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Exercise improves high-fat diet- and ovariectomy-induced insulin resistance in rats with altered hepatic fat regulation

Alexandra J. Jacobs [a], Adam L. Roskam [b], Faith M. Hummel [c], Patrick J. Ronan [d,e], Brittany K. Gorres-Martens [f,*]

[a] Biology Department, Dakota Wesleyan University, Mitchell, SD, USA
[b] Chemistry Department, Mount Marty College, Yankton, SD, USA
[c] Biology Department, Black Hills State University, Spearfish, SD, USA
[d] Veterans Affairs Research Service, Sioux Falls VA Health Care System, Sioux Falls, SD, USA
[e] Department of Psychiatry and Division of Basic Biomedical Sciences, Sanford School of Medicine, University of South Dakota, Vermillion, SD, USA
[f] Exercise and Sport Sciences Department, Augustana University, Sioux Falls, SD, USA

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ABSTRACT

A high-fat diet (HFD) and loss of endogenous estrogens increases the risk for type 2 diabetes (T2D) and insulin resistance. Although exercise is known to prevent and manage insulin resistance, the cellular mechanisms remain largely unknown, especially in the context of a combined HFD and endogenous estrogen loss via ovariectomy (OVX). This study uses female Wistar rats to assess the effect of diet, endogenous estrogens, an exercise on insulin resistance, serum hormones, hepatic AMPK, hepatic regulators of fat metabolism, and expression of signaling molecules of the brain reward pathway. The combination of the HFD/OVX increased the homeostatic model assessment of insulin resistance (HOMA-IR), the glucose-insulin (G-I) index, and the serum adiponectin and leptin values, and exercise decreased these factors. The combination of the HFD/OVX decreased hepatic pAMPK, and exercise restored hepatic pAMPK, an important regulator of fat and glucose metabolism. Furthermore, consumption of the HFD by rats with intact ovaries (and endogenous estrogens) did not result in these drastic changes compared to intact rats fed a standard diet, suggesting that the presence of estrogens provides whole body benefits. Additionally, the HFD decreased the hepatic protein expression of acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS), two proteins involved in *de novo* lipid synthesis and increased the hepatic protein expression of lipoprotein lipase (LPL), a protein involved in fat storage. Finally, exercise increased mRNA expression of the dopamine D_2 receptor and tyrosine hydroxylase in the dopaminergic neuron cell body region of the ventral tegmental area, which is a key component of the brain reward pathway. Overall, this study demonstrates that exercise prevents insulin resistance even when a HFD is combined with OVX, despite hepatic changes in ACC, FAS, and LPL.

1. Introduction

Consumption of a high-fat diet (HFD) increases the risk of adiposity and type 2 diabetes (T2D). T2D is characterized by insulin resistance, and thus, hyperglycemia and hyperinsulinemia result. In fact, the greatest risk factor of T2D is obesity, specifically, abdominal obesity (Wang et al., 2005). Both human and animal studies suggest that the presence of estrogens in females may positively modulate adiposity and blood glucose levels. Premenopausal women have lower total percent body fat and lower intraabdominal fat compared to postmenopausal women (Sites et al., 2002). Premenopausal women also have lower fasting blood glucose and insulin compared to age- and body mass index-matched post-menopausal women (Lynch et al., 2002), and estrogen replacement therapy decreases blood glucose levels in postmenopausal women with T2D (Friday et al., 2001). Thus, postmenopausal women have a greater risk for T2D and insulin resistance due to their low estrogen level.

T2D and insulin resistance results in dysregulation of physiological functions throughout the body. Most notably, high-fat feeding in male rodents classically demonstrates altered insulin signaling, decreased glucose uptake, and aberrant glucose regulation in the skeletal muscle and white adipose tissue (WAT) (Pedersen et al., 1991; Han et al., 1995; Zierath et al., 1997; Tremblay et al., 2001). However, more recent studies

* Corresponding author. Augustana University, 2001 S. Summit Ave., Sioux Falls, SD, 57197, USA.
E-mail address: brittany.gorres-martens@augie.edu (B.K. Gorres-Martens).

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focus on fat accumulation in the liver and the whole body repercussions of hepatic insulin resistance (Lallukka and Yki-Jarvinen, 2016; Seppala-Lindroos et al., 2002). Notably, the estrogen receptors are present in the liver (Chamness et al., 1975). Thus, estrogens have the potential to modulate liver metabolism. In fact, previous studies report that estrogens can alter hepatic proteins involved in *de novo* lipid synthesis (D'Eon et al., 2005; Weigt et al., 2013).

We have previously shown that, unlike males fed a HFD, female rodents fed a HFD do not demonstrate decreased glucose uptake and altered insulin signaling in the skeletal muscle and WAT (Gorres-Martens et al., 2018; Gorres et al., 1985). Thus, the present study focuses on the effects of a HFD in female rodents with and without endogenous estrogens on the liver, brain, and blood hormones. The blood hormones leptin and adiponectin are important regulators of lipid metabolism via AMPK regulation (Ahima, 2006; Minokoshi et al., 2002; Yamauchi et al., 2002). Additionally, understanding the changes in brain reward pathways may provide novel information on the effect of diet and estrogens on motivation and eating behaviors. Finally, exercise is an established means to decrease one's risk for T2D and insulin resistance (Goedecke and Micklesfield, 2014). Exercise can decrease body fat (Irwin et al., 2003) and prevent glucose intolerance (Gollisch et al., 2009). While hormone replacement therapy (HRT) may provide benefits, there are also great risks associated with HRT including breast cancer, thrombosis, and coronary heart disease (Nelson et al., 2002). Thus, assessing the benefits of exercise is an important tool for treating insulin resistance in estrogen-deplete females.

2. Materials and methods

2.1. Experimental groups

Female Wistar rats (Envigo) weighing 164 ± 2 g were kept in a temperature controlled AALAC approved vivarium at 22 ± 2 °C on a 12 h:12 h light:dark cycle and were allowed 5 days to acclimate prior to experimental procedures. Rats were fed either a standard diet (SD, 10% kcal fat; Research Diets D12450J) or a high-fat diet (HFD, 60% kcal fat; Research Diets D12492) for 10 weeks. Also, on Day 1 of the diet, some rats fed the HFD underwent bilateral ovariectomy (OVX) or sham surgery in which their ovaries remained intact (In). Furthermore, a subset of the HFD/OVX rats experienced treadmill exercise (Ex). Thus, there were four experimental groups (n = 8/group): 1) SD/In, 2) HFD/In, 3) HFD/OVX, and 4) HFD/OVX/Ex. All experimental procedures complied with NIH guidelines and were approved by the Institutional Animal Care and Use Committees at Augustana University and the University of South Dakota/Sioux Falls Veterans Affairs Health Care System.

