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Neuroanatomical, Behavioral, and Physiological Correlates of High Voluntary Wheel Running

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

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by

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DEDICATION

For all of the steadfast front-line workers honorably resisting the COVID-19 pandemic. For Ignaz Semmelweis, may we overcome the tendency to reject new evidence or knowledge because it contradicts established norms or beliefs.

ABSTRACT OF THE DISSERTATION

Neuroanatomical, Behavioral, and Physiological Correlates of High Voluntary Wheel Running

by

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Dr. Theodore Garland, Jr., Chairperson

Exercise has a myriad of benefits for brain and body health. Wheel running is an animal model of physical activity, and artificial selection for increased levels of running in rodents can reveal how genes and the environment affect exercise physiology. I examined endocannabinoids (eCBs), neuroanatomy, and reward behavior in four replicate lines of mice bred for high voluntary wheel running (High Runners; HR), compared to four non-selected Control (C) lines.

First, I examined the eCBs 2-arachidonoyl-*sn*-glycerol (2-AG) and anandamide (AEA) and eCB analogs docosahexaenoylglycerol (DHG), oleoylethanolamide (OEA), and docosahexaenoylethanolamide (DHEA) in the upper small-intestinal epithelium of male and female HR and C mice, housed with or without wheel access for six days. I found a significant 3-way interaction of linetype, wheel access, and sex for 2-AG and DHG. When compared to C

mice, lines of HR mice had lower concentrations of 2-AG. Also, jejunal epithelial 2-AG was significantly correlated with circulating 2-AG (data from a prior study in the same mice).

Second, I measured five key brain region volumes and cell densities of female HR and C mice, either given or not given wheel access for 10 weeks from weaning. The red nucleus and the hippocampus were significantly larger in HR compared to C brains, but no difference was observed for the basolateral amygdala, nucleus accumbens or ventral pallidum. The cell densities of each region were not statistically different between HR and C mice. Chronic wheel access did not affect the volume or cell density of any region.

Third, I used a behavioral test of conditioned place preference (CPP) with methylphenidate and cocaine stimuli, as well as wheel access, in three separate studies to evaluate the extent to which genetic predisposition for exercise reward is associated with increased drug or exercise reward. Both HR and C mice displayed significant CPP for cocaine and methylphenidate, but with no statistical difference between linetypes for either drug. Neither HR nor C mice conditioned to wheel access.

Altogether, I identified underlying traits that may contribute to the increased ability and/or motivation of HR mice for running ~3x more per day than C lines.

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INTRODUCTION

The *Physical Activity Guidelines Advisory Committee Scientific Report* (2018) defines physical activity, exercise, and physical fitness as distinct terms (see also Garland et al. 2011). Physical activity includes any bodily movement that increases energy expenditure above a basal level and may come in the form of exercise, which is voluntary and planned with the goal of improvement or maintenance of physical fitness. Physical fitness is characterized as "a multicomponent construct including cardiorespiratory endurance, skeletal muscle endurance, skeletal muscle power, flexibility, balance, speed of movement, reaction time, and body composition." The report explains that reduced all-cause mortality, cardiovascular disease mortality, and development of non-communicable disease(s) are associated with greater physical fitness, which is affected by both genetic factors and behavior, including physical activity. Consequently, uncovering the underlying biological determinants of physical activity behavior is of great importance for human health outcomes (Lightfoot et al. 2018).

Artificial selection experiments are useful for testing hypotheses about the effects of genetic background and/or environmental intervention(s) on both behavior and physiology. In contrast to genetic engineering (e.g., transgenic approaches), selective breeding offers a way to alter phenotypes in a way more consistent with the polygenic nature of complex traits (e.g., physical activity levels) than targeting a single or a few genes (Swallow and Garland 2005;

Garland and Rose 2009). In particular, a few rodent models have been designed to study selective breeding for traits associated with physical activity. For instance, Koch and Britton (2001) selected for high and low intrinsic aerobic endurance running capacities in lines of rats using motorized treadmills. Among many other differences, high-capacity runners (HCR) outperform low-capacity runners (LCR) in cognitive discrimination tasks (Wikgren et al. 2012) and LCR subjects score higher for cardiovascular risk factors in association with impaired mitochondrial function (Wisløff et al. 2005).

Another selection experiment by Booth and colleagues (Roberts et al. 2013) selectively bred lines of rats for high and low voluntary running (HVR and LVR) on wheels. Running wheels have been used for decades in behavioral research and are consistently used by animals in the laboratory and even when placed in nature (Sherwin 1998; Meijer and Robbers 2014). Running wheels are also a common preclinical model of human voluntary exercise (Eikelboom 1999; Garland et al. 2011; Novak et al. 2012). In the case of the LVR rats, they provide an interesting model of human inactivity, and alterations between the HVR and LVR transcriptome of the brain's reward hub, the nucleus accumbens, are suggestive of differential regulation of the mammalian reward response given variation in activity levels (Roberts et al. 2013).

In 1993, the Garland lab began a selection experiment in mice to examine voluntary exercise behavior (Swallow et al. 1998a). The original 224 outbred Hsd:ICR laboratory house mice (*Mus domesticus*) were paired randomly, and

their offspring were assigned to eight closed lines. The lines were established by randomly choosing and pairing one male and one female from each litter (no sib-mating). 10 pairs were then randomly assigned to each line, with four lines each making up the Control (C) or selected linetypes, and their offspring became generation 0. Subsequently, each of the lines propagated with at least 10 families per generation. At 21 postnatal days, offspring are weaned and housed in groups of four by line and sex. Beginning at ~6-8 weeks of age, mice are housed individually with access to a running wheel for six days. In the selected lines, the highest-running male and female from each family are selected as breeders based on the total number of revolutions run on days five and six of the six-day test [designated as High Runner (HR) lines]. In C lines, breeders are chosen without regard to wheel running.

Mice from the HR and C lines differ in several important ways. These differences include alterations ranging from the genetic and genomic level (Kelly et al. 2013; Saul et al. 2017; Hillis et al. 2020; Nguyen et al. 2020), to tissue and organ levels, to behavior. Changes have been defined in the skeletal system and musculature, the physiology of the endocrine system, in developmental processes, and pertinent to my research, the central nervous system. The purpose of my dissertation is to investigate the periphery, the brain, and behavior in three unique studies utilizing the HR mouse model.

Knowledge about the differential traits of the HR lines is abundant, but first and foremost, adult HR mice run on wheels approximately 3-times the amount of C mice in a given 24-hr period (both sexes). They achieve this mainly by running faster, rather than more frequently, especially for females. Female HR mice run more revolutions than male HR mice, but the fold difference compared to C mice is the same for both sexes. The absolute number of daily HR wheel revolutions substantially increased over the first 15-20 generations of selection (Careau et al. 2013). Subsequently, this number reached a plateau (referred to as the selection limit) between generations 17-27 depending on line and sex (Careau et al. 2013). Overall, HR running behavior has since been reasonably sustained for >90 generations, apart from strong seasonal fluctuations (see appendices to Careau et al. 2013). Also of note, when not provided wheel access, the HR mice have increased physical activity in their home cages (Malisch et al. 2009; Copes et al. 2015).

When compared to the C lines, HR mice have lower lean body mass (Swallow et al. 2001), higher endurance during forced exercise on a motorized treadmill (Meek et al. 2009), increased VO_2max (Swallow et al. 1998b; Rezende et al. 2006a; Kolb et al. 2010; Cadney et al. 2021), increased heart mass (Rezende et al. 2006b; Cadney et al. 2021), and several other adaptations beneficial to sustained, endurance-type locomotor activity. The HR lines also have low whole-body locomotor costs, quantified as the oxygen consumption required per unit of running distance (Rezende et al. 2006b). Additional traits

conducive to improved ability for wheel running include increased symmetry of HR hind limb bones, larger joint surfaces, and thicker shafts (Garland and Freeman 2005; Kelly et al. 2006; Middleton et al. 2010; Castro et al. 2021). HR mice also have skulls with distinct semicircular canal morphology, which may aid in their coordination of locomotion and head movement (Schutz et al. 2014). Differences in the HR endocrine system (Garland et al. 2016) include higher circulating corticosterone (controlling for body mass) and adiponectin concentrations (Malisch et al. 2007; Vaanholt et al. 2007) alongside significantly lower serum leptin levels (controlling for body fat; Girard et al. 2007).

Much is also known about the neurobiology of the HR mice, which might be expected to underlie increased motivation for exercise. Several studies offer evidence to support the claim that wheel running is rewarding to rodents and engages the brain's reward and motivational systems (Sherwin 1998; Vargas-Pérez et al. 2004; Greenwood et al. 2011; Novak et al. 2012). Both laboratory rats and mice will lever-press to gain access to running wheels (Kagan and Berkun 1954; Collier and Hirsch 1971; Iversen 1993; Belke 1997). When offered a 90-second running reward vs. 30 minutes, HR mice will lever-press only for the 30-minute reward, while C mice will press for both, suggesting a change in the HR motivational state (Belke and Garland 2007). When provided wheel access for six days but subsequently blocked from wheels, HR mice have significantly higher Fos-IR expression (see Sagar et al. 1988 for more about Fos protein) in the striatum, sensory and piriform cortices, lateral hypothalamus, and

paraventricular hypothalamic nucleus. In addition, the amount of running for the day prior to wheel prevention, considered an index of motivation, positively correlates with Fos-IR in these brain regions as well as prefrontal cortex (Rhodes et al. 2003). Two additional studies used a similar wheel deprivation paradigm. Malisch et al. (2009) found male HR mice spend significantly more time immobile in a forced-swim test, suggestive of anhedonia-like behavior. Saul et al. (2017) reported several differences in genetic expression within the striatum of wheel-deprived HR mice.

Pharmacological studies support the existence of an altered neurophysiology for High Runners. Of particular note, when given dopamine transporter blockers methylphenidate (Ritalin), cocaine, or GBR 12909 to allow for extended dopamine neurotransmission, C mice either did not change or increased their wheel running, while HR mice showed an acute decrease in running. Separately, dopamine receptors were tested with antagonistic drugs to ultimately find that HR mice required a higher dose of D1-type receptor blocker SCH 23390 to reduce their wheel-running behavior (Rhodes et al. 2001, 2005; Rhodes and Garland 2003). Additionally, Keeney et al. (2008, 2012) found that agonistic/antagonistic drugs of the endocannabinoid system had acute linetype- and sex-specific effects on wheel running.

Building upon the knowledge gained from previous studies in the HR selection experiment, my research tests novel hypotheses about 1) endocannabinoid concentrations in the upper small intestine and how they relate

to circulating endocannabinoids, 2) brain region volume differences in the midbrain and limbic system, and 3) conditioning behavior for place preference when provided Ritalin, cocaine, or wheel rewards.

