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Voluntary wheel running during adolescence distinctly alters running output in adulthood in male and female rats

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Abstract

Adult female rats show greater running output compared with age-matched male rats, and the midbrain dopaminergic system may account for behavioral differences in running output. However, it is unknown if the lower running output in adult males can be regulated by wheel running experience during adolescence, and whether wheel running experience during adolescence will diminish the sex differences in running output during adulthood. We therefore determined and compared the exercise output in adult male and female rats that either had initiated voluntary wheel running only during adulthood or during adolescence. Our results demonstrate that running output in adult males were significantly higher when running was initiated during adolescence, and this higher running output was not significantly different from females. Running output did not differ during adulthood in females when wheel running was initiated during adolescence or during adulthood. Higher running output in females was associated with reduced expression of tyrosine hydroxylase and hyperactivation of calcium/calmodulin-dependent protein kinase II (CaMKII) in the dorsal striatum. Notably, running during adolescence-induced higher exercise output in adult males was associated with hyperactivation of CaMKII in the dorsal striatum, indicating a mechanistic role for CaMKII in running output. Together, the present results indicate sexually dimorphic adaptive biochemical changes in the dorsal striatum in rats that had escalated running activity, and highlight the importance of including sex as a biological variable in exploring neuroplasticity changes that predict enhanced exercise output in a voluntary physical activity paradigm.

Keywords

Physical activity; tyrosine hydroxylase; MAPK-1; D1R; PSD-95; CaMKII

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1.0 Introduction

It is becoming increasingly clear that physical activity in humans is a critical component of a high-quality life (Yeung, 1996; Reed & Ones, 2006; Wipfli *et al.*, 2008; Evero *et al.*, 2012). In support, clinical studies indicate that, engaging in physical exercise activates the brain reward system, and this effect may interfere with and reduce the reinforcement produced by other stimuli such as food or illicit drugs (Wang *et al.*, 2000; Taylor *et al.*, 2007; Boecker *et al.*, 2008; Oh & Taylor, 2012). Wheel running behavior in rodents can be modeled as a form of physical exercise, and wheel running is naturally reinforcing and rewarding in rodents (Knab *et al.*, 2009; Detweiler *et al.*, 2017; Manzanares *et al.*, 2018). Notably, the findings from physical exercise in humans have been replicated in a large volume of wheel running studies in rodents, supporting the connection between physical exercise and the brain reward system (Voss *et al.*, 2013). With respect to the brain reward system, the dopaminergic system controls motivation for natural rewards and motor movement, and several studies in rodents suggest that certain aspects of dopaminergic functioning in the dorsal striatum may contribute to the biological regulation of voluntary wheel running (Knab *et al.*, 2009; Mathes *et al.*, 2010; Greenwood *et al.*, 2011; Herrera *et al.*, 2016). Combined, these studies prompt inquiry into the molecular underpinnings underlying dopaminergic alterations in the dorsal striatum in rats that demonstrate escalation of wheel running activity.

In the context of the above hypothesis, wheel running produces adaptations within the nigrostriatal and mesolimbic pathway, consistent with repeated activation of these circuits (Foley & Fleshner, 2008; Greenwood *et al.*, 2011). For example, wheel running in rodents increases synthesis and metabolism of dopamine in the dorsal striatum, an area rich in dopamine innervation (Hattori *et al.*, 1994). Dopamine has an overall stimulatory role in motor responses via D1 receptor-mediated activation of the direct pathway neurons of the basal ganglia, or D2 receptor-mediated inhibition of the indirect pathway neurons of the basal ganglia (Foley & Fleshner, 2008). However, manipulating the levels of tyrosine or the activity of tyrosine hydroxylase, the rate limiting enzyme of dopamine synthesis, does not increase exercise output (Struder *et al.*, 1998; Chinevere *et al.*, 2002; Sutton *et al.*, 2005), suggesting that intracellular mechanisms downstream of dopamine signaling may play a role in enhanced running output. Conversely, mechanistic studies demonstrate that animals that were inbred for high wheel running performance have decreased D1 receptor function and expression in concert with reduced tyrosine hydroxylase expression in the dorsal striatum, with intact D2 receptor function (Rhodes & Garland, 2003; Knab *et al.*, 2009). These findings suggest that alterations in D1 receptor signaling may mediate motivational differences for high running output in animals endogenously demonstrating higher wheel running activity.