2.2. Exercise protocol

A subset of the HFD/OVX animals were forced to exercise on a motor-driven treadmill 5 times per week. For the first two weeks after the OVX, the animals did not exercise to allow for recovery following the OVX surgery. Next, the animals exercised for 15 min per day at 35 cm/s at a 5° incline for one week. The following week, the animals exercised for 20 min per day at 45 cm/s at a 5° incline. Finally, the animals exercised for 25 min per day at 45 cm/s at a 5° incline for the remainder of the study. Rats who slowed their pace received a mild shock at the end of the treadmill to encourage continued running.

2.3. HOMA-IR and G-I index measurements

Nine weeks after starting the diet, the homeostatic model assessment of insulin resistance (HOMA-IR) and glucose-insulin (G-I) index values were determined. These measurements occurred 72 h after exercise to assess the effects of chronic exercise. After a 12-h fast, the glucose levels were measured using a drop of tail blood on a glucometer (Accu-Check Active). Tail blood was also collected for insulin measurement in heparinized microcapillary hematocrit tubes. The tubes were sealed with Critoseal and centrifuged for 5 min in a hematocrit tube centrifuge at room temperature. Plasma was collected and stored at −80 °C until the insulin levels were measured via an ELISA (ALPCO 80-INSRTU). The HOMA-IR was calculated using the following equation: (fasting insulin (μU/mL) X fasting glucose (mg/dL))/405. Following measurement of the fasting blood glucose levels, non-anesthetized rats were injected with glucose (2 g/kg; i.p.), and blood glucose levels were measured at 15, 30, 60, 90, and 120 min after the glucose injection. At each time point, tail blood was also collected for measurement of plasma insulin levels. The glucose and insulin area under the curve (AUC) for time points 0, 15, 30, 60, 90, and 120 min was determined from the blood glucose and plasma insulin values. The G-I index was calculated using the following equation: (glucose AUC X insulin AUC)/10^6.

2.4. Tissue harvest

Ten weeks after the start of the study, the rats were fasted for 12 h and then anesthetized with pentobarbital sodium (7.5 mg/100 g bw). The tissue collection occurred 72 h after the final bout of exercise. Thus, this study measures changes at the tissue level due to chronic exercise. The liver was removed, frozen in liquid nitrogen, and stored at −80 °C. Blood was collected into 1.5 mL tubes from the thoracic cavity after removal of the heart. The blood was allowed to clot for 30 min at room temperature and then placed on ice. The blood was spun at 16,000 RCF, and serum was collected and stored at −80 °C for future analysis of blood hormones. The rats were decapitated, and brains were removed and frozen in isopentane cooled on dry ice. The brains were then wrapped in parafilm and stored at −80 °C until analysis.

2.5. Serum hormone measurements

The serum leptin and adiponectin levels were measured via an ELISA according to the manufacturer's instructions for leptin (EZRL-83K; Millipore) and adiponectin (22-ADPRT-E01; ALPCO).

2.6. Western blot analysis

Approximately 50 mg of liver tissue was homogenized in cell extraction buffer (ThermoFisher FNN0011) supplemented with 200 mM PMSF (Fisher BP231), 200 mM NaF (Sigma S6776), 200 mM sodium orthovanadate (Sigma S6508), and protease inhibitor cocktail according to the manufacturer's instructions (Sigma P-2714) at a ratio of 50 mg liver:750 μl lysis buffer. The homogenized samples were rotated at 4 °C for 30 min and then centrifuged at 3,000 rpm for 20 min at 4 °C. The supernatant was removed, and the protein concentration was determined by the Bradford Bio-Rad Protein Assay Kit II (Bio-Rad 5000002). The samples were mixed with 4X Bolt LDS sample buffer (ThermoFisher B0007) and 10X Bolt sample reducing agent (ThermoFisher B0009) and heated to 70 °C for 10 min according to the manufacturer's instructions. The samples (75 μg protein) were ran on 8% Bolt bis-tris gels (ThermoFisher NW00087BOX) according to the manufacturer's instructions. The protein was transferred to a PVDF membrane using the Pierce Power Blotter, blocked for 1 h at room temperature in 5% milk in TBST, and then incubated in a primary antibody with gentle shaking on a rocker at 4 °C overnight. The primary antibodies against acetyl-CoA carboxylase (ACC; 3662), fatty acid synthase (FAS; 3189), pAMPKα T172 (2535), AMPKα (2603), and α-tubulin (9099) were purchased from Cell Signaling Technology and used according to the manufacturer's instructions. The primary antibodies against lipoprotein lipase (LPL; 373759) and the secondary antibody mouse IgG kappa binding protein conjugated to horseradish peroxidase (m-IgGκ BP-HRP; 516102) were purchased from Santa Cruz Biotechnology and used according to the manufacturer's instructions. The secondary antibody peroxidase AffiniPure donkey anti-rabbit IgG (H + L) (711-035-152) was purchased from Jackson Immuno Research and used according to the manufacturer's instructions.

The proteins were detected using SuperSignal west pico PLUS chemiluminescent substrate (ThermoFisher 34577) and visualized using a UVP ChemStudio imager. The membrane was stripped with restore plus Western blot stripping buffer (ThermoFisher 46430) for 15 min at 37 °C according to the manufacturer's instructions. The membranes that were used to detect phosphorylated proteins were re-probed for the total protein, and the membranes that were used to detect non-phosphorylated proteins were re-probed for tubulin. The protein bands were quantified using ImageJ densitometry.