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CHAPTER 1

Effects of selective breeding, voluntary exercise, and sex on endocannabinoid levels in the mouse small-intestinal epithelium

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Abstract

The endocannabinoid (eCB) system in the gut communicates with the body and brain as part of the homeostatic mechanisms that affect energy balance. Although perhaps best known for its effects on energy intake, the eCB system also regulates voluntary locomotor behavior. Here, we examined gut eCB concentrations in relation to voluntary exercise, specifically in mice selectively bred for high voluntary wheel running behavior. We measured gut eCBs in four replicate non-selected Control (C) lines and four replicate lines of High Runner (HR) mice that had been selectively bred for 74 generations based on the average number of wheel revolutions on days 5 and 6 of a 6-day period of wheel access when young adults. On average, mice from HR lines run voluntarily on wheels ~3-fold more than C mice on a daily basis. A recent study showed that circulating levels of primary endocannabinoids 2-arachidonoyl-s*n*-glycerol (2-AG) and anandamide (AEA) are altered by six days of wheel access, by acute wheel running, and differ between HR and C mice in sex-specific ways (Thompson et al. 2017). We hypothesized that eCBs in the upper small-intestinal epithelium (i.e., proximal jejunum), a region firmly implicated in eCB signaling, would differ between HR and C mice (linetype), between the sexes, between mice housed with vs. without wheels for six days, and would covary with amounts of acute running and/or home-cage activity (during the previous 30 minutes). We used the same 192 mice as in Thompson et al. (2017), half males and half females, half HR and half C (all 8 lines), and half either given or not given access

to wheels for six days. We assessed the eCBs, 2-AG and AEA, and their analogs docosahexaenoylglycerol (DHG), docosahexaenoylethanolamide (DHEA), and oleoylethanolamide (OEA). Both 2-AG and DHG showed a significant 3-way interaction of linetype, wheel access, and sex. In addition, HR mice had lower concentrations of 2-AG in the small-intestinal epithelium when compared to C mice, which may be functionally related to differences in locomotor activity or to differences in body composition and/or food consumption. Moreover, the amount of home-cage activity during the prior 30 min was a negative predictor of 2-AG and AEA concentrations in mucosa, particularly in the mice with no wheel access. Lastly, 2-AG, but not AEA, was significantly correlated with 2-AG in plasma in the same mice.

Introduction

Endocannabinoids (eCBs) are lipid-derived signaling molecules that bind and activate G-protein-coupled cannabinoid type-1 (CB_1) and type-2 (CB_2) receptors found throughout the body. The eCBs, their receptors, and their biosynthetic and degradative enzymes – collectively termed the eCB system – play an integral role in homeostasis, including the control of energy balance, body composition, and appetite regulation and food intake, through mechanisms that include indirect and direct control of afferent vagus nerve signals to the brain from the gastrointestinal tract (DiPatrizio et al. 2013, 2015; DiPatrizio 2016, 2021; Argueta and DiPatrizio 2017; Avalos et al. 2020). Notably, a variety of studies suggest that overactive eCB signaling contributes to diet-induced obesity (Engeli et al. 2005; Côté et al. 2007; Izzo et al. 2009; Matias et al. 2012; DiPatrizio et al. 2013; Perez and DiPatrizio 2018), which precedes and impacts metabolic conditions, cardiovascular disease, diabetes, and increased risk of cancer(s) and infection from communicable diseases, including COVID-19 (Alberca et al. 2020; Dietz and Santos-Burgoa 2020). More specifically, the proximal small intestine, in contrast to the brain and other peripheral locations, is implicated as a key regulatory region for eCB control of feeding behavior and resultant body composition (DiPatrizio et al. 2011; Argueta et al. 2019; Avalos et al. 2020; see for example, DiPatrizio 2021).

On the other hand, energy expended through physical activity and voluntary exercise are important components of the overall energy budget in both

humans and rodents (Garland et al. 2011), and the eCB system has also been linked to both physical activity and exercise (Dietrich and McDaniel 2004; Keeney et al. 2008, 2012; Raichlen et al. 2012, 2013; Fuss et al. 2015; Brellenthin et al. 2017; Thompson et al. 2017; Crombie et al. 2018). Given the eCB system's role in exercise and energy homeostasis, further research aimed at identifying relationships between gut-brain eCB signaling and exercise is needed. The present study is the first to measure gut eCB content in the context of voluntary exercise behavior and artificial selection for voluntary exercise. We aimed to evaluate levels of the primary eCBs, the monoacylglycerol 2-arachidonoyl-*sn*-glycerol (2-AG), and the fatty acid ethanolamide, arachidonoyl ethanolamide (AEA, anandamide), as well as their lipid analogs docosahexaenoyl glycerol (DHG), docosahexaenoylethanolamide (DHEA), and oleoylethanolamide (OEA) in the upper small-intestinal epithelium of mice from lines selectively bred for high voluntary wheel-running behavior (High Runner; HR mice) as compared with those from non-selected Control (C) lines (Swallow et al. 1998; Thompson et al. 2017). We also quantified sex differences and the effects of six days of opportunity to run on wheels.

The five analytes we examined have varying functions relevant to the gut-brain axis (e.g., appetite, reward) and control of voluntary locomotor behavior. Compared to other lipid-derived signaling molecules, more is known about the endocannabinoids 2-AG and AEA, the primary subjects within the existing body of eCB research. However, in the present study, we also quantified two other

fatty acid ethanolamides, OEA and DHEA. In contrast to appetite-stimulating properties of AEA, OEA promotes satiety and fat catabolism, and suppression of food intake (Fu et al. 2007; Piomelli 2013; Bowen et al. 2017; Brown et al. 2017). DHEA is lesser studied; however, it has been reported to be involved in glucose balance and CB_1 receptor expression in myoblasts (Kim et al. 2014), and modulating cytokine release of interleukin-6 in the periphery (Meijerink et al. 2015). In addition, we measured levels of the monoacylglycerol, DHG, an ω-3 analog of 2-AG (an ω-6 eCB), which has anti-inflammatory properties (Dotsey et al. 2017; Price et al. 2018).

Three studies in the selectively bred HR lines of mice have examined the eCB system. When systemically injected with the CB_1 antagonist rimonabant, HR females decreased running more than C females over the following hour, while males showed no linetype difference (Keeney et al. 2008). Moreover, treatment of both female and male HR mice with the CB_1 agonist, WIN 55,212-2, led to decreased running when compared to C mice (Keeney et al. 2012). Thompson et al. (2017) measured plasma 2-AG and AEA in HR and C mice of both sexes, with or without wheel access for six-days (we collected upper small-intestinal epithelium from the same individual mice). In brief, females had lower plasma 2-AG than males, with a main effect of sex but not linetype. 2-AG was also lower in mice that received wheel access, except in C males (Thompson et al. 2017). Additionally, the amount of prior running during the previous 30 min before plasma collection was not a significant predictor of 2-AG concentrations.

Females had higher levels of AEA than males, and there was an interaction between wheel access and linetype indicating that wheel access increased AEA in C mice, while decreasing AEA in HR males. In contrast to 2-AG, the amount of prior running was a positive predictor and home-cage activity was a negative predictor of plasma AEA concentrations. Based on these previous studies of HR mice (Keeney et al. 2008, 2012; Thompson et al. 2017), we expected sex effects and possible interactions of linetype, sex, and wheel access for jejunal 2-AG and AEA, but analogs DHG, DHEA, and OEA were novel targets of study for the HR mice.

Materials & Methods

1. Ethical approval

All experimental procedures were approved by the UC Riverside Institutional Animal Care and Use Committee.

2. Selection experiment

Outbred Hsd:ICR mice (*Mus domesticus*) were obtained from Harlan Sprague Dawley (Indianapolis, Indiana, USA) and randomly separated into eight closed lines for a long-term artificial selection experiment that was begun in 1993 (Swallow et al. 1998). Four lines became the selected High Runner (HR) linetype and the other four were designated as Control (C) lines. At sexual maturity, all mice are provided access to Wahman-type running wheels (1.12 m

circumference) for six days, with wheel-running revolutions recorded in 1-min bins for ~23 hours per day (1 hour used to download data and check mice). Mice in the HR lines are chosen to breed based on their average number of wheel revolutions on days five and six of this period, and within-family selection is used (Swallow et al. 1998). By approximately generation 20, HR lines reached selection limits at which they ran ~2.5-3-fold more revolutions per day as C mice on average (Garland 2003; Kolb et al. 2010; Careau et al. 2013).

3. Tissue sample collection

Adult mice (N = 192, sampled from all 8 lines) from generation 74, half HR and half C, half male and half female, were either allowed access to wheels for six days or kept without access to wheels. All mice were fed a standard diet (Teklad Rodent Diet W-8604, 14% kJ from fat, 54% kJ from carbohydrates, and 32% kJ from protein, no added sugars [less than ~9% naturally occurring sugars by weight, mostly from grains]). Wheel revolutions for mice with wheels were recorded for 23 hours per day, and home-cage activity was recorded for all mice for ~23 hours per day (see Thompson et al. (2017) for wheel and activity data). We did not choose to provide locked wheels for the mice without wheel access, as HR mice climb more than C mice when given locked wheels (Koteja et al. 1999). On day 6 animals were anesthetized with isoflurane, and blood plasma (Thompson et al. 2017) and jejunum mucosa scrapings were preserved. Specifically, jejunum was rapidly collected, washed with ice-cold phosphate-

buffered saline (PBS) on ice, sliced longitudinally, scraped with a glass slide to obtain mucosa, then frozen in liquid nitrogen. Animals were on a reversed photoperiod, with lights off from 0700 h to 1900 h, so that sampling could occur during the time of peak wheel running. Sampling occurred from ~0900 h to 1300 h (from 2-6 hours after lights off). Mice were 71-91 days old at the time of sampling.

4. Ultra-performance liquid chromatography/tandem mass spectrometry

Frozen jejunum mucosa samples were weighed and subsequently homogenized in 1.0 mL of methanol solution containing internal standards (d4-FAEs, d5-2-AG 0.26 mM, 19:2 DAG). Lipids were extracted with chloroform (2.0 mL) and washed with 0.9% saline (0.9 mL). Organic phases were collected and fractionated by open-bed silica gel column chromatography as previously described (DiPatrizio et al. 2011). Eluted fractions were dried under N^2 and reconstituted in 0.1 mL of methanol:chloroform (9:1) for liquid chromatography/tandem mass spectrometry analyses. Lipids were analyzed using a Waters Acquity I-Class Ultra Performance Liquid Chromatography system coupled to a Waters TQS-micro Triple Quadrupole Mass Spectrometer, as previously described (Wiley et al. 2021). Lipids were separated using an Acquity UPLC BEH C18 column (50 x 2.1 mm; i.d. 1.7 µm), eluted by a gradient of methanol (0.25% acetic acid, 5 mM ammonium acetate) in water (0.25% acetic acid, 5mM ammonium acetate) (from 80 to 100% methanol in 2.5 minutes, 100%

2.5-3.0 minutes, 100-80% 3.0-3.1 minutes) at a flow rate of 0.4 mL/minute. Column temperature was kept at 40° C and samples maintained in the sample manager at 10° C. Argon was used as the collision gas.