The most often cited molecular mechanism in the dorsal striatum underlying D1 receptor signaling-induced motivation for natural and drug rewards is activity of mitogen activated protein kinase (MAPK-1; (Mizoguchi *et al.*, 2004; Shiflett *et al.*, 2010; Cui *et al.*, 2011; Xu & Kang, 2014; Goto *et al.*, 2015) and reviewed in (Shiflett & Balleine, 2011)). Interestingly, the activity of MAPK-1 does not play a role in rewarding effects of wheel running behavior (Yokota *et al.*, 2001; Bachstetter *et al.*, 2014), suggesting alternate molecular mechanisms. For example, it is clear that the activity of calcium/calmodulin-dependent protein kinase II

(CaMKII) plays a significant role in biological regulation of voluntary wheel running (Yokota *et al.*, 2001; Bachstetter *et al.*, 2014), however, it is unknown whether activity of CaMKII in the dorsal striatum could be associated with escalated patterns of wheel running behavior. Furthermore, the effects of wheel running on the expression of plasticity-associated proteins, including D1 receptors, MAPK-1 and CaMKII in the dorsal striatum in male and female rats have not been examined. Given the robust evidence from rodent studies that female rats have higher running output than males (Eikelboom & Mills, 1988; Jones *et al.*, 1990; Hancock & Grant, 2009; Rosenfeld, 2017), it is interesting to note that sex differences in running output and the associations with altered plasticity in the dorsal striatum have been minimally explored (Mathes *et al.*, 2010). Next, several studies suggest that wheel running initiated during adulthood has significant protective effects on brain plasticity and behavior (for reviews, see (Nithianantharajah & Hannan, 2006; Gomez-Pinilla & Hillman, 2013; Voss *et al.*, 2013; Kelly, 2015)), however, it is not known whether wheel running initiated during adolescence produces similar protective effects in the adult brain (Hueston *et al.*, 2017; Dahlin *et al.*, 2019). The current study, therefore, investigated the effect of voluntary wheel running on expression of D1 receptors, MAPK-1 and CaMKII in the dorsal striatum in adult male and female rats, where wheel running was either initiated during adolescence or during adulthood. We hypothesized that running output during adolescence will be higher in male and female rats compared with running output during adulthood. Next, we speculated that the enhanced running output will be associated with increased activity of CaMKII in the dorsal striatum. Our results indicate that the activity of CaMKII is higher in adult female rats that had escalated running activity, and this effect may be important for overall sex differences in exercise output levels.

2.0 Materials and Methods

2.1 Animals

Experimental procedures were conducted in strict adherence to the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH publication number 85–23, revised 1996) and approved by the Institutional Animal Care and Use Committee of The Scripps Research Institute and VA San Diego Healthcare System. Adolescent and adult male and female GFAP-TK Long Evans rats ((Snyder *et al.*, 2016); bred at Scripps Research and VA Vivarium), were housed in a temperature-controlled (22°C) vivarium on a 12 h/12 h light/dark cycle (lights on at 9:00 P.M.) with *ad libitum* access to food and water. To avoid litter effects, which can result in a variety of problems with data interpretation, each experimental group had two to three animals (of each sex) per litter, and every litter was equally represented in each experimental group. At either 6 weeks of age (adolescent; based on age definition by (Ojeda *et al.*, 1980; Spear, 2000)) or 10 weeks (adult), rats assigned to the voluntary exercise group were moved into individual housing with *ad libitum* access to a running wheel (Nalgene activity wheels 34.5 cm diameter × 9.7 cm wide with magnetic switches connected to a PC for monitoring). The total number of revolutions was recorded in 10 minute bins and summed for each week (VitalView, Minimitter Inc.).

