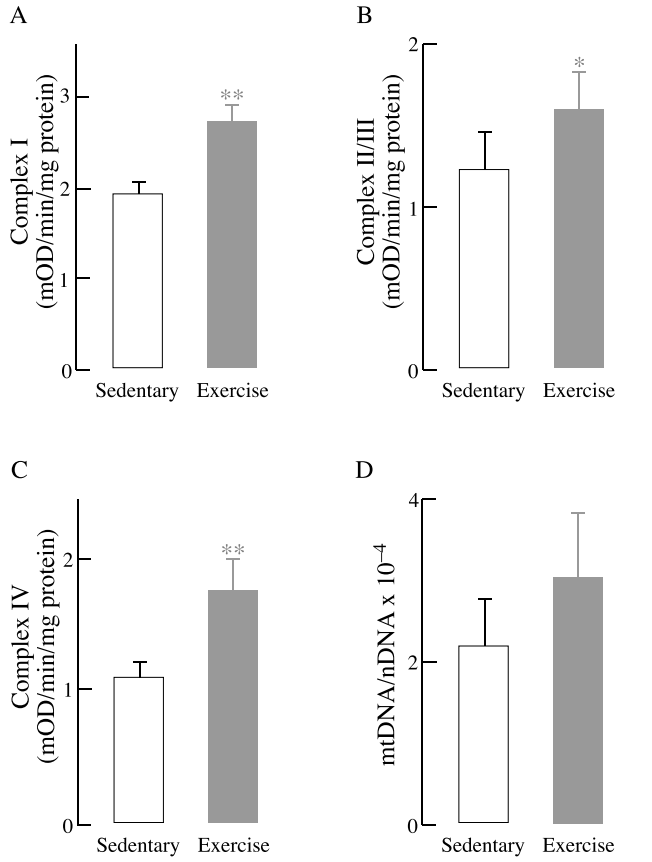

Fig. 2. *Effect of Treadmill Exercise on Mitochondrial Electron Transfer Complexes and Mitochondrial Biogenesis.* (A) Complex I; (B) Complex II/III; (C) Complex IV; (D) mitochondrial biogenesis expressed as mtDNA/nDNA values. Experimental conditions as described in the Materials and Methods section. $n = 8$; $^{*}p < 0.05$, $^{**}p < 0.01$.

$p < 0.05$ (Fig. 4B). Hence, exercise results in stimulation of the Akt pathway of insulin signaling and inhibition of the JNK pathway (Fig. 4C).

3.7. Motor behavior on the rotarod

The motor behavior in HD mice was evaluated on the accelerating rotarod using latency to fall (expressed as seconds, s). Exercise (37.0 ± 8.6 s) significantly improved latency to fall in HD mice by 73% compared to the sedentary (21.3 ± 3.5 s) ($p < 0.05$) group (Fig. 5).

4. Discussion

Exercise, incorporating components of skill and aerobic, is emerging as an important component of the standard of care in a number of neurological disorders including PD and mild cognitive impairment (MCI) [31,32]. In HD a small number of clinical trials in patients have demonstrated feasibility and some mild but transient symptomatic improvement [33,34]. We have previously shown that exercise, in the form of intensive treadmill running, initiated during the prodromal phase of disease (at 2–6 months of age) in the CAG_{140} KI mouse model modifies disease progression manifesting in improvement in a number of behaviors including anxiety, depression, and motor [20]. In addition, the time course of the emergence of HTT protein aggregate pathology was attenuate. Others have also reported the beneficial effects of exercise in rodent models of HD [35–37]. Reports of early lifestyle behaviors that incorporate physical activity may impact disease progression in humans by delaying or altering the onset of symptoms [38–40]. Despite encouraging findings, the underlying mechanisms by which exercise may impact disease progression and symptomology remain unknown, especially in neurodegenerative disorders such as HD.

In both the pre-symptomatic and symptomatic phases of HD there is evidence of deficits in neuroenergetics including mitochondrial dysfunction manifesting in a reduction in ATP production, excessive generation of ROS, and failure to efficiently buffer calcium [41]. Impairment in mitochondrial function can have devastating impact on neuronal function especially in synaptic neurotransmission, circuit connectivity, and the maintenance of neuroenergetic homeostasis. It is becoming evident that synaptic dysfunction manifests early in disease and progresses during the prodromal phase, years before typical motor behaviors of HD emerge [42,43]. Deficits in energy metabolism may in fact precede neuropathology and the loss of cells observed at autopsy [44].