5. Statistical analyses

Following previous studies using these eight lines of mice (Swallow et al. 1998; Rhodes et al. 2000; e.g., Thompson et al. 2017), jejunum endocannabinoid concentrations were analyzed by nested analysis of variance (SAS Procedure Mixed). Line nested within linetype (HR vs. C) was a random effect and we used covariates of age (mice were 71-91 days old) and the time of day that tissue sampling occurred. Another factor used in the current analyses was mini-muscle status (determined at dissection). The mini-muscle phenotype is caused by a recessive allele that, when homozygous, reduces triceps surae and total hindlimb muscle mass by ~50% and has pleiotropic effects on numerous other traits (Garland et al. 2002; Kelly et al. 2013). We ran this analysis for all five lipids (2-AG, AEA, DHG, DHEA, and OEA) using all mice (n = 183-190), mice with wheel access (n = 90-92), and mice without wheel access (n = 91-95).

Following Thompson et al. (2017), we repeated the preceding analyses with physical activity covariates: amount of wheel running (revolutions/unit time) and home-cage activity (HCA) in the 30 minutes prior to sampling. For the analysis of all mice, we used both covariates and assigned running values of zero to mice without wheel access. Then we performed separate analyses for

the mice with wheels using both running and HCA covariates, and for the mice without wheel access using only the HCA covariate. We used 30 minutes of activity for consistency with Thompson et al. (2017), who computed the number of wheel revolutions in each minute before plasma and tissue sampling, from 1 to 10 min before, and then in 10-min bins from 10 to 120 min before sampling. After examining models using each of these alternative covariates, they determined that 30 minutes provided the best fit. In the Thompson et al. (2017) analysis, it was established that these physical activity covariates could be significant predictors of plasma endocannabinoid concentrations.

Dependent variables were transformed when needed to improve the normality of residuals. Residuals that were >3 standard deviations above or below the mean were excluded from analyses. Main effects were considered statistically significant when $P \leq 0.05$. Following Thompson et al. (2017), interactions of main effects were considered significant when $P \leq 0.10$ because the power to detect interactions is generally substantially lower than for detecting main effects in ANOVAs (Wahlsten 1990, 1991). Least squares means and associated standard errors from SAS Procedure Mixed were inspected to determine the directions of main effects and interactions. In addition, for some pairwise comparisons of subgroup means, we refer to differences of least squares means from SAS Procedure Mixed.

A total of 429 P values are presented herein and within Appendix A Tables, representing all of the primary results (excluding time of day, which was

a nuisance variable). Based on the positive False Discovery Rate (pFDR) procedure as implemented in SAS Procedure Multtest, an appropriate cutoff would be ~P = 0.007 to control the false discovery rate at 0.05. However, simulations to explore statistical power indicate generally deflated Type I error rates for linetype comparisons in this selection experiment for $\alpha = 0.05$ (Castro et al. 2021). Thus, for simplicity, all P values reported in the text are the nominal ones, not adjusted for multiple comparisons.

Results

Of the five endocannabinoids analyzed in the small-intestinal epithelium in the present study, 2-AG had the highest concentrations for all mice, with group averages between 54-88 nmol/g (Fig. 1.1 shows transformed least squares from SAS analyses). DHG was second highest with group average concentrations falling between 8.4-13.5 nmol/g (Fig. 1.2). AEA and DHEA concentrations were the lowest, measured in the picomole range of 16.4-19.6 pmol/g (Appendix A Fig. 1: AEA $\log_{10}$-transformed data and Appendix A Fig. 2: DHEA data transformed by raising to the 0.4 power). OEA concentrations were 172-201 pmol/g (Appendix A Fig. 3; data transformed by raising to the 0.5 power). In comparison to intestinal tissue, Thompson et al. (2017) reported (in the same mice) plasma AEA levels between 0.18-0.22 pmol/mL and 2-AG levels between 62-98 pmol/mL. Mini-muscle status (see Methods), age, and time of day were included as factors or covariates in all analyses. Time of day was not a statistically significant predictor

for intestinal eCB concentrations in any group or analysis. Appendix A Table 1 shows a summary of all statistical analyses.

1. Wheel running and HCA

Wheel revolutions and home-cage activity (HCA) for Day 5 of the experiment are reported in Thompson et al. (2017). Briefly, as expected, HR mice ran significantly more than C mice (P = 0.0004), but neither the effect of sex (P = 0.1431) nor the sex-by-linetype interaction (P = 0.1576) were significant. For HCA, mice with wheel access had significantly reduced activity levels; the reduction was greater for HR mice than for the C mice; and females and HR mice always had higher HCA than males or C mice, respectively.

2. Jejunum 2-AG concentrations

a. 2-AG without physical activity covariates

HR mice had lower levels of 2-AG in their jejunum mucosa than did C mice (Table 1.1; P = 0.0491) in all four experimental groups (Fig. 1.1). A three-way interaction among wheel access, sex, and linetype also occurred (P = 0.0857): levels of 2-AG were lowest for HR females with no wheel access and highest for C males with wheel access. When examining the pairwise comparisons, for these two groups the difference was significant (P = 0.0336). The difference between HR and C females with no wheel access was also significant (P = 0.0376).

Separate analyses of the mice with (n = 92) and without (n = 95) wheels (not including physical activity covariates; results not shown) indicated that HR mice had significantly lower 2-AG levels in their jejunum only when they had wheel access (P = 0.0094 for mice with wheels, P = 0.1720 for mice without wheels) (Appendix A Table 1). Mini-muscle status, age, and time of day were not significant for 2-AG when data were analyzed without running or HCA covariates (Appendix A Table 1).

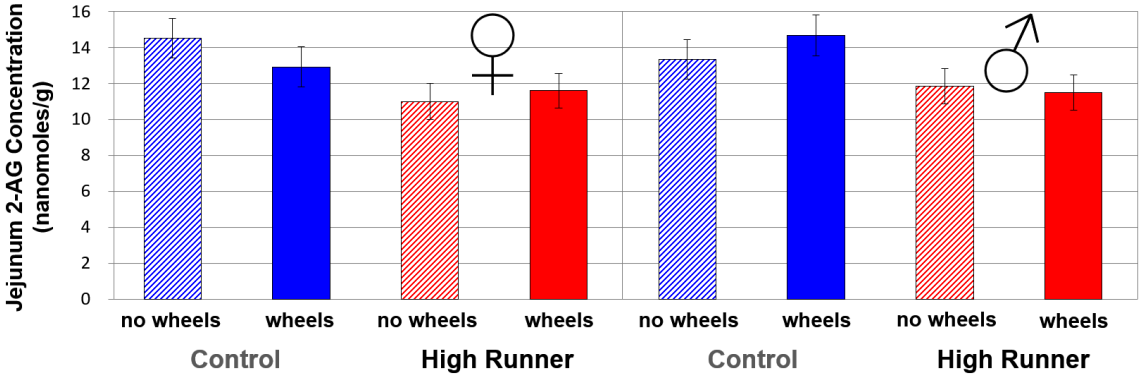

Figure 1.1. Concentrations of 2-AG in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 190). Overall, 2-AG was higher in C mice than in High Runner mice (P = 0.0491) and a three-way interaction among wheel access, sex, and linetype was present (P = 0.0857). Values are LS means ± standard errors from SAS Proc Mixed for data transformed by raising to the 0.6 power. See Table 1.1 for full statistical analysis.

b. 2-AG with physical activity covariates

For this analysis, the amount of wheel running and HCA in the 30 minutes prior to cardiac puncture and tissue sampling were included as covariates (raised to the 0.4 power and 30 min for consistency with Thompson et al. (2017)). For the mice housed with wheel access (n = 92), neither wheel running nor home-

cage activity were significant predictors of 2-AG concentrations (Appendix A Table 1). However, similar to results reported above, HR mice had significantly lower 2-AG levels in their jejunum mucosa than C mice (P = 0.0296), with no effect of sex, nor a sex-by-linetype interaction. For the mice housed without wheels (n = 95), HCA was a significant negative predictor of 2-AG levels (P = 0.0333), with no effect of linetype, sex or a sex-by-linetype interaction.

To test whether acute physical activity had a different effect than the five days of wheel access [following Thompson et al. (2017) and Copes et al. (2015)], an additional analysis was performed for each analyzed eCB using the activity covariates, in which we included the mice housed without wheel access but we assigned them values of zero for their wheel running. In our 2-AG analysis (n = 186), the three-way interaction reported in Table 1.1 (Fig. 1.1) remained significant (P = 0.0597; Table 1.2); however, except for C males, levels of 2-AG were lower in mice with wheel access. The prior 30 minutes of HCA was a significant negative predictor of jejunum 2-AG (P = 0.0208), but wheel running was not.

3. Jejunum AEA concentrations

a. AEA without physical activity covariates

Levels of AEA in the jejunum were roughly uniform across all groups (16.6-19.6 pmol/g), with no significant main effects or interactions (Table 1.1; Appendix A Fig. 1) and no effect of mini-muscle status, age, or time of day (Table

1.1). In the separate analyses of the mice with (n = 90) and without (n = 91) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (results not shown). However, mini-muscle mice had significantly higher AEA levels than non-mini muscle mice in the wheel-access group (P = 0.0454).

b. AEA with physical activity covariates

For mice with wheel access (n = 90), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of AEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Appendix A Table 1). For the mice housed without wheels (n = 91; results not shown), HCA was a significant negative predictor of AEA levels (P = 0.0145), with no effect of sex, linetype or their interaction. For the analysis of all mice (n = 180; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of covariates (Table 1.2), though 30 min of HCA tended to negatively predict AEA concentrations (P = 0.0705).

4. Jejunum DHG concentrations

a. DHG without physical activity covariates

The 2-AG endocannabinoid analog DHG showed a three-way interaction (P = 0.0529; Table 1.1; Fig. 1.2). Levels of DHG were higher in mice with wheel access, except for C females. When examining the pairwise comparisons, results were similar to those for 2-AG: for females without wheels, HR tended to have lower DHG (P = 0.0514); and HR females without wheels had lower DHG than C males with wheels (P = 0.0609). Mini-muscle status, age, and time of day were not significant predictors of DHG concentrations when data for all mice were analyzed without running or HCA covariates (Table 1.1).

In separate analyses of the mice with (n = 92) and without (n = 95) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (Appendix A Table 1). Mini-muscled mice housed without wheels tended to have lower DHG levels than non-mini-muscled mice (P = 0.0506).