2.2 Tissue Collection

Following cessation of voluntary exercise (or age-matched for sedentary controls), within one hour of removal from running wheel cages, rats were briefly anesthetized with isoflourane, then rapidly decapitated and the brain was immediately removed. The right hemisphere was postfixed in 4% paraformaldehyde for immunohistochemistry and the left hemisphere was snap frozen for Western blotting.

2.3 Immunohistochemistry

For immunohistochemistry, the tissue was sliced in 40 μm sections along the coronal plane on a cryostat. Two sections through the dorsal striatum (1.60 and 1.00 mm from bregma; Figure 3a–b) and two sections enriched with the substantia nigra (–5.80 and –6.04 mm from bregma; Figure 3c–d) were mounted on Superfrost® Plus slides and dried overnight. Sections were stained for tyrosine hydroxylase (TH, 1:2,000, catalog # AB152, EMD Millipore Corporation), followed by biotin-tagged secondary antibody and visualized with DAB. For morphometric analysis of the density of TH fiber projections, images from the dorsal striatal sections were captured at 10x using a Zeiss AxioImager A2 system equipped with Zeiss AxioCam MRc camera. Images were converted to gray scale, white-balanced images with ImageJ software (NIH). Next, the TH fibers were contoured using the polygonal selection feature. A circular area in the corpus callosum was used to quantify non-specific/background staining. The maximum and minimum threshold for all the images was set to 130 and 90, respectively. The area stained (% area) and the background was measured; specific staining was calculated by subtracting the background.

TH immunoreactive cells were examined and quantified with a Zeiss AxioImagerA2 microscope equipped with MicroBrightField Stereo Investigator software, a three-axis Mac 5000 motorized stage, a Zeiss AxioCam MRc camera, PCI color frame grabber, and computer workstation. Live video images were used to draw contours delineating the substantia nigra. The fields of the brain regions for quantification were traced at 25x magnification. A $150 \times 150 \mu\text{m}$ frame was placed over the regions of interest using the Stereo Investigator stereology platform. The frame was systematically moved over the tissue to cover the entire contoured area and the labeled cells in the region falling entirely within the borders of the contour were marked and quantified. Immunoreactive cells were quantified unilaterally (absolute cell counting in the area contoured for analysis) and data are represented as number of cells per mm^2 of the region.

2.4 Western Blot Analysis

The left hemisphere and was quickly frozen in dry ice-cooled isopentane and stored at -80°C until further processing. Dorsal striatal (1.7 to 0.48 mm from bregma; (Paxinos & Watson, 2007)) tissue punches were collected from 500 μm thick sections and stored at -80°C until further processing (Figure 4a). Procedures optimized for measuring both phosphoproteins and total proteins was performed as previously described (Kim *et al.*, 2014; Galinato *et al.*, 2015; Navarro & Mandyam, 2015; Staples *et al.*, 2015). Tissue was homogenized in a refrigerated bead mill homogenizer (Next Advance) in buffer (320 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mM EDTA, 1% SDS, with Protease Inhibitor Cocktail and Phosphatase Inhibitor Cocktails II and III diluted 1:100; Sigma), heated at 95