2.7. Brain microdissection

Whole brains were mounted in optimal cutting temperature compound and frozen on dry ice. Frozen brains were sectioned at 300 μm using a cryostat microtome (CryoStar NX70, ThermoFisher Scientific). Slices were very briefly thaw-mounted onto microscope slides by warming the underside of the slide with a gloved finger. The slides were stored at −80 °C until further processing. Brain regions were dissected using a modified Palkovits technique with the aid of brain atlases (Palkovits and Brownstein, 1998; Paxinos and Watson, 1998) as previously described (Arendt et al., 2013, 2014). Briefly, slides were placed onto a Peltier freezing stage (PhysiTemp, Clifton, NJ) set at −25 °C, and the nucleus accumbens (NAc) and ventral tegmental area (VTA) were microdissected with the aid of a dissecting microscope (Leica EZ4) using a sterile brain punch (19–24 gauge NIH Style Neuro Punches, Fine Science Tools, Inc; Foster City, CA) connected by flexible tubing to a 10 cc syringe.

2.8. qRT-PCR mRNA quantification

Tissue pellets were gently expelled into 1.5 mL tubes with 75 μl of Quaizol lysis buffer from the Qiagen RNeasy Lipid Tissue Mini Kit (74804). RNA was extracted using the RNeasy Lipid Tissue Mini Kit according to manufacturer's instructions. To avoid DNA contamination, all samples were subjected to on-column DNase digestion using the RNase-Free DNase Set (Qiagen) and eluted into a total of 20 μl of RNase free ddH_2O. The RNA was quantified using the Qubit™ 2 Fluorometer (Invitrogen™) using the Qubit™ RNA HS Assay Kit (Invitrogen™). The RNA was converted into cDNA using the SuperScript™ III First-Strand Synthesis SuperMix for qRT-PCR (Invitrogen™) in 20 μl reactions according to manufacture recommendations, followed by RNase H treatment. TaqMan® Gene Expression Assays were used for all qPCR assays. All measurements were taken using an Applied Biosystems® 7500 Real-Time PCR system (LifeTechnologies, CA, USA) and analyzed using the Life Technologies Sequencing Detection Software v1.5.1 for the dopamine D_1 receptor (Rn03062203_s1), the dopamine D_2 receptor (Rn00561126_m1), tyrosine hydroxylase (TH; Rn00562500_m1), and GAPDH (Rn99999916_s1). Amplification was performed using the following parameters: 10 min at 95 °C; 40 cycles of 15 s at 95 °C, and 60 °C for 60 s. Expression assays were carried out in 20 μl reactions using 10 μl of 2x TaqMan™ Universal Master Mix II, no UNG (Applied Biosystems™), 1 μl of 20x TaqMan® Gene Expression Assay, and 9 μl cDNA/water. The qPCR reactions for each animal were performed in triplicate, and the results from individual reactions were averaged. Changes in gene expression were determined using the $2^{-\Delta\Delta CT}$ method. Fold induction was determined as compared to the SD/In (control) animals.

2.9. Statistical analysis

The data were statistically analyzed using IBM SPSS Statistics version 24. Normality was determined using the Shapiro–Wilk test. Normally distributed data were analyzed using a one-way ANOVA and a Tukey post-hoc test. Non-normally distributed data were analyzed using Kruskal–Wallis testing. The data are presented as the mean ± SE with n = 8/group.

3. Results

3.1. The combination of the HFD/OVX increases the HOMA-IR and G-I index

The HOMA-IR uses the fasting blood glucose an insulin values together to assess insulin resistance, with higher values indicative of greater insulin resistance. After nine weeks of high-fat feeding and OVX, the HOMA-IR was significantly increased in the HFD/OVX group compared to all other groups ($p < 0.05$; Fig. 1A). Notably, when the HFD/OVX rats experienced exercise (HFD/OVX/Ex), the HOMA-IR value was not significantly greater than the control group (SD/In). Additionally, high-fat feeding in the presence of intact ovaries (HFD/In) did not increase the HOMA-IR. These data suggest that the combination of the HFD/OVX places females at a greater risk for insulin resistance; however, chronic exercise decreases this risk.

A similar trend occurred with the G-I index, a value calculated from the area under the curve for the glucose and insulin values during a glucose tolerance test. The G-I index value represents the individual's ability to control blood glucose and insulin levels throughout a glucose tolerance test, with lower values indicative of better glucose and insulin regulation. The HFD/OVX group had a significantly greater G-I index compared to the SD/In group ($p < 0.05$; Fig. 1B). Chronic exercise decreased the G-I index, and the G-I index of the HFD/In group was not

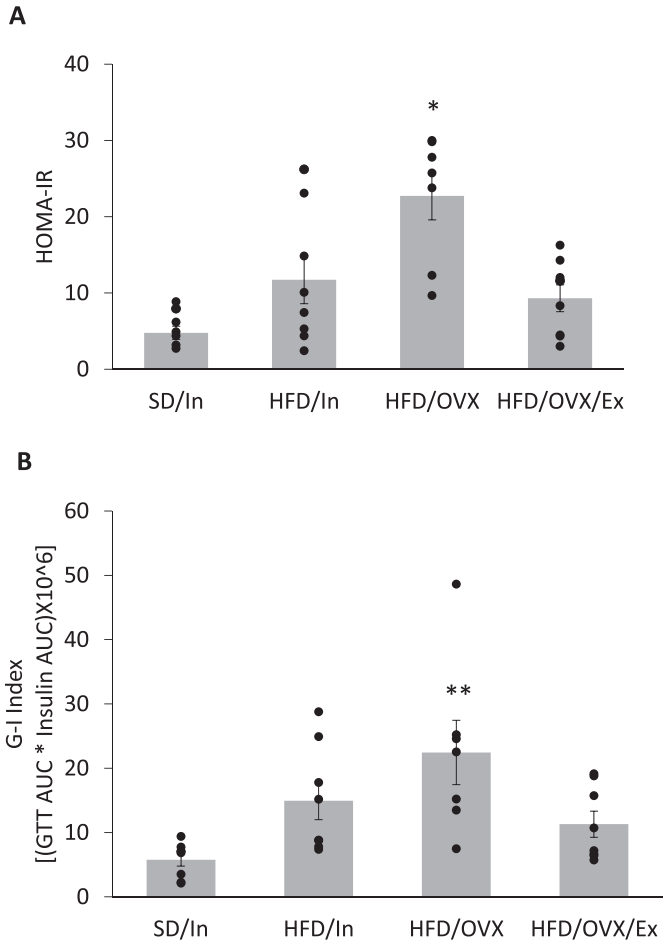

Fig. 1. The combination of the HFD/OVX increases the HOMA-IR and G-I index. Nine weeks following the start of the study, the fasting blood glucose and fasting plasma insulin levels were determined and used to calculate the HOMA-IR values (A). The G-I Index (B) was calculated from the area under the curve values for glucose and insulin during a glucose tolerance test. The data are presented as the mean ± SE. *$p < 0.05$ vs. all other groups; **$p < 0.05$ vs. SD/In.

greater than the SD/In group. These data suggest that both chronic exercise and the presence of endogenous estrogens aid in blood glucose and insulin regulation.