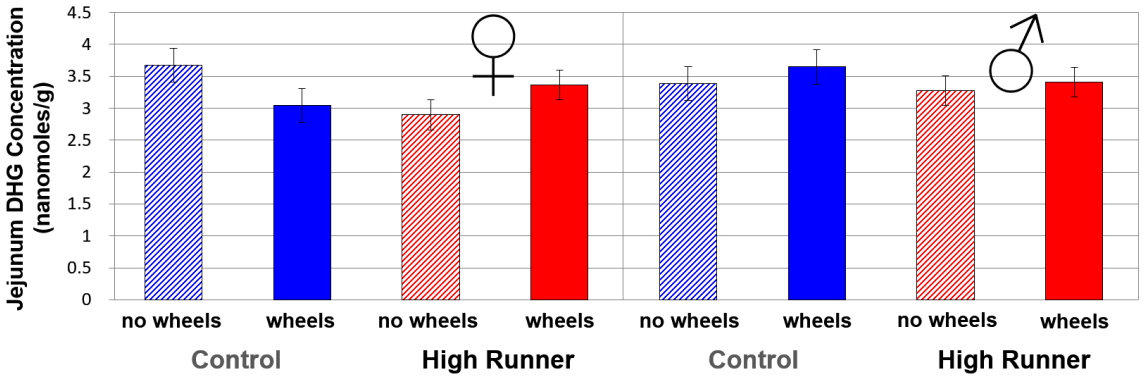

Figure 1.2. Concentrations of DHG in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 190). There was a three-way interaction among wheel access, sex, and linetype (P = 0.0529). Values are LS means ± standard error from SAS Proc Mixed for data transformed by raising to the 0.5 power. See Table 1.1 for full statistical analysis.

b. DHG with physical activity covariates

For mice with wheel access (n = 92), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of DHG levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Appendix A Table 1). For the mice housed without wheels (n = 95), HCA was not a significant predictor of DHG levels, with no effect of sex, linetype or their interaction. However, mini-muscle mice had significantly lower jejunum DHG than non-mini-muscled mice (P = 0.0242).

For the analysis of all mice (n = 187; values of zero assigned to the mice without wheel access), the three-way interaction observed previously without covariates (Table 1.1), remained significant (P = 0.0551; Table 1.2), and levels of DHG were higher in mice with wheel access, except for C females. There were

no other main effects or interactions of wheel access, sex, and/or linetype (Table 1.2). Neither wheel running nor HCA were significant predictors of jejunum DHG (Table 1.2).

5. Jejunum DHEA concentrations

a. DHEA without physical activity covariates

Mice with wheel access tended to have higher levels of the AEA analog DHEA (P = 0.0593; Table 1.1) in all four experimental groups (Appendix A Fig. 2). In the separate analyses of mice with (n = 91) and without (n = 95) wheels (not including physical activity covariates) we found no effect of sex, linetype or their interaction (Appendix A Table 1).

b. DHEA with physical activity covariates

For mice with wheel access (n = 91), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of DHEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Appendix A Table 1). For the mice housed without wheels (n = 95), HCA tended to negatively predict DHEA levels (P = 0.0683), with no effect of sex, linetype or their interaction. For the analysis of all mice (n = 186; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of covariates (Table 1.2).

6. Jejunum OEA concentrations

a. OEA without physical activity covariates

Male mice tended to have higher OEA levels in their jejunum mucosa than females (P = 0.0845; Table 1.1; Appendix A Fig. 3). In the separate analyses of the mice with (n =94) and without (n = 92) wheels (not including physical activity covariates), we found no effect of sex, linetype or their interaction (Appendix A Table 1).

b. OEA with physical activity covariates

For mice with wheel access (n = 92), neither wheel running (raised to the 0.4 power) nor home-cage activity (also raised to the 0.4 power) in the 30-minutes prior to tissue sampling were significant predictors of OEA levels in jejunum mucosa, with no effect of sex, linetype or their interaction (Appendix A Table 1). For mice housed without wheels (n = 94), HCA tended to be a negative predictor of OEA levels (P = 0.0552), with no effects of sex, linetype or their interaction. For the analysis of all mice (n = 187; values of zero assigned to the mice without wheel access), we found no main effects of wheel access, sex, linetype, their interactions, or effects of physical activity covariates (Table 1.2).

7. Correlations between eCBs

Following Thompson et al. (2017), we examined the relationship between jejunum and circulating levels of eCBs by a variety of measures. First, we

calculated Pearson correlation coefficients for the raw values for jejunum and plasma analyte concentrations in all mice (statistical outliers removed). In the jejunum, all five analytes were significantly intercorrelated (Table 1.3: all $P < 0.01$), whereas 2-AG and AEA were uncorrelated in plasma ($r = 0.083$, $n = 184$, $P = 0.261$). Between tissues, only one correlation was statistically significant (plasma 2-AG and jejunum OEA, $r = -0.176$, $P = 0.016$).

Second, we analyzed the correlations for jejunum analytes within each of the eight experimental subgroups (n = 21–24 per group) and found values ranging from −0.10 to +0.91, with 78 of 80 values being greater than zero, and 44 of the 78 significant at $P < 0.05$ (Appendix A Table 2). Jejunum 2-AG and DHG were highly correlated within all eight groups (all 2-tailed $P <0.01$), as were jejunum AEA and OEA (all $P <0.05$). HR females without wheels were the only group that showed all five analytes to be significantly correlated (all $P <0.01$). Of the 80 possible correlations between plasma 2-AG and AEA and jejunal values for our five analytes, only six were statistically significant ($P <0.05$, five negative, one positive: Appendix A Table 3).

Third, the plasma and jejunum LS means for 2-AG in the eight experimental groups, when analyzed with the 30 min of previous wheel-running and HCA as covariates, were positively correlated ($r = 0.745$, $P = 0.0339$; Fig. 1.3). When we repeated the analysis without the physical activity covariates, the relationship was weaker and not significant (figure not shown; $r = 0.633$; $P = 0.0922$). For AEA, the corresponding values, with and without wheel-running and

HCA covariates, were not correlated (figures not shown; r = 0.284 and P = 0.4953 with covariates; r = 0.384 and P = 0.3472 without covariates).

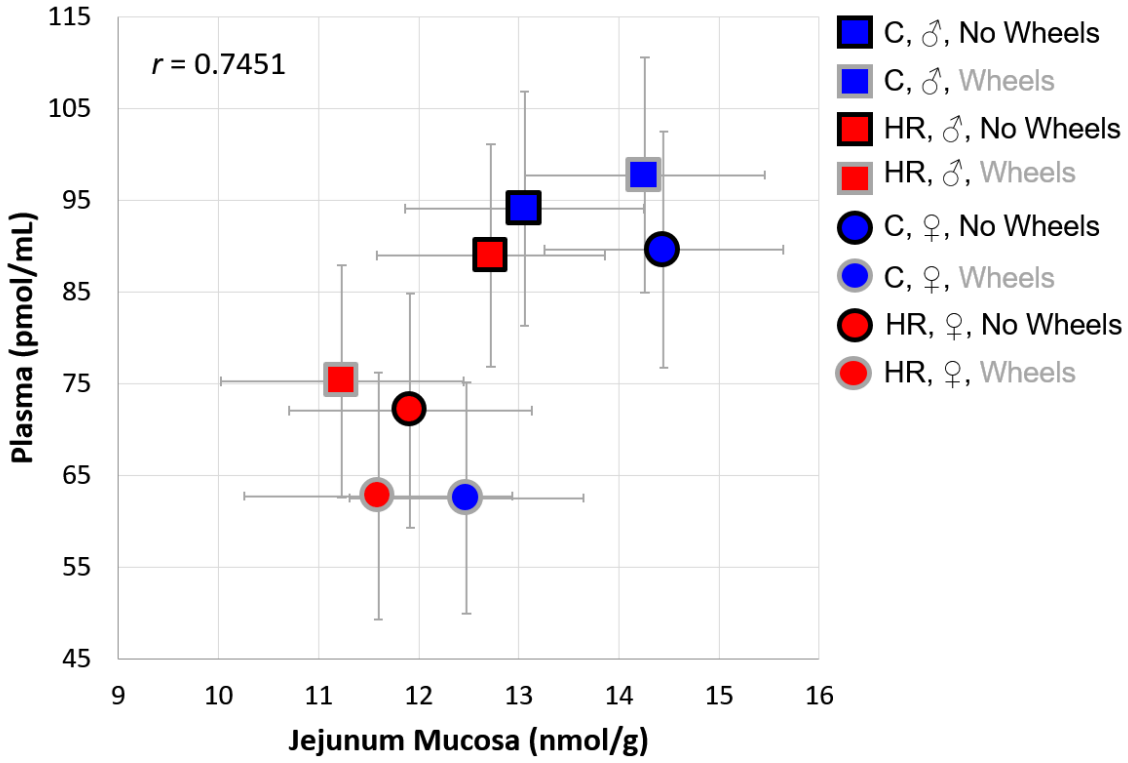

Figure 1.3. Correlation between group means for plasma (from Thompson et al. 2017) and jejunum mucosa 2-AG. Values are LS means ± standard error from SAS Proc Mixed with the amount of wheel running and HCA in the 30 mins prior to sampling as covariates (from our Table 1.2 and Section 3.4 of Thompson et al. 2017). Jejunum data was transformed by raising to the 0.6 power. The correlation is statistically significant (P = 0.0339).

Fourth, we computed simple mean values for each of the eight subgroups (linetype by sex by wheel access; Appendix A Table 4) and found two statistically significant correlations: between jejunum AEA and DHG (r = 0.919, P = 0.001), and between jejunum AEA and OEA (r = 0.721, P = 0.044). No correlations were present between the simple subgroup means of jejunum vs. plasma eCBs.

Discussion

Whether genetic background, sex differences, and/or exercise modulate levels of eCBs in the gut, specifically the proximal small intestine, is unknown. We used a unique, artificial selection mouse model to test how levels of eCBs and their related lipid messengers in the small-intestinal epithelium differ between *(i)* lines of mice selectively bred for high voluntary wheel-running behavior vs. non-selected Control lines, *(ii)* males vs. females, and *(iii)* mice provided six days of wheel access vs. no wheel access. We also tested for possible interactive effects and for correlations with the amount of acute physical activity during the 30 min prior to tissue extraction. We found evidence for decreased 2-AG concentrations in the jejunum mucosa of selectively bred High Runner vs. Control mice, along with three-way interactions for 2-AG and its ω-3 lipid analog DHG. In addition, the amount of home-cage activity during the prior 30 min was a negative predictor of 2-AG and AEA concentrations in intestinal epithelium, particularly in the mice with no wheel access. Furthermore, intestinal 2-AG, but not AEA, was significantly correlated with 2-AG in circulation in the same mice (Thompson et al. 2017).