The purpose of this study was to explore the potential impact of exercise, in the form of intensive running on a motorized treadmill, on markers of HD disease in a progressive mouse model at an age when typical motor symptoms emerge. In the CAG_{140} KI mouse model, motor symptoms begin to manifest at 12–14 months of age and slowly progress, thus allowing a window of opportunity by which an intervention, such as exercise, can be applied. While the potential molecular targets in energy production are numerous and complex, we focused on specific markers of metabolism and mitochondrial function known to be impacted in HD. In these studies we focused on NO due to its key role in regulating metabolism and the facts it is known to be dysfunctional in HD [11] and its expression be elevated through exercise [8,45].

We summarize graphically our working model based on our findings in the HD mouse model in Fig. 6. Exercise leads to the generation of NO, which is stored as nitrosothiols and entails Akt activation of eNOS as a major mechanism [46–48]. One function of NO is to inhibit TG cross-

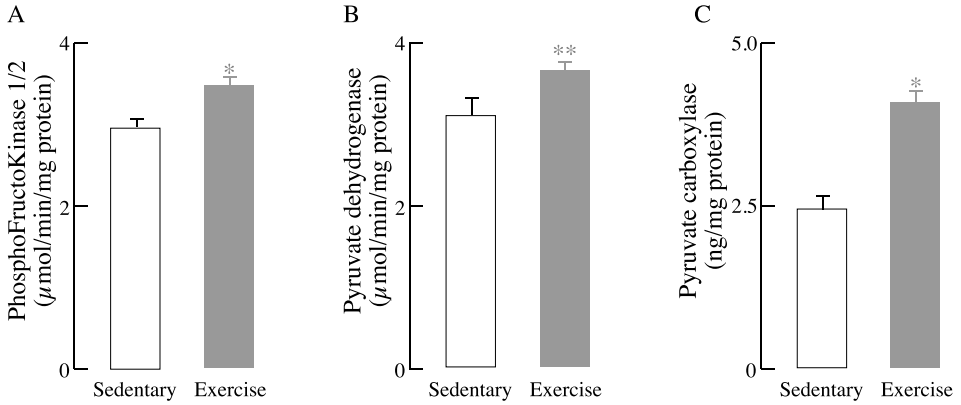

Fig. 3. *Effect of Exercise on Glucose and Pyruvate Metabolism.* (A) PFK1/2 activity; (B) Pyruvate dehydrogenase (PDH) activity. (C) Pyruvate carboxylase content. Experimental conditions as described in the Materials and Methods section. $n = 8$; $^{*}p < 0.05$, $^{**}p < 0.01$.

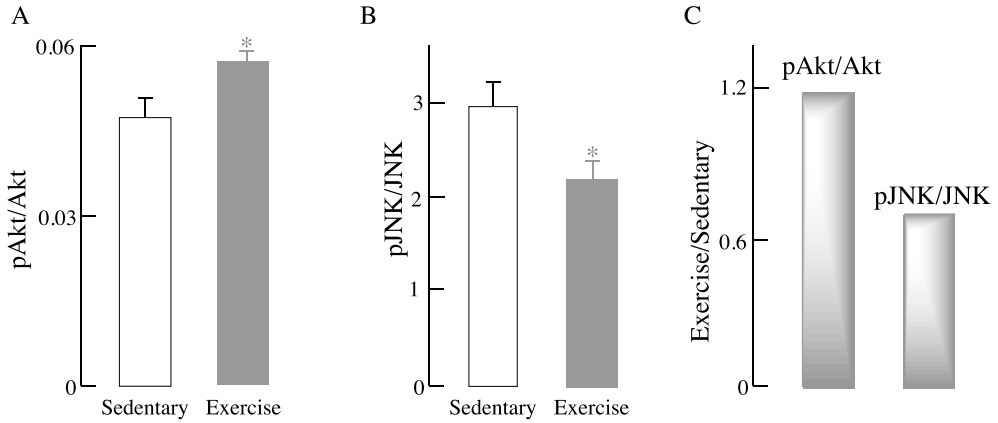

Fig. 4. *Effect of Exercise on Akt and JNK Expression.* Experimental conditions as described in the Materials and Methods section. $n = 8$; $^{*}p < 0.05$, $^{**}p < 0.01$.