3.2. The combination of the HFD/OVX increases the serum adiponectin and leptin levels

Ten weeks following the start of the study, the serum adiponectin and leptin levels were measured via an ELISA. Adiponectin was significantly greater in the HFD/OVX group compared to the SD/In group ($p < 0.05$; Fig. 2A). However, the HFD/In groups did not have significantly greater levels compared to the SD/In group. Thus, while the HFD alone did not alter the adiponectin levels in female rats, the combination of the HFD/OVX increased the adiponectin levels. Additionally, when exercise was added to the HFD/OVX group, the serum adiponectin levels were not greater than the SD/In group.

A similar trend occurred with the serum leptin values. Leptin was significantly lower in the SD/In group compared to all other groups ($p < 0.05$; Fig. 2B); thus, high-fat feeding in the presence of intact ovaries (HFD/In) significantly increased the leptin levels. Furthermore, the HFD/OVX group had significantly greater leptin levels compared to the HFD/OVX/Ex group ($p < 0.05$; Fig. 2B), suggesting that chronic exercise can prevent the increased leptin induced by high-fat feeding and OVX.

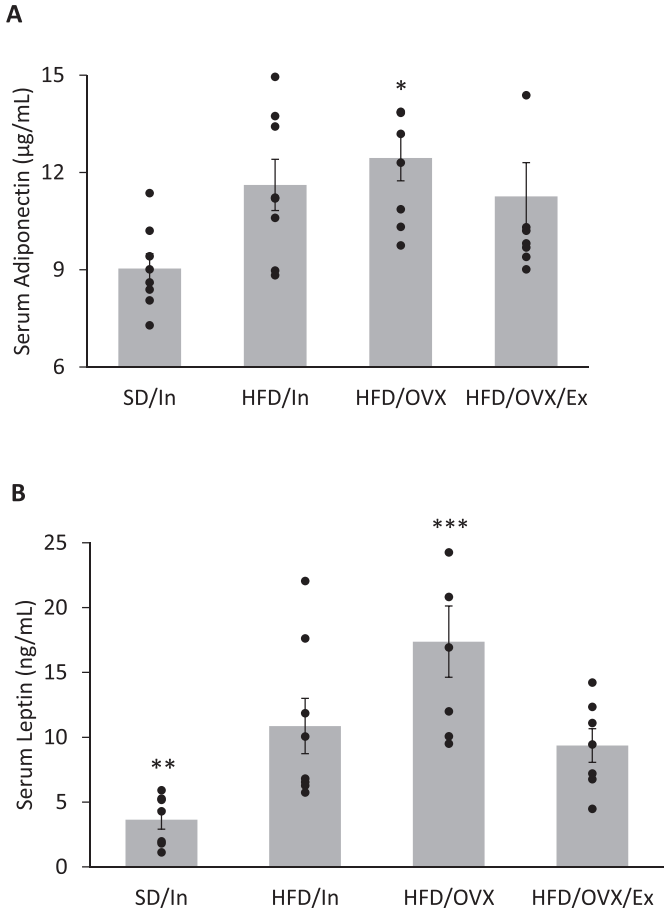

Fig. 2. The combination of the HFD/OVX increases the serum adiponectin and leptin levels. Ten weeks following the start of the study, fasting blood samples were collected, and the serum adiponectin (A) and leptin (B) levels were measured via an ELISA. The data are presented as the mean ± SE. *$p < 0.05$ vs. SD/In; **$p < 0.05$ vs. all other groups; ***$p < 0.05$ vs. HFD/OVX/Ex.

3.3. The combination of the HFD/OVX decreases hepatic pAMPK

At the end of the 10-week study, Western blot analysis measured hepatic pAMPK. AMPK is an energy sensing enzyme that stimulates fatty acid oxidation when it is activated via phosphorylation. The HFD/OVX group had significantly lower pAMPK compared to the SD/In group ($p < 0.05$; Fig. 3). Notably, the HFD/OVX/Ex group did not have reduced pAMPK. Thus, the combination of the HFD/OVX may decrease the utilization of fats as fuel, and chronic exercise restores the utilization of fats as fuel.

3.4. Alterations in hepatic proteins involved in fat metabolism

At the end of the 10-week study, Western blot analyses measured hepatic proteins involved in fat metabolism. ACC and FAS are two protein involved in *de novo* lipid synthesis. ACC and FAS were significantly greater in the SD/In group compared to all three groups fed the HFD ($p < 0.05$; Fig. 4A and $p < 0.01$; Fig. 4B, respectively). Thus, the consumption of a HFD appears to downregulate *de novo* lipid synthesis in the liver, regardless of ovarian status or exercise. LPL, a protein that stimulates fat storage, was significantly greater in all three groups fed the HFD compared to the SD/In group ($p < 0.01$; Fig. 4C).

3.5. Dopaminergic system gene expression in the brain reward pathway

qRT-PCR was performed on microdissected nucleus accumbens and ventral tegmental area tissues for tyrosine hydroxylase (rate-limiting enzyme for dopamine anabolism), and dopamine receptors D_1 and D_2. While the expression of dopamine D_1 and D_2 receptors and tyrosine hydroxylase (TH) mRNA were unaltered in the nucleus accumbens (Fig. 5A–C), there were significant changes in the ventral tegmental area. A HFD alone caused no change in D_1 mRNA expression, but when the HFD was combined with OVX, D_1 mRNA expression was significantly decreased ($p < 0.05$; Fig. 6A). There was no effect of exercise on D_1 mRNA expression. For both of D_2 and TH, the HFD/OVX/Ex group had significantly greater mRNA expression compared to all other groups ($p < 0.05$; Fig. 6B and C). The most significant difference was found in the HFD/OVX/Ex group for TH; an almost 6-fold change over all other groups.