Previous studies of eCBs in the gut or in relation to exercise

ECBs, their biosynthetic and degradative enzymes, and their receptors have been studied in both human and rodent central and peripheral tissues. Experiments have separately examined eCB system components in the intestine

or given exercise, but not together. In the intestine, several studies of diet and food intake in rodents have quantified various components of the eCB system (Izzo and Sharkey 2010; DiPatrizio and Piomelli 2015; DiPatrizio 2016), leading to the discovery that efferent vagal signals to the jejunum result in eCB production and receptor binding in the mucosal epithelium. These cannabinoids can then inhibit production of the satiety peptide cholecystokinin (CCK), thereby significantly altering the afferent gut-to-brain control of feeding (DiPatrizio 2016).

One other recent study by Guida et al. (2020) evaluated intestinal eCBs in a study of vitamin D deficiency and pain processing where mice received tibial and common peroneal nerve injury. In the colon, but not small intestine, vitamin D deficient mice had reduced 2-AG levels and palmitoylethanolamide (PEA) treatment elevated AEA, OEA, and DHEA in vitamin D normal mice with nerve injury. The authors interpreted their results as suggesting that vitamin D deficiency may be accompanied by increased pain and inflammation caused by reduced eCB signaling at intestinal CB1 and CB2 receptors. They also suggest that PEA may provide amelioration (Guida et al. 2020). Future studies could examine the relationship of exercise-related pain paired with the gut eCB system.

Importantly, exercise can affect the gastrointestinal tract, digestion, and the gut-brain axis in various ways (Bunt 1986; Moses 1990; Migrenne et al. 2006; Royes 2020). Exercise studies have primarily used plasma or saliva samples and they vary by acute vs. chronic paradigms and by exercise intensity and duration. Generally for humans, plasma AEA (Sparling et al. 2003; Raichlen et

al. 2013; Brellenthin et al. 2017; Crombie et al. 2018) and OEA (Crombie et al. 2018) are increased following exercise, and only sometimes is 2-AG increased (Brellenthin et al. 2017; Crombie et al. 2018). DHG and DHEA have not been previously quantified in an exercise context. We compared our results of gut eCBs to the plasma results in Thompson et al. (2017) and the across-tissue similarities and differences we found are discussed below in section 4.2.

Our novel pairing of exercise and eCB measurement in the intestine limits comparisons with previous studies. Even considering "control" conditions (i.e., adults on "standard" diet, freely-fed), male mice have varying concentrations of 2-AG, AEA, DHG, DHEA, and OEA in the intestine (Izzo et al. 2009; Igarashi et al. 2015; Argueta and DiPatrizio 2017; Perez and DiPatrizio 2018). Some of this variability is likely related to experimental details, such as age, the %kcal from fat in the standard diet, the use of metabolic cages that prevent coprophagia, or homogenization of whole jejunum vs. the mucosal layer. Overall, the 2-AG, AEA, and DHEA concentrations observed in the present study are within the range of values reported previously, but the group mean concentrations of DHG and OEA are lower.

Comparisons of gut and plasma endocannabinoids in this experiment and Thompson et al. (2017)

We studied the same individual mice as Thompson et al. (2017) used to measure circulating endocannabinoids. Therefore, we compared 2-AG and AEA

concentrations in small-intestinal epithelium and plasma by examining the statistical results reported here (Sections 3.2.1-3.3.2) with those reported in Thompson et al. (2017). In general, most of the 2-AG and AEA effects found in the proximal small intestine vs. the plasma differed. Notably, the significant main effect of sex on circulating 2-AG was absent from jejunum mucosa (Tables 1.1 and 1.2). With regard to AEA, the wheel access-by-linetype interaction, where wheel access significantly lowered plasma AEA levels in all mice but much more so in HR lines than in C lines, was not observed in the jejunum (Table 1.2). Additionally, the previous 30 minutes of running was a highly significant positive predictor of plasma AEA, whereas the previous amount of running did not covary with jejunal mucosa AEA (Table 1.2).

The one obvious similarity for jejunum and plasma (Thompson et al. 2017) was the three-way interaction between linetype, sex, and wheel access for concentrations of 2-AG (our Sections 3.2-3.3 and their Sections 3.3-3.4). A three-way interaction present in both tissues and a statistically significant positive correlation between jejunum and plasma 2-AG (at the level of our eight experimental groups; Fig. 1.3) suggests that gut and plasma 2-AG may be regulated by common pathways and/or acting synergistically in the eCB system.

Previous research indicates a correlation of eCBs across tissues is plausible. Argueta and DiPatrizio (2017) found that 2-AG and AEA concentrations are elevated in both plasma and jejunum mucosa of male C57BL/6 mice fed ad-libitum WD for 60 days when compared to their SD

counterparts. Their study suggests that local signaling by eCBs in the upper small intestine, along with those in circulation, may both interact with feeding- and reward-related pathways in the brain. Thus, our 2-AG across-tissue correlation (Fig. 1.3) might also suggest that the periphery-to-brain communication of 2-AG may be modulated by an interaction of genetic background, biological sex, and access or no access to running wheels.

Another interesting difference between jejunum and plasma is their respective correlations between 2-AG and AEA. In the jejunum, 2-AG and AEA were considerably more positively correlated than in the plasma (Table 1.3). Further, besides a slight negative relation between plasma 2-AG and jejunum OEA, none of our raw analyte values were correlated with the 2-AG or AEA from Thompson et al. (2017).

As our study quantified the three additional analytes DHG, DHEA, and OEA, we also tested for intercorrelations between all five lipid messengers. Overall, our analyses indicate that raw values of all five intestinal analytes are positively related (n = 182-190; Table 1.3) but not when tested as the simple means for the eight experimental groups (n = 8; Appendix A Table 4). The highest correlation between the raw values of two analytes was between jejunum 2-AG and its ω-3 counterpart DHG (Table 1.3: $n = 190$, $r = 0.800$, $p << 0.0001$), and 2-AG and DHG were also significantly correlated within each of the eight experimental groups (Appendix A Table 2). These findings may involve the

biosynthetic and/or degradative enzymes acting on both of the monoacylglycerols.

Linetype differences in relation to activity effects

In combined analyses, mice from the selectively bred HR lines had lower 2-AG in their jejunum mucosa than C mice for both sexes and housing conditions, although the magnitude of this difference varied, as evidenced by a three-way interaction (Table 1.1, Fig. 1.1). Separate analyses of mice housed with or without wheel access indicated that HR mice had lower 2-AG only with wheel access (sections 3.2.1 and 3.2.2.). Thus, the magnitude of the linetype difference varies with both sex and physical activity. A study of males found that HR mice also have greater rates of lipid oxidation than C mice during exercise at 66% of VO_2max (Templeman et al. 2012), an intensity of exercise that occurs during voluntary wheel running (Rezende et al. 2005). The lipids metabolized for energy during sustained, aerobically supported exercise (as in mouse wheel running) may be drawn from various pools, including white adipose tissue (May et al. 2017; Mika et al. 2019) and skeletal muscle (McClelland 2004), in addition to digestion in the small intestine (Jones and Havel 1967; Terjung et al. 1982; Horowitz and Klein 2000). If the synthesis of 2-AG involves precursors that are also used during exercise-induced lipid oxidation, then perhaps jejunal 2-AG might be depleted during exercise in HR mice. However, eCBs in the intestine are likely acting locally and are produced and degraded locally. To date, we

have no evidence that precursors and eCBs in the intestine may escape into circulation (e.g., see Wiley et al. 2021).

When all mice were analyzed with physical activity covariates, the prior 30 min of HCA was a significant negative predictor of 2-AG (i.e., more activity in their home-cage was associated with less 2-AG and vice versa; Table 1.2). In the analyses of mice without wheels, 2-AG, AEA, DHEA, and OEA were also negatively predicted by HCA in the 30 minutes before tissue samples were extracted. Given that the eCB system is physiologically extensive and linked to many other bodily systems, these results suggest that varying levels of rodent activity in their home cages could be a confounding factor in various studies (e.g., pharmacological studies using drugs that affect locomotion) by way of small intestinal lipid messengers.

Lastly, mice with wheel access tended to have higher levels of DHEA (Table 1.1; Appendix A Fig. 2). DHEA is a lesser-studied fatty acid ethanolamide, and research is just beginning to elucidate its physiological role(s). For example, Kim et al. (2014) treated myoblasts *in vitro* with DHEA, which resulted in higher CB_1, GLUT1, and insulin receptor mRNA expression as well as higher glucose uptake compared to controls. In addition, Meijerink et al. (2015) confirmed *in vitro* that DHEA modulates release of cytokine interleukin 6 (IL-6) in peritoneal macrophages after LPS stimulation. These findings suggest possible mechanisms (e.g., glucose metabolism, or tissue inflammation) that could connect wheel running to DHEA levels.

Sex differences in small intestine eCBs

Considering only the control groups in the present study (i.e., mice from either genetic linetype housed without wheels), we found no statistically significant sex differences for levels of eCBs and other related lipids included in our analysis (based on differences of LS Means from SAS Proc Mixed) (results not shown). However, in combined analyses of all mice, we did find evidence for interactive effects that included sex for both 2-AG and DHG (Tables 1.1 and 1.2).

To our knowledge, only one previous study of small-intestinal eCB concentrations in mice included both sexes. Perez and DiPatrizio (2018) also measured 2-AG, AEA, DHG, DHEA, and OEA in small-intestinal epithelium (jejunum mucosa) of offspring from dams fed a standard (SD) or a Western-style diet (WD, high fat and sucrose), but did not compare the sexes. Based on their Table 2, female offspring from SD dams had higher concentrations of all five analytes in small-intestinal epithelium than male SD offspring. The same pattern was apparent for offspring from WD dams, with the exception of AEA. However, t-tests indicate that female offspring had significantly higher mucosal 2-AG than the male offspring from the WD dams (t = 2.836; df = 11, P = 0.0162), and a similar trend was present for 2-AG in offspring from SD dams (t = 1.9340; df = 14; P = 0.0736). Another sex-difference they report (their page 7) comes from 2-AG and AEA concentrations measured in the dams during their pre-gestation phase (i.e., adult female mice on SD or WD for 10 weeks). When compared to control mice maintained on SD (i.e., low-fat/no sucrose), female WD mice had

reduced levels of 2-AG and increased levels of AEA in plasma; however, no differences were detected in levels of 2-AG or AEA in the upper small-intestinal epithelium. This result is in contrast to male mice maintained on WD for 60 days (Argueta and DiPatrizio, 2017), which had elevated levels of 2-AG and AEA in both upper small-intestinal epithelium and plasma, when compared to mice maintained on SD control. The underlying molecular mechanisms for these sex-dependent discrepancies are unknown and their elucidation will be important for future investigations.