degrees C for five minutes, and stored at -80 degrees C until determination of protein concentration by a detergent-compatible Lowry method (Bio-Rad, Hercules, CA). Samples were mixed (1:1) with a Laemmli sample buffer containing β -mercaptoethanol. Each sample containing protein from one animal was run (20 μ g per lane) on 10% SDS-PAGE gels (Bio-Rad) and transferred to polyvinylidene fluoride membranes (PVDF pore size 0.2 μ m). Blots were blocked with 2.5% (for phosphoproteins) or 5% milk (w/v) in TBST (25 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (v/v)) for one hour at room temperature and were incubated with the primary antibody for 16–20 h at 4 °C: Antibody to phosphorylated-p44/42 MAPK (pMAPK-1) at Thr202/Tyr204 (mouse monoclonal, 1:500, Cell Signaling cat# 9106S, molecular weights 44/42 kDa); total MAPK-1 (rabbit polyclonal, 1:500, Cell Signaling cat# 9102, molecular weights 44/42 kDa); pCamKII Tyr-286 (rabbit polyclonal, 1:200, Abcam cat# ab5683, molecular weight 50 kDa); total CaMKII (rabbit polyclonal, 1:200, Abcam cat# ab52476, molecular weight 47 kDa); D1R (rabbit polyclonal, 1:500, Abcam cat# ab20066, molecular weight 48 kDa); PSD-95 (rabbit polyclonal, 1:500, Millipore cat# 04–1066, molecular weight 95 kDa). Blots were then washed three times for 5 min in TBST, and then incubated for 1 h at room temperature with horseradish peroxidase–conjugated goat antibody to mouse or rabbit in TBST. Following subsequent washes, immunoreactivity was detected using SuperSignalWest Dura chemiluminescence detection reagent (Thermo Scientific, Waltham, MA, USA) and images were collected using a digital imaging system (Azure Imager c600, VWR, Radnor, PA, USA). For normalization purposes, membranes were incubated with 0.125% coomassie stain for 5 min and washed three times for 5–10 min in destain solution (Welinder & Ekblad, 2011; Thacker *et al.*, 2016). Densitometry was performed using ImageJ software (NIH). The signal value of the band of interest following subtraction of the background calculation was then expressed as a ratio of the corresponding coomassie signal (following background subtraction). This ratio of expression for each band was then expressed as a percent of the adult male sedentary rat included on the same blot.

2.5 Statistical analysis

Body weight was monitored weekly for all subjects beginning at onset of voluntary exercise (age-matched for sedentary controls). Weight gained during the duration of the experiment was analyzed by a three-way ANOVA with age, sex and physical activity the between-subject independent variables and weight gained the dependent variable. Running output was monitored in ten minute bins and analyzed as weekly output over a period of 8 weeks. Weekly running activity was statistically assessed utilizing a mixed-model two-way ANOVA with age-group as the between-subject and week of activity as the within-subject independent factors and number of wheel revolutions the dependent factors. Post-hoc comparisons (Fisher's LSD) were performed comparing male and female, adolescent and adult wheel activity on each week, as the weekly average running output within each age group to the week one average output. Differences in density of proteins or number of immunoreactive cells were analyzed by one-way ANOVA. For Western blotting, data analysis was performed on raw density values and graphs are represented as percent change from adult male sedentary control. Pearson's correlations were used to examine the relationship between wheel running output and pCaMKII expression. Data are expressed as mean $\pm$ SEM and were analyzed using GraphPad Prism v7.0 or SPSS v20. Values of p

0.05 were considered statistically significant. Graphs were generated using GraphPad Prism v7.0 software.

3.0 Results

3.1 Male rats weigh more than female rats during adolescence and adulthood; Running reduces body weight in male and females

Rats were weighed once weekly during the exercise period (or time matched for sedentary controls); body weight is presented in Figure 1. Three-way ANOVA with age, sex and running activity as independent factors and body weight as the dependent factor did not detect a significant sex $\times$ age $\times$ running interaction [$F(1, 283)=1.8$, $p=0.17$] or age $\times$ running interaction [$F(1, 283)=0.6$, $p=0.41$], however, detected a significant sex $\times$ age interaction [$F(1, 283)=9.6$, $p=0.002$], and sex $\times$ running interaction [$F(1, 283)=9.7$, $p=0.002$]. A significant main effect of sex [$F(1, 283)=301$, $p<0.001$], age [$F(1, 283)=134$, $p<0.001$] and running [$F(1, 283)=19$, $p<0.001$] was revealed. Post-hoc analysis showed higher body weight in males compared with females in weeks 7 through 13 ($p<0.05$; Figure 1), and higher body weight in sedentary females vs. running females and higher body weight in sedentary males vs. running males in weeks 9 through 13 (Figure 1).