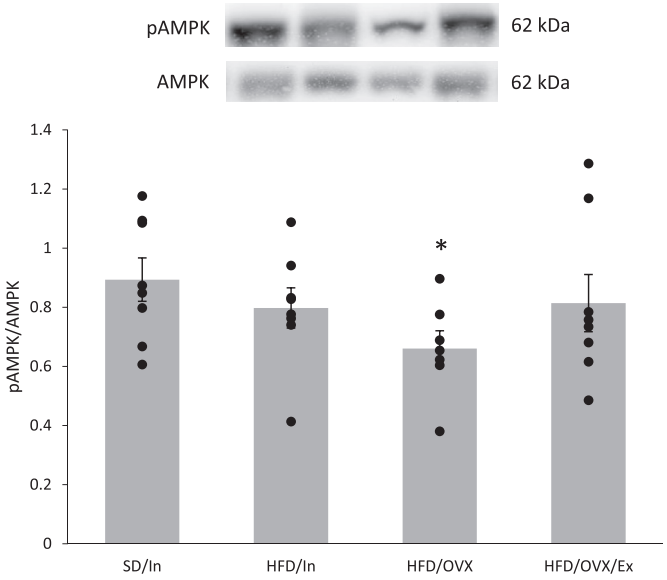

Fig. 3. The combination of the HFD/OVX decreases hepatic pAMPK. At the end of the 10-week study, the liver was collected for Western blot analysis, and the protein level of pAMPK was measured. The data are presented as the mean ± SE. *$p < 0.05$ vs. SD/In.

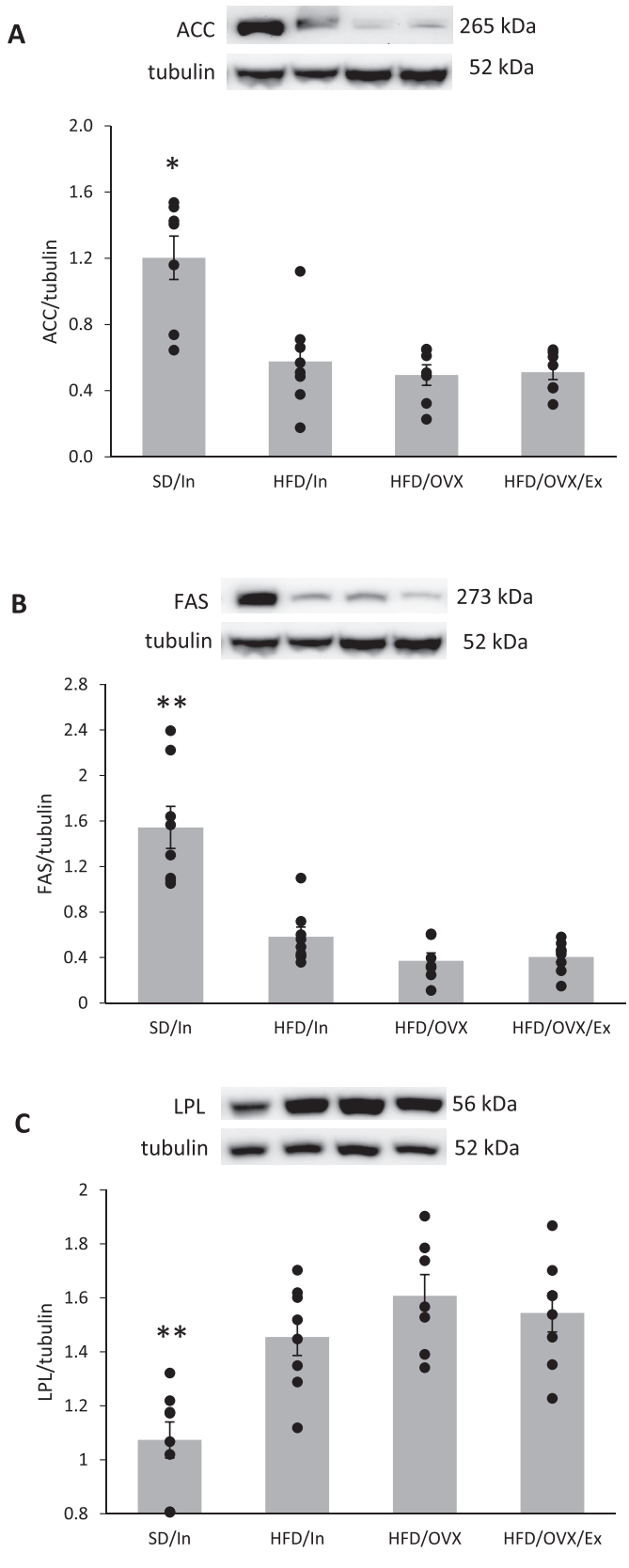

Fig. 4. Alterations in hepatic proteins involved in fat metabolism. At the end of the 10-week study, the liver was collected for Western blot analysis. The protein levels of ACC (A), FAS (B), and LPL (C) were measured. The data are presented as the mean ± SE. *p < 0.05 vs. all other groups; **p < 0.01 vs. all other groups.