Future directions

With the addition of our study, research has found that sex differences, exercise, and genetic background may, together or on their own, modify the eCB system. Bidirectional gut-brain communication is a key component of the eCB system of particular relevance (DiPatrizio 2021). Our results may have implications for the gut-brain axis, particularly the link between proximal small intestine, midbrain, and dorsal striatum (Han et al. 2018), brain regions critical for motivated behaviors (Palmiter 2008; Bissonette and Roesch 2016). The interaction between voluntary exercise and the gut-brain axis may indeed be modulated by the eCB system and/or its control of dopamine function (Covey et al. 2017), but further research is required.

Different components of the eCB system, including enzymes and cannabinoid receptors, are feasibly causing, and responding, to varying

concentrations of gut eCBs. For example, lower levels of 2-AG observed in the small-intestinal epithelium of HR mice may be a result of increased activity of the degradative enzymes for 2-AG and other monoacylglycerols (e.g., monoacylglycerol lipase, MGL), and/or decreased activity of the biosynthetic monoacylglycerol enzyme, diacylglycerol lipase (DGL). Consequently, less available 2-AG might mean less activity at CB_1, and thus altered afferent signals to the CNS. Future studies should investigate receptor expression and binding and compare the two possible mechanisms underlying the decreased 2-AG in HR mice, i.e., a decreased rate of 2-AG synthesis (from 1-stearoyl-2-arachidonoyl-*sn*-glycerol [SAG]) via DGL or an increased rate of degradation (via MGL into arachidonic acid and glycerol).

Finally, future studies should examine how acute vs. chronic voluntary exercise may change eCB concentrations and communication in the proximal small intestine, and could also incorporate differences in diet (e.g., Western diet). Rodents fed a Western diet have significantly altered peripheral eCB system profiles and eCB endogenous activity at peripheral CB_1 receptors is crucial for driving hyperphagia (Argueta and DiPatrizio 2017). Furthermore, Western diet causes a large increase in daily wheel running of HR mice (over many weeks), with little or no effect in C mice (Meek et al. 2010), demonstrating the importance of genetic history interacting with diet. More broadly, a better understanding of how eCBs in the gut respond to genetic and environmental factors may be essential to addressing issues of obesity, including non-communicable diseases

(e.g., metabolic syndrome) to increased risk of infection from communicable diseases (e.g., COVID-19).

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Table 1.1. Three-way analysis of jejunum mucosa concentrations of 2-AG, AEA, DHG, DHEA, and OEA with no physical activity covariates (n = 183-190). See Fig. 1.1 and 1.2 for graphical representations of adjusted group means of 2-AG and DHG, respectively.

	2-AG			**AEA**			**DHG**			**DHEA**			**OEA**		
	n = 190			*n = 183*			*n = 190*			*n = 189*			*n = 190*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Sex	1,6	0.33	0.5847	1,6	1.56	0.2580	1,6	1.5	0.2669	1,6	0.21	0.6611	1,6	4.26	0.0845
Linetype	1,6	6.05	**0.0491**	1,6	0.05	0.8301	1,6	1.02	0.3508	1,6	0.08	0.7824	1,6	0.11	0.7496
Wheel access	1,6	0.00	0.9997	1,6	0.75	0.4208	1,6	0.12	0.7376	1,6	5.39	0.0593	1,6	1.41	0.2799
Sex x Wheel access	1,6	1.08	0.3391	1,6	0.01	0.9235	1,6	1.18	0.3189	1,6	0.35	0.5763	1,6	0.15	0.7096
Sex x Linetype	1,6	0.01	0.9357	1,6	0.01	0.9245	1,6	0.03	0.8639	1,6	0.37	0.5659	1,6	0.10	0.7641
Linetype x Wheel access	1,6	0.05	0.8288	1,6	1.21	0.3144	1,6	2.32	0.1786	1,6	0.05	0.8342	1,6	1.50	0.2670
Sex x Linetype x Wheel access	1,6	4.22	**0.0857**	1,6	0.71	0.4315	1,6	5.79	**0.0529**	1,6	0.02	0.9024	1,6	0.23	0.6466
Mini-muscle	1,148	0.15	0.6977	1,141	0.37	0.5434	1,148	0.02	0.8796	1,147	0.36	0.5504	1,148	0.24	0.6277
Age	1,148	2.34	0.1279	1,141	2.20	0.1404	1,148	2.57	0.1110	1,147	1.40	0.2394	1,148	6.63	**0.0110**
Time of day	1,148	0.01	0.9269	1,141	0.13	0.7238	1,148	0.39	0.5334	1,147	1.78	0.1837	1,148	0.02	0.8996

Table 1.2. Analysis of covariance of jejunum mucosa concentrations of 2-AG, AEA, DHG, DHEA, and OEA with wheel running and HCA covariates (n = 180-187), where mice without wheel access had running values set to zero.

	2-AG			AEA			DHG			DHEA			OEA		
	n = 186			*n = 180*			*n = 187*			*n = 186*			*n = 187*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Sex	1,6	0.10	0.7616	1,6	0.64	0.4531	1,6	0.88	0.3842	1,6	0.39	0.5543	1,6	2.87	0.1414
Linetype	1,6	2.89	0.1398	1,6	0.06	0.8223	1,6	0.07	0.7966	1,6	0.07	0.7988	1,6	0.02	0.8919
Wheel access	1,6	0.28	0.6158	1,6	0.34	0.5805	1,6	0.48	0.5143	1,6	0.00	0.9533	1,6	0.17	0.6954
Sex x Wheel access	1,6	1.13	0.3288	1,6	0.00	0.9796	1,6	1.49	0.2681	1,6	0.21	0.6617	1,6	0.12	0.7406
Sex x Linetype	1,6	0.00	0.9844	1,6	0.02	0.8825	1,6	0.00	0.9874	1,6	0.27	0.6207	1,6	0.01	0.9138
Linetype x Wheel access	1,6	0.22	0.6558	1,6	0.06	0.8119	1,6	2.25	0.1840	1,6	0.15	0.7106	1,6	0.86	0.3898
Sex x Linetype x Wheel access	1,6	5.37	**0.0597**	1,6	0.60	0.4689	1,6	5.64	**0.0551**	1,6	0.00	0.9648	1,6	0.19	0.6798
Mini-muscle	1,142	0.20	0.6546	1,136	0.30	0.5873	1,143	0.10	0.7556	1,142	0.36	0.5508	1,143	0.14	0.7100
Age	1,142	1.90	0.1699	1,136	2.46	0.1190	1,143	2.55	0.1124	1,142	1.37	0.2438	1,143	6.80	**0.0101**
Time of day	1,142	0.11	0.7424	1,136	0.35	0.5523	1,143	0.46	0.4981	1,142	2.03	0.1560	1,143	0.01	0.9107
Running in previous 30 min	1,142	0.16	0.6919	1,136	0.20	0.6519	1,143	1.14	0.2873	1,142	0.83	0.3635	1,143	0.05	0.8216
HCA in previous 30 min	1,142	5.46	**0.0208**	1,136	3.32	0.0705	1,143	1.01	0.3171	1,142	0.90	0.3453	1,143	0.82	0.3680

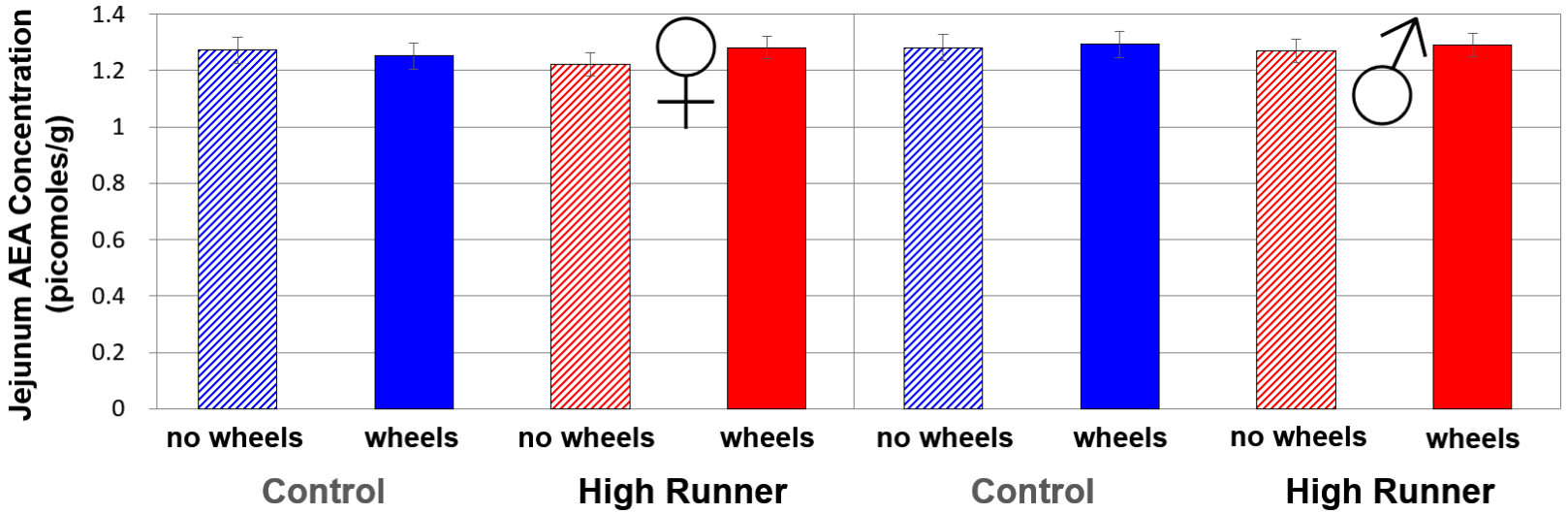
Table 1.3. Pearson correlations of raw values for all mice between 2-AG, AEA, DHG, DHEA, and OEA in jejunum mucosa, as well as plasma endocannabinoids (from Thompson et al. 2017). P values are for 2-tailed tests.