3.2 Running Output is higher in adult males when wheel running is initiated during adolescence

Running output as revolutions per week was monitored for the duration of the exercise period (Figure 2). Repeated measures ANOVA was used to compare running output in adolescent females and males, with sex and age as the between-subjects independent factor, week of running the within-subject independent factor, and average wheel revolutions per week as the dependent factor.

Three-way ANOVA demonstrated a significant sex $\times$ age $\times$ running output interaction [$F(3, 156)=3.2$, $p=0.025$], a significant sex $\times$ age interaction [$F(1, 156)=55.7$, $p<0.0001$], a significant sex $\times$ running output interaction [$F(7, 156)=5.12$, $p<0.0001$] and a age $\times$ running output interaction [$F(7, 156)=5.12$, $p<0.0001$]. Post-hoc analysis revealed higher running output in adolescent male runners during weeks 10 to 13 compared to adult male runners ($p<0.05$).

Repeated measures two-way ANOVA analysis was used to compare running output in adolescent females and males, with sex as the between-subjects independent factor, week of running the within-subject independent factor, and average wheel revolutions per week as the dependent factor. A significant main effect of sex [$F(1, 88)=15.2$, $p=0.0002$], significant main effect of weeks [$F(7, 88)=35.8$, $p<0.0001$], and a significant interaction of the two factors (sex $\times$ week) [$F(7, 88)=2.15$, $p=0.04$] was revealed. Subsequent post-hoc comparisons demonstrated the female runners demonstrated greater running output compared to males during weeks 8 and 13 ($p<0.05$; Figure 2). Next, repeated measures two-way ANOVA analysis was used to compare running output in adult females and males, with sex as the between-subjects independent factor, week of running the within-subject independent factor, and average wheel revolutions per week as the dependent factor. A

significant main effect of sex [$F(1, 44)=193.8, p<0.0001$], significant main effect of weeks [$F(3, 44)=23.4, p<0.0001$], and a significant interaction of the two factors (sex $\times$ week) [$F(3, 44)=11.9, p<0.0001$] was revealed. Subsequent post-hoc comparisons demonstrated that female runners have greater running output compared to males during weeks 10 to 13 ($p<0.05$; Figure 2).

3.3 TH expression and number of TH neurons following voluntary exercise in male and female rats

Rats were euthanized following the completion of the running period, and their striatal tissue was processed for immunohistochemical analysis (Figure 3). The density of TH fibers in the dorsal striatum, in relation to age-matched sedentary controls, in males and females is presented in Figure 3. First, two-way ANOVA revealed significant sex $\times$ running output interaction [$F(1, 14)=5.04, p=0.04$] and a main effect of running [$F(1, 14)=19.9, p<0.001$]. Post-hoc analysis showed reduced density of TH fibers in the dorsal striatum in adult female runners and sedentary males compared with sedentary females ($p<0.05$). Second, one-way ANOVA was performed to determine whether running initiated adulthood and running initiated during adolescence produced distinct changes in the density of TH in the dorsal striatum. One-way ANOVA did not reveal any significant effect of running in males (n.s.).

The number of TH neurons in the substantia nigra, in relation to age-matched sedentary controls, in males and females is presented in Figure 3. Two-way ANOVA revealed significant main effect of sex [$F(1, 14)=6.9, p=0.01$]. One-way ANOVA was performed to determine whether running initiated adulthood and running initiated during adolescence produced distinct changes in the number of TH neurons in the substantia nigra. One-way ANOVA did not reveal any significant effect of running in males (n.s.).

3.4 Expression of plasticity related proteins in the dorsal striatum following voluntary exercise in male and female rats

Rats were euthanized following the completion of the running period, and their dorsal striatal tissue was processed for Western blotting (Figure 4a). The density of total and activated CaMKII, total and activated MAPK-1, D1R and PSD-95 in the dorsal striatum, in relation to age-matched male sedentary controls, in males and females is presented in Figure 4.