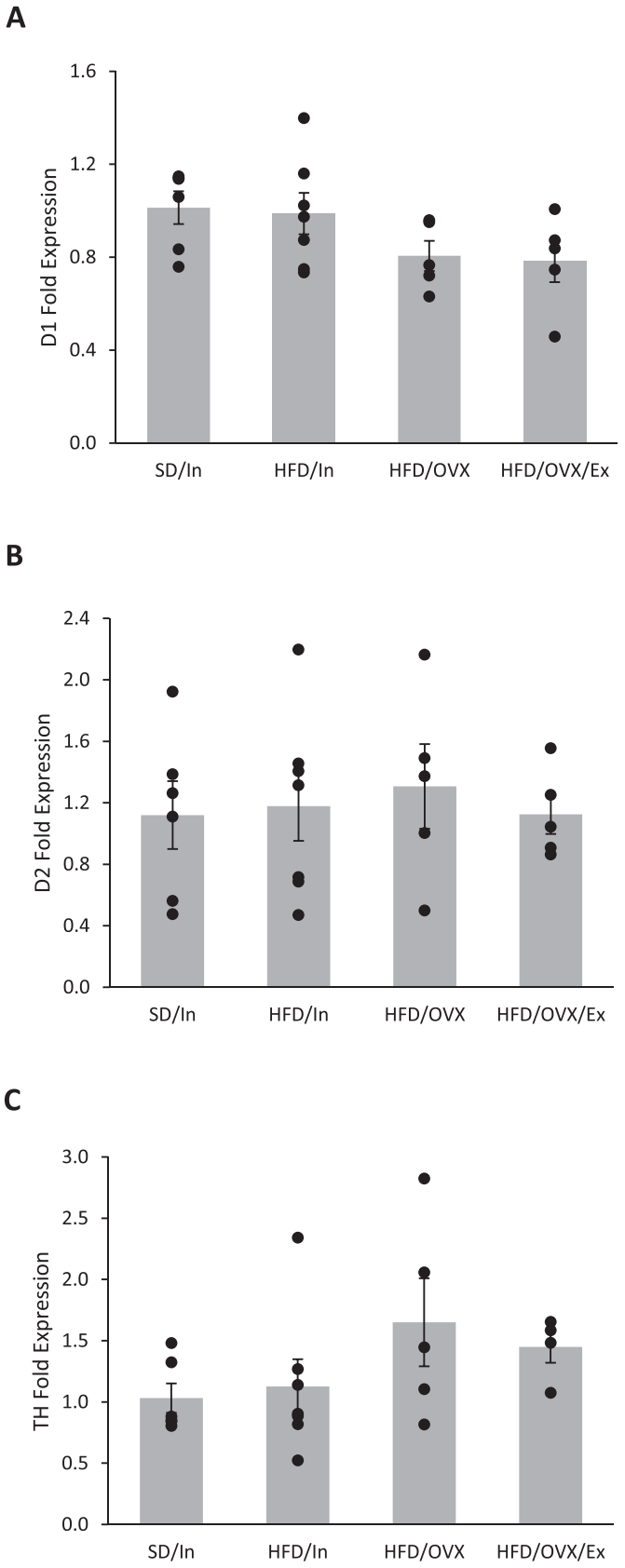

Fig. 5. Gene expression in the nucleus accumbens of the brain. At the end of the 10-week study, the brain was collected. The nucleus accumbens was isolated, and the dopamine D_1 (A) and D_2 (B) receptors and tyrosine hydroxylase (C) mRNA levels were measured. The data are presented as the mean ± SE.

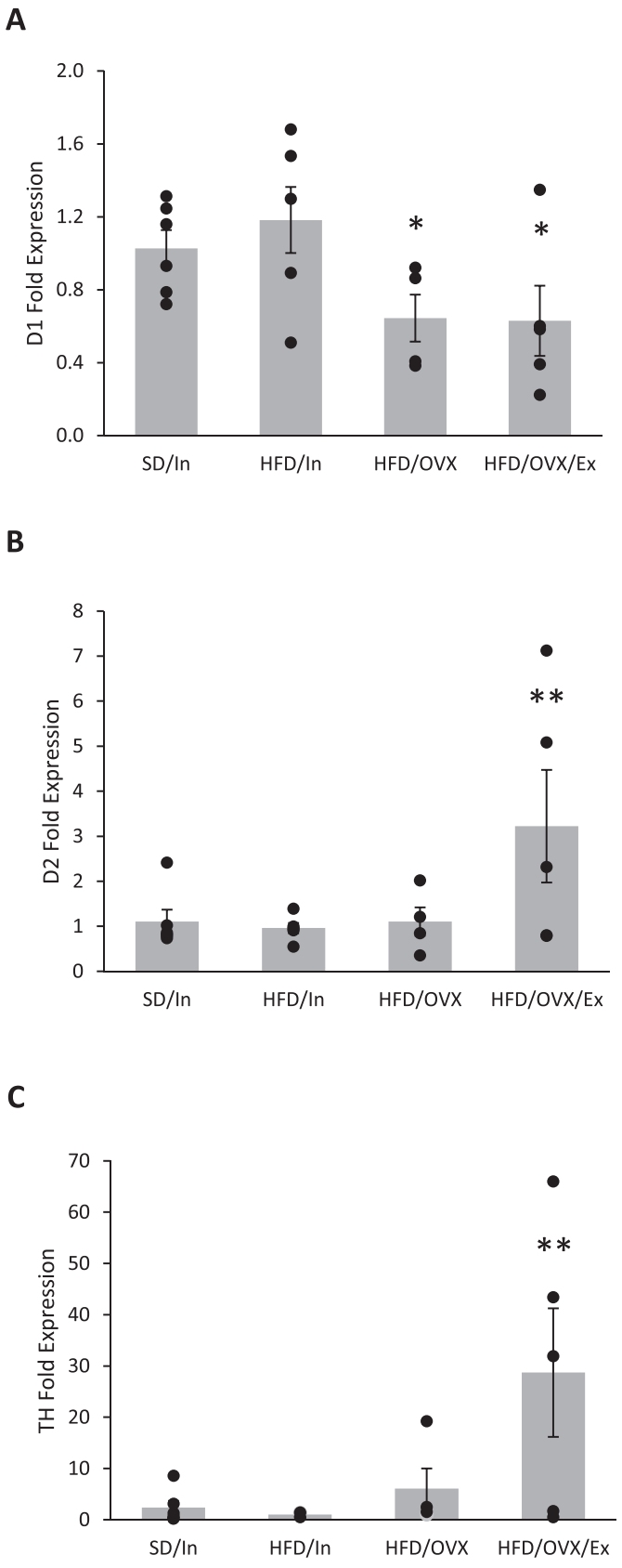

Fig. 6. Gene expression in the ventral tegmental area of the brain. At the end of the 10-week study, the brain was collected. The ventral tegmental area was isolated, and the dopamine D_1 (A) and D_2 (B) receptors and tyrosine hydroxylase (C) mRNA levels were measured. The data are presented as the mean ± SE. *$p < 0.05$ vs. HFD/In; **$p < 0.05$ vs. all other groups.

4. Discussion

High-fat feeding and obesity lead to an inability to regulate blood glucose levels. Since more than 70% of the blood glucose is disposed into the skeletal muscle (Bjornholm and Zierath, 2005), numerous studies focus on metabolic alterations in the skeletal muscle, especially the insulin signaling pathway. While obesity and high-fat feeding in male rodents certainly lead to decreased activation of the skeletal muscle insulin signaling pathway and decreased glucose uptake (Han et al., 1995; Zierath et al., 1997; Tremblay et al., 2001), we've recently shown that the insulin signaling pathway is not altered in the skeletal muscle or white adipose tissue (WAT) of female rodents fed a HFD, even when combined with OVX (Gorres-Martens et al., 2018). However, high-fat feeding and/or OVX can certainly alter other physiological functions that lead to metabolic dysregulation. The increased HOMA-IR and GI index values in the current study indicate that metabolic dysregulation does occur in the HFD/OVX rats, despite normal insulin signaling in the skeletal muscle and WAT. Thus, this paper provides important and novel data in the rat model on changes in blood hormones, the liver, and the brain that may account for the aberrations in blood glucose and insulin regulation, despite relatively healthy skeletal muscle.