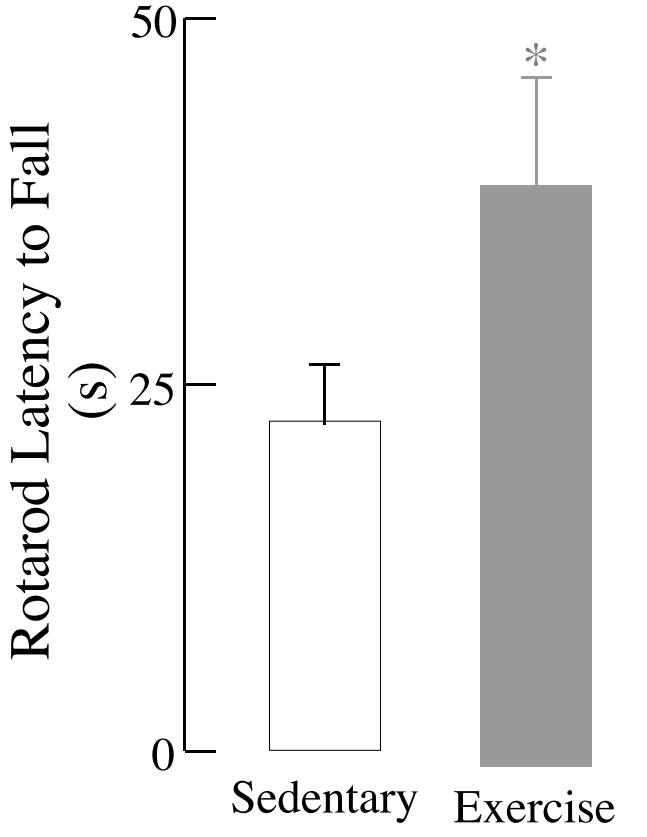

Fig. 5. *Motor Behavior as Assessed by Rotarod Test.* Rotarod latency to fall, expressed in sec. Behavioral testing as described in the Materials and Methods section. $n = 8$; $^{*}p < 0.05$.

linking activity by S-nitrosylation of Cys_{277} in the catalytic core of the enzyme [23]. Typically, TG catalyzes Ca^{2+}-dependent protein deamidation, transamidation, and cross-linking activities through addition of the ϵ-amino group of a lysyl residue to the carboxamide moiety of a glutaminyl residues to form an isopeptide bond (cross-link) and ammonia [49,50]. The majority of TG-driven cross-linking occurs in mitochondria [23,51]. TG activity has been shown to be elevated while aconitase activity is decreased in pre-clinical rodent models and CSF and postmortem brain samples of HD patients [52–55] and is involved in the pathogenesis of mitochondrial dysfunction in HD due to the formation of high molecular weight aggregates of aconitase through these cross-linking reactions [50,56]. Although a complete understanding of why TG activity is elevated in HD and other neurodegenerative diseases is unknown, several mechanisms have been proposed [24,57,58]. TG may promote protein aggregates resulting in the generation of amyloid plaques in AD, nuclear inclusions in HD, and Lewy bodies in PD that may enhance cytotoxicity in this disorder [24,58–60]. Additionally, TG may sensitize cells to apoptosis thus promoting cell death [25,61]. Evidence supporting the role of TG in HD originates from a TG knockout R6/1 HD mouse model that reported reduction in neuronal death, improved behavior, and prolonged survival [62]. In our studies, we observed decreased TG activity in brain mitochondria with exercise in HD mice. We also observed a systemic effect of exercise with decreased TG activity in the liver; however, the impact of such changes on CNS targets has yet to be established.

Aconitase catalyzes the isomerization of citrate to isocitrate in the TCA cycle. The aberrant activity of TG in HD is associated with mitochondrial dysfunction, in part due to aconitase inactivation by cross-linking [24]. Currently, it is unclear whether aconitase acts as an acyl acceptor, donor, or both, and unclear as to which mitochondrial proteins aconitase is bound as second substrates including itself. The increase of brain $[NO_2^- + NO_3^-]$ following treadmill training supports the notion that the observed decrease in brain mitochondrial TG activity is potentially a consequence of its inhibition by increased NO, thus preventing its crosslinking inactivation of aconitase. It is unlikely that peroxynitrite [63] or superoxide anion [64] are involved in aconitase inhibition because they elicit an irreversible inactivation of the iron-sulfur cluster upon loss of labile iron in the enzyme [65]. Thus, the likely mechanism is that exercise induces an inverse relationship in which aconitase activity is preserved through NO inactivation of TG in brain mitochondria. Exercise significantly improved aconitase activity in the HD mouse model, which impacted downstream levels of reducing equivalents (i.e. NADH and NAD^+). These data confirm earlier reports of inhibition of TG subjected to a NO flow from a NO-donor such as S-nitroso-N-acetylpenicillamine (SNAP) [15].