Two-way ANOVA of the density of pCaMKII revealed significant sex $\times$ running interaction [$F(2, 25)=4.7, p=0.01$], a main effect of sex [$F(1, 25)=8.3, p=0.008$] and a main effect of running [$F(2, 25)=6.3, p=0.005$]. Post-hoc analysis showed enhanced pCaMKII in the dorsal striatum in adult and adolescent female runners compared with sedentary females ($p<0.05$), and adolescent male runners compared with sedentary males ($p<0.05$). Two-way ANOVA of the density of tCaMKII revealed significant sex $\times$ running interaction [$F(2, 25)=4.7, p=0.01$] and a main effect of running [$F(2, 25)=3.2, p=0.05$]. Post-hoc analysis showed reduced tCaMKII in the dorsal striatum in adolescent male runners compared with sedentary males ($p<0.05$). Two-way ANOVA of the density of pMAPK-1 and tMAPK-1 did not show any significant differences. Two-way ANOVA of the density of D1R did not detect a sex $\times$ running interaction [$F(2, 25)=0.5, p=0.5$], however, detected a main effect of sex [$F(1,$

25)=4.5, $p=0.04$] and main effect of running [$F(2, 25)=3.3$, $p=0.05$]. Two-way ANOVA of the density of PSD-95 did not show any significant differences between any groups.

3.5 Relationship between running output and pCaMKII expression in the dorsal striatum

Pearson's correlations revealed no relationship between wheel running (running output from the last day) and pCaMKII expression in male and female rats (males: $R^2 = 0.01$, $p = 0.76$; females: $R^2 = 0.06$, $p = 0.44$). These findings suggest that running output *per se* enhanced activity of CaMKII and the amount of running was not predictive of the amount of expression of pCaMKII.

4.0 Discussion

The primary goal of this work was to identify differences in running output in adult male and female rats, when access to wheel running was initiated during adolescence or during adulthood. Our secondary goal was to identify an associated mechanism in the dorsal striatum that could be correlated with enhanced running output. We first found that male and female rats have a similar running output when wheel running is initiated during adolescence. This finding is in agreement with previously published studies in rodents (Hancock & Grant, 2009; Gallego *et al.*, 2015). We next report that female rats have a higher running output compared to males when wheel running is initiated during adulthood. This finding is also in agreement with previously published studies (Eikelboom & Mills, 1988; Jones *et al.*, 1990; Hancock & Grant, 2009; Rosenfeld, 2017). We add to these findings to report that the sex differences in running output is abolished in adult male and female rats when running was initiated during adolescence. For example, our findings revealed that wheel running initiated during adolescence enhanced running output during adulthood in males, whereas wheel running initiated during adolescence failed to show a similar enhancement in running output in females, indicating that this effect was sex specific. Additional findings from postmortem tissue analysis revealed that higher running output in adult female rats was associated with enhanced activity of CaMKII in the dorsal striatum. Furthermore, adolescent-initiated running in adult males increased running output to the levels of adult females in concert with increased activity of CaMKII in the dorsal striatum. To the best of our knowledge, this is the first study to investigate the impact of adolescent-initiated wheel running on running output in adulthood in male and female rats, and alterations in the plasticity related proteins in the brain region implicated in motivation for voluntary wheel running behavior.

Adult female rats escalated their running activity and showed significantly more running output than adult male rats, when wheel running was initiated during adulthood. Furthermore, the escalated wheel running in adult females was reflective of the magnitude of activated CaMKII in the dorsal striatum. From a translational perspective, this sex difference in running output is potentially an important observation, as much of our understanding of how running influences molecular and cellular components of reward and motivation is derived from analysis of male subjects (Novak *et al.*, 2012). It is important to note that adolescent-initiated wheel running enhanced running output in adult males to a degree similar to adult females. More notably, wheel running during adolescence produced higher