In our current study, the combined HFD/OVX state resulted in increased HOMA-IR and GI index values, and chronic exercise along with the HFD/OVX decreased the HOMA-IR and GI index values. A previous study shows that OVX alone can increase the GI index, which was ameliorated by exercise (Saengsirisuwan et al., 2009). Our study supports this data and adds to the literature by adding the combined HFD/OVX physiological state, which models the physiological state of an increasing number of individuals worldwide. With respect to the HOMA-IR, a previous study shows that OVX alone did not increase the HOMA-IR (Zoth et al., 2010). However, our study adds important information to the literature by showing that combining a HFD with OVX does increase the HOMA-IR, which was ameliorated with chronic exercise. Thus, the presence of endogenous estrogens helps regulate blood glucose and insulin levels, but the loss of endogenous estrogens combined with a HFD results in dysregulation, suggesting altered metabolic regulation at the tissue level as well.

Adiponectin and leptin are important regulators of whole body lipid metabolism (Ahima, 2006).

Adiponectin is secreted from the adipose tissue and is often inversely associated with obesity (Arita et al., 1999); however, other studies report no change in serum adiponectin with high-fat feeding (Ludgero-Correia et al., 2012; Metz et al., 2016). In our study, high-fat feeding alone (HFD/In) didn't alter the serum adiponectin levels, but the combination of the HFD/OVX increased the serum adiponectin levels. In fact, several studies demonstrate that the loss of estrogens resulted in increased serum adiponectin (D'Eon et al., 2005; Ludgero-Correia et al., 2012; Gavrila et al., 2003; Leung et al., 2009). Thus, the increased adiponectin in the HFD/OVX group in our study is likely due to the loss of estrogens. Adiponectin is thought to promote insulin sensitization and prevent insulin resistance (Yamauchi et al., 2002). Thus, the increased adiponectin in the HFD/OVX group may provide protective effects against skeletal muscle and WAT insulin resistance despite consumption of a HFD, which has been shown previously (Gorres-Martens et al., 2018). Alternatively, during obesity or high-fat feeding of male rodents, the adiponectin levels do not increase (Arita et al., 1999; Metz et al., 2016), and skeletal muscle and WAT insulin resistance occur. The estrogens' ability to influence adiponectin levels may contribute to the dichotomy seen between male and females consuming a HFD and the tissues effected.

Leptin is secreted from the adipose tissue and acts on the brain to induce satiety. Serum leptin levels are directly correlated with obesity, with serum leptin levels increasing as obesity increases (Metz et al., 2016). We also show that the HFD increased the serum leptin levels. Furthermore, our study demonstrates that chronic exercise decreases the serum leptin levels, which is supported by other studies showing similar data (Metz et al., 2016; Kohrt et al., 1996; Zoth et al., 2012). This

decrease in serum leptin with chronic exercise is likely the result of the decreased adiposity in exercise-treated HFD/OVX rats, as previously published (Gorres-Martens et al., 2018).

Both adiponectin and leptin can activate AMPK in the skeletal muscle, and AMPK then stimulates skeletal muscle lipolysis, prevents lipotoxicity, and promotes insulin sensitivity (Minokoshi et al., 2002; Yamauchi et al., 2002). Thus, one may hypothesize that increased adiponectin and leptin may decrease the HOMA-IR and GI index values. However, in our study, we saw increased adiponectin and leptin in the HFD/OVX group, which also demonstrated the greatest HOMA-IR and G-I Index values, indicating insulin resistance in this group. Although adiponectin and leptin levels were increased in the HFD/OVX group, we did not see increased AMPK activation in this group. In fact, pAMPK was reduced in the HFD/OVX group, which likely lead to the increased HOMA-IR and GI index values. Notably, reduced pAMPK is often seen with high-fat feeding (Prakash et al., 2019). Additionally, the absence of estradiol results in decreased pAMPK (D'Eon et al., 2005; Rogers et al., 2009). Thus, while OVX can increase adiponectin levels and adiponectin can activate AMPK, the presence of the HFD and OVX in our study had a greater impact on regulating AMPK activation, leading to decreased pAMPK in the HFD/OVX group. This decrease in pAMPK likely explains the increased HOMA-IR and G-I Index values, as decreased pAMPK leads to insulin resistance. However, when chronic exercise was used as a treatment (HFD/OVX/Ex), the pAMPK values were restored, and the HOMA-IR and G-I Index values were reduced. Thus, chronic exercise promotes activation of hepatic AMPK even in the context of a HFD combined with OVX, and whole body regulation of blood glucose and insulin levels improved.

Little is known about the regulation of hepatic ACC, FAS, and LPL expression by diet and estrogen status. A previous study shows that high-fat feeding decreased the hepatic FAS expression in female rats (Weigt et al., 2013), and another study suggests that E_2 treatment in OVX mice fed a standard diet decreases the hepatic FAS and ACC mRNA expression (D'Eon et al., 2005). Similarly, our study shows that the HFD decreased the hepatic FAS and ACC protein expression, although the ovarian status and exercise did not alter the hepatic FAS and ACC levels. Additionally, our data supports previously published data showing that a HFD downregulates ACC and FAS protein expression in the WAT regardless of ovarian status and exercise (Gorres-Martens et al., 2018). Therefore, our data suggest that in the context of a HFD, endogenous estrogens and exercise may not be able to alter *de novo* lipid synthesis. Similarly, our data show that the increased hepatic LPL protein expression was due to the HFD and was not altered by endogenous estrogens or exercise. Previous studies have measured LPL expression in the WAT and skeletal muscle, but not in the liver. Thus, our study adds important information to the literature as LPL regulation in the liver differs from that in the WAT and skeletal muscle. In rodents fed a standard diet, E_2 treatment after OVX and the presence endogenous estrogens decreased the LPL expression in the WAT and increased the skeletal muscle LPL expression (D'Eon et al., 2005; Weigt et al., 2013). In rodents fed a HFD, endogenous estrogens decreased the LPL expression in the WAT compared to OVX rodents (Gorres-Martens et al., 2018). The ability of estrogens to decrease LPL in the WAT is thought to be beneficial, as the fat storage capability is reduced with decreased LPL. However, fat storage in the liver also affects whole body glucose regulation and insulin sensitivity, and our study shows that the HFD increases hepatic LPL expression, which may increase hepatic fat storage. These changes in the liver add novel information to the literature regarding an organ that needs to be assessed and understood to fully explain the whole body changes occurring.