Correlations			Jejunum Mucosa					Plasma	
			2-AG	AEA	DHG	DHEA	OEA	2-AG	AEA
Jejunum Mucosa	2-AG	Pearson Correlation		0.372**	0.800**	0.295**	0.417**	-0.038	0.098
		Sig. (2-tailed)		2.10E-07	1.58E-43	3.66E-05	2.09E-09	0.599	0.183
		N	190	183	190	189	190	189	185
	AEA	Pearson Correlation			0.403**	0.515**	0.684**	-0.089	0.115
		Sig. (2-tailed)			1.57E-08	1.07E-13	1.44E-26	0.234	0.126
		N		183	183	182	183	182	178
	DHG	Pearson Correlation				0.216**	0.440**	-0.081	-0.023
		Sig. (2-tailed)				0.003	2.13E-10	0.268	0.759
		N			190	189	190	189	185
	DHEA	Pearson Correlation					0.586**	-0.087	0.05
		Sig. (2-tailed)					8.72E-19	0.238	0.496
		N				189	189	188	184
	OEA	Pearson Correlation						-0.176*	0.048
		Sig. (2-tailed)						0.016	0.520
		N					190	189	185
Plasma	2-AG	Pearson Correlation							0.083
		Sig. (2-tailed)							0.261
		N						189	184
	AEA	Pearson Correlation							
		Sig. (2-tailed)							
		N							185

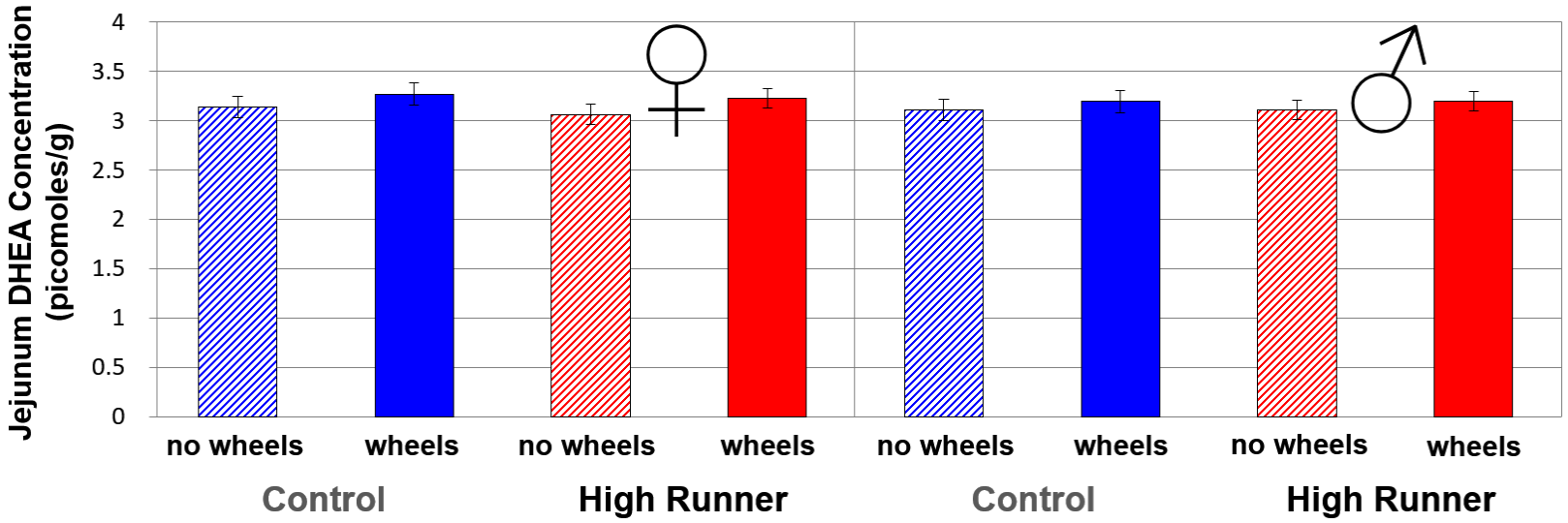
** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

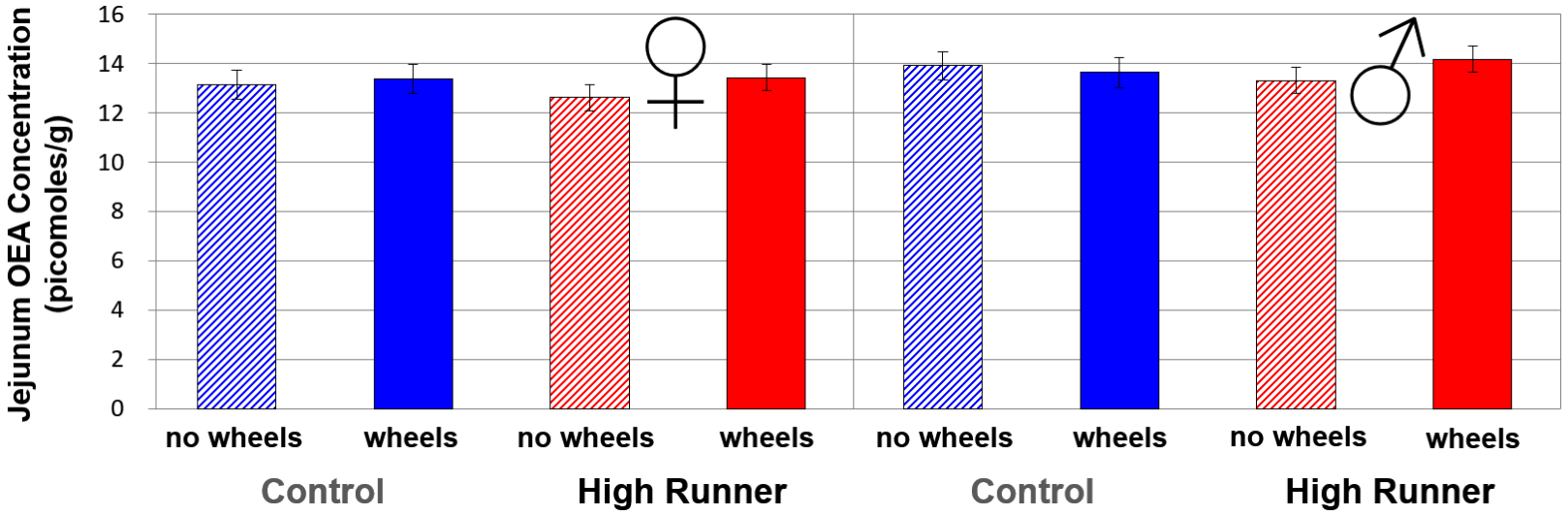
APPENDIX A



Appendix A Figure 1. Concentrations of AEA in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 183). No statistically significant differences between groups was found. Values are LS means ± standard error from SAS Proc Mixed for log_{10}-transformed data. See Table 1.1 for full statistical analysis.



Appendix A Figure 2. Concentrations of DHEA in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 189). No statistically significant differences between groups was found, although mice with wheel access tended to have higher values (P = 0.0593). Values are LS means ± standard error from SAS Proc Mixed for data transformed by raising to the 0.4 power. See Table 1.1 for full statistical analysis.



Appendix A Figure 3. Concentrations of OEA in the jejunum mucosa collected during peak activity on the 6th night of wheel running (n = 190). No statistically significant differences between groups was found, although male mice tended to have higher values (P = 0.0845). Values are LS means ± standard error from SAS Proc Mixed for data transformed by raising to the 0.5 power. See Table 1.1 for full statistical analysis.

Appendix A Table 1. Summary of statistical analyses from SAS Proc Mixed. Separate analyses are detailed in text of Chapter 1 Results.

SAS RESULTS SUMMARY OF P-VALUES													
SAS Analysis	**n**	**Wheel Acces**	**Sex**	**Linetype**	**Wheel Access x Sex**	**Wheel Access x Linetype**	**Sex x Linetype**	**Wheel Access x Sex x Linetype**	**Mini-Muscle**	**Age**	**Time of Day**	**RUN 30**	**HCA 30**
2AG_All	190	0.9997	0.5847	**0.0491**	0.3391	0.8288	0.9357	**0.0857**	0.6977	0.1279	0.9269	-	-
AEA_All	183	0.4208	0.2580	0.8301	0.9235	0.3144	0.9245	0.4315	0.5434	0.1404	0.7238	-	-
DHG_All	190	0.7376	0.2669	0.3508	0.3189	0.1786	0.8639	**0.0529**	0.8796	0.1110	0.5334	-	-
DHEA_All	189	0.0593	0.6611	0.7824	0.5763	0.8342	0.5659	0.9024	0.5504	0.2394	0.1837	-	-
OEA_All	190	0.2799	0.0845	0.7496	0.7096	0.2670	0.7641	0.6466	0.6277	0.0110	0.8996	-	-
2AG_All_with_RUN_HCA	186	0.6158	0.7616	0.1398	0.3288	0.6558	0.9844	**0.0597**	0.6546	0.1699	0.7424	0.6919	**0.0208**
AEA_All_with_RUN_HCA	180	0.5805	0.4531	0.8223	0.9796	0.8119	0.8825	0.4689	0.5873	0.1190	0.5523	0.6519	0.0705
DHG_All_with_RUN_HCA	187	0.5143	0.3842	0.7966	0.2681	0.1840	0.9874	**0.0551**	0.7556	0.1124	0.4981	0.2873	0.3171
DHEA_All_with_RUN_HCA	186	0.9533	0.5543	0.7988	0.6617	0.7106	0.6207	0.9648	0.5508	0.2438	0.1560	0.3635	0.3453
OEA_All_with_RUN_HCA	187	0.6954	0.1414	0.8919	0.7406	0.3898	0.9138	0.6798	0.7100	0.0101	0.9107	0.8216	0.3680
2AG_WHEELS	92	-	0.1881	**0.0094**	-	-	0.1934	-	0.1204	0.8340	0.6719	-	-
AEA_WHEELS	90	-	0.2602	0.6929	-	-	0.5566	-	**0.0454**	0.6066	0.6303	-	-
DHG_WHEELS	92	-	0.1134	0.4753	-	-	0.2255	-	0.0927	0.4777	0.9803	-	-
DHEA_WHEELS	91	-	0.4149	0.5212	-	-	0.9217	-	0.2483	0.6731	0.0570	-	-
OEA_WHEELS	92	-	0.2679	0.9823	-	-	0.7741	-	0.4236	0.3636	0.4981	-	-
2AG_WHEELS_with_RUN_HCA	92	-	0.2082	**0.0296**	-	-	0.1937	-	0.1271	0.8787	0.7393	0.6043	0.8757
AEA_WHEELS_with_RUN_HCA	90	-	0.3495	0.8472	-	-	0.5050	-	0.0559	0.6502	0.7637	0.9871	0.5696
DHG_WHEELS_with_RUN_HCA	92	-	0.1544	0.9417	-	-	0.1787	-	0.1100	0.5271	0.9971	0.3798	0.8283
DHEA_WHEELS_with_RUN_HCA	91	-	0.5147	0.2975	-	-	0.7799	-	0.1956	0.7254	0.0610	0.3894	0.8838
OEA_WHEELS_with_RUN_HCA	92	-	0.2936	0.9145	-	-	0.8210	-	0.4236	0.3828	0.5257	0.7577	0.9535
2AG_NO_WHEELS	95	-	0.7960	0.1720	-	-	0.2993	-	0.6045	0.0615	0.9562	-	-
AEA_NO_WHEELS	91	-	0.3960	0.6860	-	-	0.7139	-	0.8178	<0.0001	0.1092	-	-
DHG_NO_WHEELS	95	-	0.8438	0.3008	-	-	0.1828	-	0.0506	0.0371	0.2725	-	-
DHEA_NO_WHEELS	95	-	0.9663	0.9144	-	-	0.6974	-	0.8957	0.0352	0.5914	-	-
OEA_NO_WHEELS	94	-	0.1085	0.3602	-	-	0.7735	-	0.3746	<0.0001	0.8644	-	-
2AG_NO_WHEELS_with_HCA	95	-	0.4385	0.5842	-	-	0.3767	-	0.3312	0.0700	0.8201	-	**0.0333**
AEA_NO_WHEELS_with_HCA	91	-	0.6093	0.4317	-	-	0.9098	-	0.7170	<0.0001	0.1524	-	**0.0145**
DHG_NO_WHEELS_with_HCA	95	-	0.9451	0.7464	-	-	0.2166	-	**0.0242**	0.0402	0.3095	-	0.1909
DHEA_NO_WHEELS_with_HCA	95	-	0.6675	0.5485	-	-	0.8080	-	0.6735	0.0215	0.5751	-	0.0683
OEA_NO_WHEELS_with_HCA	94	-	0.2296	0.7353	-	-	0.9758	-	0.1468	<0.0001	0.9284	-	0.0552

Appendix A Table 2. Pearson Correlations of raw values within experimental groups between 2-AG, AEA, DHG, DHEA, and OEA in jejunum mucosa (n = 21–24 per group).