running output in adult males, and this was also associated with enhanced activity of CaMKII in the dorsal striatum. We also performed correlations between running output and activated CaMKII and did not detect any relationships, suggesting that greater amount of sustained running activity *per se* enhanced activity of CaMKII and was independent of the amount of running in the high performers. A lack of correlation may be due to the fact that the duration of activity of CaMKII influenced by synaptic plasticity lasts for minutes to hours *in vivo* (Fukunaga *et al.*, 1993; Murakoshi *et al.*, 2017). Nevertheless, the finding of enhanced activity of CaMKII is important because, previous observations from *in vitro* studies indicate that dopamine efflux is regulated by cytosolic Ca^{2+} (Gnegy *et al.*, 2004). In addition, CaMKII is regulated by cytosolic Ca^{2+} , and is abundant in striatal neurons (Choe & Wang, 2001). Specifically, CaMKII has been implicated in maintaining synaptic plasticity in striatal medium-sized spiny neurons (Klug *et al.*, 2012), and is involved in the establishment of reward-based behaviors (Li *et al.*, 2008). Furthermore, CaMKII regulates dopamine efflux in response to amphetamines (Fog *et al.*, 2006; Steinkellner *et al.*, 2012), whereas, CaMKII-knock out mice have two-fold higher extracellular dopamine concentrations, indicating that CaMKII acts as a bidirectional modulator of dopamine release (Steinkellner *et al.*, 2014). Enhanced activity (phosphorylation) of CaMKII is also correlated with increases in dopamine release in the striatum (Riday *et al.*, 2012). Taken together, the activation (hyperphosphorylation) of CaMKII in the dorsal striatum in females can be indicative of altered dopamine release in response to escalated wheel running activity, and could be associated with alterations in TH expression and functionality of D1 receptors (Rhodes & Garland, 2003; Knab *et al.*, 2009).

In the context of the above relationship, it is notable that lower levels of TH in the dorsal striatum has been correlated with higher running output in mice selectively bred for high activity (Knab *et al.*, 2009). This could indicate depletion of dopamine production in animals with higher running output, and may be important for the escalated patterns of activity, as seen in females in our study. While decreased or depleted dopaminergic function in animals endogenously demonstrating higher running output is contradictory to other studies that have demonstrated a direct relationship between dopamine depletion in the striatum and decreased locomotor activity (Tolliver & Carney, 1994; Leng *et al.*, 2004), these findings highlight the importance of understanding the motivational factors involved in biology of wheel running behavior. It is also possible that sex differences in the dopamine system may directly mediate differences in running output, instead of wheel running activity itself acting to alter the dopamine system (Yoest *et al.*, 2018a; Yoest *et al.*, 2018b).

These findings, while intriguing, must be presented with a caveat. We must acknowledge that enhanced sensitivity of CaMKII activity to exercise observed in female rats and adult male rats that initiated wheel running during adolescence is likely due, at least in part, to the reduction in the amount of CaMKII activity measured in adult male rats that initiated wheel running during adulthood. There is possibly a threshold of minimal running output which must be achieved in order for the CaMKII modulating effects of exercise to be observable, and the adult male rats that initiated wheel running during adulthood did not breach this threshold. Therefore, future studies to address this would require a forced exercise paradigm during adulthood in adult male rats, which was avoided in the present work to avoid the potential for stress effects subsequent to forced activity. Next, while the rats in our study

were individually housed in wheel equipped cages, new technological advances have provided methods to monitor wheel activity in pair housed cages. Radio frequency identification in a new method that implants electronic microchips into animals, such that their individual activity can be monitored over an extended period of time (Hoy *et al.*, 2010; Schuch *et al.*, 2019). Notably, wheel running output does not significantly differ between rats that are individually housed versus pair housed with wheel access, at least when evaluated for days (O'Connor & Eikelboom, 2000). Therefore, these findings suggest that, while running output may be similar in conditions of individual versus pair housing conditions, the enriched environment associated with pair housing may have significant effects on brain plasticity (Kempermann *et al.*, 2010). Lastly, we acknowledge that other neurotransmitter systems in the dorsal striatum may have played a role in the enhanced running output seen in adult male rats when running activity was initiated during adolescence. For example, wheel running enhances presynaptic GABA release in the striatum via a cannabinoid CB1 receptor-mediated mechanism, suggesting that changes in the sensitivity of striatal CB1 receptors represent a common synaptic correlate of the activation of the central reward pathway (De Chiara *et al.*, 2010).