The preservation of aconitase activity further improves the generation of reducing equivalents in the TCA cycle. NAD^+ was significantly increased while NADH was not in exercised HD mice, which was interpreted as the kinetic control exerted by the high electron flow through respiratory complexes I, II/III, and IV. Although deficiencies of all mitochondrial complexes have been reported in preclinical and clinical HD, the majority of these are seen in complex II and III [12–14,54,56]. However, the effect of exercise seems to affect complexes I to IV of the respiratory chain. The neurotransmitter glutamate has also been associated with metabolic dysfunction in HD [66], albeit the substantial increase elicited by exercise was not statistically significant. Supporting a high formation of reducing equivalents in the TCA cycle are (i) faster glycolysis by conversion of glucose to pyruvate upon increased PFK1/2 activity; (ii) increased delivery of acetyl-CoA to the cycle upon increased PDH activity; and (iii) an increased anaplerotic mechanism sustained by way of pyruvate carboxylase levels. PFK and PDH as well as glucose uptake and GLUT transporter

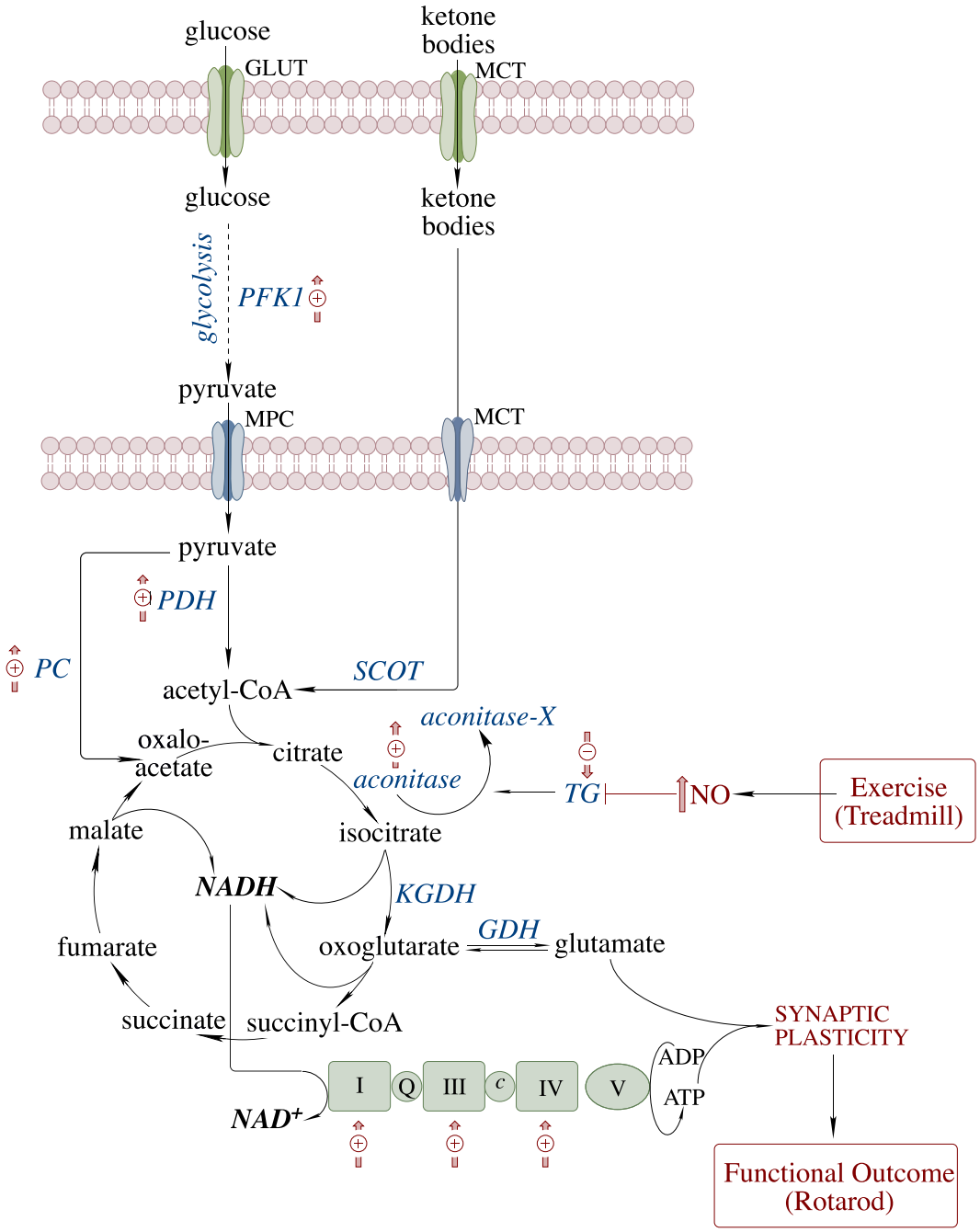

Fig. 6. *Effects of Treadmill Exercise on Metabolic Parameters.* The figure summarizes the findings in this study. Positive effects of treadmill training (resulting in brain [NO_2^- + NO_3^-] are indicated by a (+) arrow. Inhibition by a (−) arrow. MPC, mitochondrial pyruvate carrier; MCT, monocarboxylate transporter; PFK1/2, phosphofructokinase 1 and 2. PDH, pyruvate dehydrogenase; SCOT, succinyl-CoA transferase; TG, transglutaminase.

expression are impaired in HD human brain in caudate, putamen, and globus pallidus and in the striatum of R6/2 HD mouse model [54].

It is well-established that exercise impacts positively glucose transporter expression and insulin signaling through phosphorylation of Akt in skeletal muscle [67,68]. Akt insulin signaling facilitates the translocation of GLUT 3/4 in brain to the plasma membrane, promotes glycolysis, and enhances mitochondrial function through Akt translocation into mitochondria [69]. Insulin signaling plays a pivotal role not only in peripheral tissues, but also the CNS where it participates in neuronal survival, synaptic plasticity, memory and learning [29,30]. Insulin signaling is also regulated by the stress-responsive Jun-N-terminal kinase (JNK) and both signaling processes interact to regulate metabolic homeostasis. Findings from this study showed that Akt activity was increased and JNK activity was decreased with exercise. These data may confirm previous reports that endogenous NO negatively regulates JNK activation by S-nitrosylation [70]. Likewise, the significance of Akt/JNK homeostasis in brain aging and HD has been reported [71–73].