Thus far, our data suggest that consumption of a HFD and estrogen status drastically influence the liver, blood hormones, and whole body glucose and insulin regulation. Ultimately, understanding the neurological mechanisms underlying the drive and motivation for consuming a HFD may provide valuable information. The ventral tegmental area and nucleus accumbens are brain regions involved in the reward pathway that contain dopaminergic neurons. Tyrosine hydroxylase is the rate limiting enzyme in the dopamine synthesis pathway (Kaushik et al., 2007), and the D_1 and D_2 receptors are the primary dopamine receptors that mediate dopamine's function.

Previous studies both support and oppose the ability of high-fat feeding to heighten the reward response to the feeding behavior. Lee et al. demonstrate that high-fat feeding in mice increased the tyrosine hydroxylase mRNA expression in the hypothalamus (Lee et al., 2010). Another study demonstrates increased activity of the dopaminergic neurons in the ventral tegmental area and nucleus accumbens in response to high-fat feeding, which would stimulate feelings of motivation and reward towards consuming a HFD (Martel and Fantino, 1996). It is well accepted that a HFD activates opioid pathways downstream of dopamine signaling in the NAc, much like drugs of abuse (Baldo et al., 2010), suggesting enhanced dopamine signaling. However, other studies show that obese rats have a decreased response in their reward pathway (Johnson and Kenny, 2010), and consumption of a high-fat diet decreased the mesolimbic (VTA and NAc) dopamine function (Davis et al., 2008). This would suggest negative feedback whereby enhanced dopamine signaling downregulates function. In our study, consumption of the HFD alone did not alter the expression of the dopamine receptors or tyrosine hydroxylase in the NAc or the VTA. However, there was an effect of estrogen status (OVX) and chronic exercise in the VTA. Loss of estrogens caused decreased D_1 expression in the VTA of HFD rats, regardless of whether they exercised or not. However, exercise led to enhanced expression of both D_2 and TH in this region.

Less is known about the effects of estrogens on the mesolimbic reward pathway in response to high-fat feeding. However, addictive food behaviors share similar overlapping reward pathways in the brain and behavioral processes with drug addiction (Volkow et al., 2008; Kelley et al., 2005), and the literature suggests that estrogens modulate VTA sensitivity to reward related stimuli. In slice preparations, ethanol-induced firing of VTA dopamine neurons was highest in mice during diestrus II when estradiol levels are the highest. Additionally, blocking estradiol receptors in the VTA inhibits ethanol-induced firing (Vandegrift et al., 2017). Thus, estradiol increases the rewarding properties of ethanol, and the same may be true in response to food consumption, especially high fat and high sugar diets, since the consumption of food and drugs stimulate similar reward pathways (Baldo et al., 2010). Our data from this study partially supports this hypothesis as we saw greater D_1 mRNA expression in the VTA of intact rats compared to OVX rats. While a previous study reports that the presence of estrogens in mice increased the tyrosine hydroxylase mRNA expression in the midbrain (Ivanova and Beyer, 2003), we did not see any changes in tyrosine hydroxylase expression in the VTA or NAc between the intact and OVX rats.

The dopamine system in the brain can regulate motor movement and motivation and reward towards exercise (Knab and Lightfoot, 2010). Previous studies also suggest that exercise promotes the function and plasticity of dopaminergic neurons in models of Parkinson's disease (Chen et al., 2018), and mice selectively bread for voluntary wheel running demonstrate increased D_2 receptor expression in the hippocampus (Bronikowski et al., 2004). Decreased D_2 expression in the VTA has been implicated in driving addiction in humans (de Jong et al., 2015). In our study, exercise increased D_2 expression in the VTA, perhaps providing a protective mechanism against addictive behaviors. We also show that chronic exercise increased TH mRNA expression in the VTA, suggesting that treadmill exercise may increase dopamine release in the VTA terminal regions such as the NAc, mPFC, and amygdala, which may be important for motivation and drive towards the exercise behavior. Previous studies suggest that the increased TH expression in the VTA after repeated cocaine administration is a key factor mediating behavioral sensitization to cocaine, which is important for the development of cocaine addiction (Sorg et al., 1993; Morgan et al., 2006). Likewise, the increased VTA TH expression induced by chronic exercise reported in this study may be important for developing a sensitization to the rewarding effects of exercise.

One limitation of the brain data we present in this paper is that mRNA levels were measured rather than protein levels due to the small amount

of brain tissue available. It is important to note that mRNA expression does not always correlate with protein expression. Additionally, the rats experienced forced treadmill exercise rather than voluntary exercise. Forced exercise may induce stress on the brain and result in an altered response of the reward pathway.

Our study certainly demonstrates benefits of chronic exercise including increased hepatic pAMPK and decreased serum leptin, which occurred concurrently with decreased HOMA-IR and GI index values, suggesting an improved overall health status. Additionally, the liver remains an important organ to consider when assessing the cellular mechanisms contributing to insulin resistance. Changes in metabolic proteins in the liver may or may not differ from the changes occurring in the same proteins in the skeletal muscle and WAT. Finally, since estrogens certainly play a role in certain aspects of metabolic regulation and the brain's reward pathway, future studies should work to better understand the interaction between estrogens and a HFD to elucidate the risks and treatment methods for insulin resistance. Furthermore, the data presented in this study pertain to the rat model. More studies are need to assess whether this information pertains to humans.

Author contributions

Alexandra J. Jacobs: Validation, Investigation, Writing – Original Draft, Visualization. **Adam L. Roskam:** Validation, Investigation, Writing – Original Draft, Visualization. **Faith M. Hummel:** Investigation. **Patrick J. Ronan:** Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision. **Brittany K. Gorres-Martens:** Conceptualization, Methodology, Formal Analysis, Investigation, Resources, Writing – Original Draft, Supervision, Funding Acquisition.

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crphys.2020.06.001.

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