In summary, our findings reveal effects of sex and onset of exercise initiation on specific modulation of striatal CaMKII activity following unlimited access to voluntary wheel running. Future studies stemming from this work should include investigations into the emotional and behavioral correlates of these findings to better attribute the molecular changes in plasticity related proteins to a quantifiable and translational output (O'Leary *et al.*, 2019a; O'Leary *et al.*, 2019b).

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Highlights:

- Adolescent-initiated wheel running abolishes sex differences in running output in adults
- Running output predicts alterations in TH in the dorsal striatum in adult rats
- Escalated patterns of running output is associated with hyperphosphorylation of CaMKII

Figure 1. Weekly body weight (g) of male and female rats that were sedentary or experienced wheel running.

Body weight increased steadily across weeks in male and female rats. Number of animals in each group is indicated in parenthesis. Data is indicated as mean $\pm$ S.E.M. * $p < 0.05$ vs. sedentary females, # $p < 0.05$ vs. Adult female runners, * $p < 0.05$ vs. sedentary males, # $p < 0.05$ vs. Adult male runners; * $p < 0.05$ vs. males.

Figure 2. Running output of male and female adolescent and adult rats. Running output is indicated as wheel rotations per week and was collected at 10-min intervals. Number of animals in each group is n=6 adult males, n=7 adult females, n=8 adolescent males and n=5 adolescent females. Data is indicated as mean $\pm$ S.E.M. *p<0.05 vs. adult male runners, #p<0.05 vs adolescent male runners, *p<0.05 vs. adult male runners.

Figure 3. TH expression in the dorsal striatum and number of TH cells in the substantia nigra in runners and sedentary controls.

(a-b) Representative photomicrographs of DAB stained sections containing the dorsal striatum (a-b) and substantia nigra (c-d). (a-b) TH stained sections of the dorsal striatum (a-b) from one sedentary female (a) and one sedentary male (b) rat. (c-d) Representative TH stained section of the substantia nigra from one sedentary female. Box in (c) is zoomed in in (d). Arrow in (d) points to a TH-labeled cell. Scale bar in d applies (a-d); in a-c = 20 μ m; ind = 200 μ m. cc, corpus callosum. (e-f) Quantitative analysis of TH fibers (e) and TH immunoreactive cells (f). Number of animals in each group is n=4 sedentary females, n=5 sedentary males, n=5 adult female runners, n=4 adult male runners and n=4 adolescent male runners. Data is indicated as mean $\pm$ S.E.M. %p<0.05 significant interaction; &p<0.05 main effect of sex; \$p<0.05 main effect of running; *p<0.05 vs. sedentary females and #p<0.05 vs. sedentary females by posthoc analysis.

Figure 4. Plasticity related proteins in the dorsal striatum in runners and sedentary controls. Dorsal striatal tissue was collected from all animals. a) Schematic representation of dorsal striatum section adapted from the Paxinos and Watson rat atlas. Tissue punches were collected from 500 μ m thick sections represented by blue circles in the site of dorsal striatal tissue. b) Representative western blots and associated coomassie staining in all groups. c-f) Quantitative analysis of all proteins as percent of sedentary age-match males in each blot. Number of animals in each group is n=4 sedentary females, n=5 sedentary males, n=6 adult female runners, n=4 adult male runners, n=5 adolescent female runners and n=4 adolescent male runners. Data is indicated as mean $\pm$ S.E.M. %p<0.05 significant interaction; &p<0.05 main effect of sex; \$p<0.05 main effect of running. *p<0.05 vs. adult male runner; #p<0.05 vs. sedentary females.