There are some limitations of these studies. Whole brain homogenates were used for analysis due to limitations in the number of aged HD mice, thus not allowing delineation of changes in metabolic markers in either a circuit specific or cell specific manner. For example, we found that pyruvate carboxylase (PC), which converts pyruvate to oxaloacetate, the acceptor of acetyl-CoA in the TCA cycle is elevated with exercise. Pyruvate carboxylase is expressed in astrocytes, and astrocytes are the sole *de novo* synthesizers of glutamate from glucose in the CNS

[28]. Astrocytes may also be playing other roles as a consequence of exercise including metabolism and synaptogenesis as we have recently demonstrated through treadmill running in mice [74]. While we examined motor behavior in these studies to show the benefits of exercise, we have not yet experimentally linked NO with behavioral outcomes. Future studies blocking or enhancing NO expression will address this issue.

In conclusion, our findings support the beneficial effects of exercise in a rodent model of HD even during the period of onset of motor symptoms. We summarize a working model for the findings in our study as shown in Fig. 6. This metabolic platform entailing NO-derived inhibition of TG activity and concomitant rescue of aconitase activity, increase in glycolysis and mitochondrial oxidative metabolism, that impinge and modulate energy metabolism. These pathways attempt to provide mechanistic insights between the initial input (treadmill exercise) and the functional outcome (improved motor behavior on the rotarod) mediated by the increase in brain NO [NO_2^- + NO_3^-]. While the impact of exercise may target a wide spectrum of molecular components, a limited number of targets such as NO may prove pivotal in mediating a cascade of effects including pathophysiology and energy metabolism all potentially impacting synaptic connectivity. The goal is to identify these critical components and to develop optimal interventions targeting both pharmacological therapeutic modalities as well as the parameters of exercise that are most effective. The increased expression and activity of protein deacetylases, sirtuins, are well-established consequences of physical exercise [75–78]. Although sirtuins affect several cellular pathways, their involvement as drivers of mitochondrial biogenesis is a salient feature: sirtuin activity and AMPK are required to activate the co-activator of mitochondrial biogenesis, PGC1α, which, in turn induces the expression of several transcription factors engaged in mitochondrial biogenesis [79]. Although we cannot rule out an involvement of sirtuins in this study, the mDNA/nDNA values shown in Fig. 2D (reflecting mitochondrial biogenesis) were not statistically significant, thus tempering sirtuin involvement in the beneficial effects of exercise reported here.

Author statement

Charles Caldwell: Investigation, Methodology, Validation, Resources, Formal analysis.

Giselle Petzinger: Formal analysis.

Michael Jakowec: Conceptualization, Writing – review & editing.

Enrique Cadenas: Conceptualization, supervision, project administration, writing, review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cbi.2019.108907.

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