	Control				High Runner			
	Females		Males		Females		Males	
	No Wheels	Wheels	No Wheels	Wheels	No Wheels	Wheels	No Wheels	Wheels
2AG x AEA	**0.434***	0.301	0.159	0.302	**0.633****	0.397	**0.418***	0.335
2AG x DHG	**0.758****	**0.804****	**0.741****	**0.784****	**0.907****	**0.845****	**0.879****	**0.910****
2AG x DHEA	**0.542****	0.021	0.372	0.375	**0.607****	0.217	0.235	0.239
2AG x OEA	0.199	**0.575****	0.105	**0.523****	**0.591****	**0.444***	**0.678****	**0.431***
AEA x DHG	0.413	-0.092	0.292	0.359	**0.650****	0.402	0.413	0.390
AEA x DHEA	0.424	0.413	0.340	**0.406***	**0.829****	**0.492***	0.344	**0.708****
AEA x OEA	**0.539***	**0.558****	**0.691****	**0.768****	**0.699****	**0.699****	**0.745****	**0.788****
DHG x DHEA	0.332	-0.100	0.184	0.010	**0.617****	0.163	0.184	0.242
DHG x OEA	0.258	**0.407***	0.227	0.404	**0.557****	**0.535****	**0.679****	0.402
DHEA x OEA	**0.554****	**0.526****	**0.433***	**0.662****	**0.690****	**0.514***	0.301	**0.829****

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Examining the jejunal raw value intercorrelations within the eight groups, 78 of 80 were positive, and 44 were statistically significant (Appendix A Table 2). However, the average strength of the correlations varied among groups. For example, all 10 possible correlations were significantly positive for HR females without wheel access, whereas only 3 of 10 were significant for C males without wheels. The causes of these differences among groups requires further study.

Appendix A Table 3. Pearson Correlations of raw values within experimental groups between jejunum and plasma analytes (n = 21–24 per group).

	Control				High Runner			
	Females		Males		Females		Males	
	No Wheels	Wheels	No Wheels	Wheels	No Wheels	Wheels	No Wheels	Wheels
Gut2AG x Plasma2AG	-0.095	-0.140	-0.273	0.286	-0.106	-0.068	-0.316	-0.128
GutAEA x Plasma2AG	-0.186	-0.052	-0.132	-0.028	0.165	-0.206	**-0.425***	-0.248
GutDHG x Plasma2AG	0.000	-0.155	**-0.439***	0.236	-0.040	-0.054	**-0.436***	-0.204
GutDHEA x Plasma2AG	-0.014	-0.026	0.019	-0.056	0.275	-0.205	-0.213	-0.287
GutOEA x Plasma2AG	-0.138	-0.166	-0.328	-0.093	-0.106	-0.027	**-0.572****	-0.304
Gut2AG x PlasmaAEA	-0.066	-0.107	0.071	0.256	-0.279	0.310	-0.254	0.332
GutAEA x PlasmaAEA	0.281	0.029	-0.085	0.256	-0.094	0.395	-0.067	0.068
GutDHG x PlasmaAEA	-0.012	-0.051	-0.274	0.226	-0.337	0.346	**-0.456***	0.133
GutDHEA x PlasmaAEA	0.045	-0.357	0.200	0.371	-0.100	0.073	0.028	0.091
GutOEA x PlasmaAEA	0.141	-0.239	0.089	**0.429***	0.090	0.016	-0.167	0.007

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Appendix A Table 4. Pearson Correlations of simple means for our eight experimental groups between jejunum analytes and plasma 2-AG and AEA from Thompson et al. (2017).

Correlations			Jejunum Mucosa					Plasma	
			2-AG	AEA	DHG	DHEA	OEA	2-AG	AEA
Jejunum Mucosa	**2-AG**	Pearson Correlation		0.458	0.676	-0.034	0.266	0.487	0.487
		Sig. (2-tailed)		0.254	0.066	0.937	0.525	0.222	0.221
		N	8	8	8	8	8	8	8
	AEA	Pearson Correlation			0.919**	0.364	0.721*	0.313	-0.023
		Sig. (2-tailed)			0.001	0.376	0.044	0.450	0.956
		N		8	8	8	8	8	8
	DHG	Pearson Correlation				0.163	0.632	0.530	-0.033
		Sig. (2-tailed)				0.700	0.093	0.177	0.938
		N			8	8	8	8	8
	DHEA	Pearson Correlation					0.419	-0.647	0.370
		Sig. (2-tailed)					0.302	0.083	0.368
		N				8	8	8	8
	OEA	Pearson Correlation						0.310	-0.246
		Sig. (2-tailed)						0.454	0.557
		N					8	8	8
Plasma	**2-AG**	Pearson Correlation							-0.394
		Sig. (2-tailed)							0.334
		N						8	8
	AEA	Pearson Correlation							
		Sig. (2-tailed)							
		N							8

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

CHAPTER 2

Female mice from lines selectively bred for high voluntary wheel-running behavior have enlarged hippocampus and red nucleus

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Abstract

Uncovering relationships between neuroanatomy, behavior, and evolution is important for understanding the underlying factors that control human brain function. Voluntary exercise is one key behavior that affects both neuroanatomy and brain evolution. Our study used an ongoing model of artificial selection, in which mice were bred for high voluntary wheel-running behavior for more than 60 generations, yielding four replicate lines of High Runner (HR) mice that run ~3-fold more per day than four replicate non-selected Control (C) lines. Adjusting for body mass, HR mice have heavier whole brains, non-cerebellar brains, and larger midbrains than C mice. Using high resolution microscopy to image, we tested the hypothesis that HR mice would have greater volumes and/or cell density in five key brain regions from either the midbrain or limbic system. In addition, half of the mice were given 10 weeks of wheel access from weaning, and we predicted that wheel access would increase the volumes of the examined brain regions. Both the red nucleus (RN) of the midbrain and the hippocampus (HPC) were significantly larger in HR compared to C mice, with no effect of chronic wheel access. Volumes of the basolateral amygdala (BLA), nucleus accumbens (NA), and ventral pallidum (VP) did not differ between HR and C mice, nor between mice housed with or without wheels. Cell density was unchanged between linetypes or wheel access groups. A larger red nucleus in HR mice may indicate enhanced control of limb movement, be related to dopamine signaling, or involved in mechanisms of pain modulation. Additionally,

a larger hippocampus, important for motivated behaviors, might contribute to an increased motivation to exercise.

Introduction

The "mosaic" model of brain evolution posits that “the size of individual structures can be linked to special behavioral capacities" (Finlay et al. 2001) and contrasts with a model of coordinated structural evolution in which the size of the whole brain evolves with little change in the relative size of individual regions (Finlay and Darlington 1995; Barton and Harvey 2000). In mammals and other vertebrates, various studies comparing species have found that sizes of individual regions often do correlate with behavioral ecology (Krebs et al. 1989; Hutcheon et al. 2002; Gonzalez-Voyer and Kolm 2010; Swanson et al. 2012; Liao et al. 2015; DeCasien and Higham 2019; Muller and Montgomery 2019). For instance, species of food-storing birds that use long-lasting spatial memories for food retrieval have a larger hippocampal complex relative to their body size as compared with species that do not store food (Krebs et al. 1989). As another example, species of insectivorous bats that rely on echolocation when pursuing prey have larger auditory nuclei than phytophagous bats (Hutcheon et al. 2002). In a third example, the anterior cerebrum of Carnivora species is negatively predicted by forelimb use and home range size (Swanson et al. 2012).

Artificial selection provides a means to select upon a behavior of interest and use the resultant evolved organisms to test hypotheses about correlated evolution of the brain and its component parts. Uncovering and recording organismal changes in a “top-down” fashion, from an altered behavior to potentially altered organs to tissues to proteins to DNA, may ultimately provide

insights about specific genetic mechanisms that underlie individual and species differences in brain morphology and function. The artificially selected behavior in the present study is voluntary exercise in mice provided running wheels (Swallow et al. 1998; Rhodes et al. 2005). Importantly, Meijer and Robbers (2014) showed that wheel running is not only a rodent behavior and not just an artifact of captivity; rather, wheels placed in nature were frequently used by wild rodents, indicating that wheel running is a motivated and elective behavior (see also Sherwin 1998; Novak et al. 2012; Greenwood and Fleshner 2019).

The mouse model used here includes four replicate selectively bred High Runner (HR) lines and four non-selected Control (C) lines (Swallow et al. 1998). Once reaching selection limits, mice from the HR lines have run ~2.5-3 times as many revolutions per day as those from C lines (Careau et al. 2013). HR mice run more intermittently on wheels and, in addition to hyperactive wheel running, they are more active in home cages when wheels are not available. Not surprisingly, the capacity for aerobic exercise is enhanced in the HR mice (Rezende et al. 2006; Meek et al. 2009; Kolb et al. 2010). Various other behaviors have also been shown to differ between HR and C mice, including: smaller nest building by HR mice when housed either with or without wheels (Carter et al. 2000); less turning by HR mice in an open-field test (Bronikowski et al. 2001); lower latencies to attack crickets by HR male mice in a test of predatory aggression (Gammie et al. 2003); more time spent in the closed arms of an elevated plus maze by HR male mice suggesting increased anxiety

(Singleton and Garland 2019); more active behaviors and less grooming by HR male mice, regardless of wheel access (Waters et al. 2013).

Two previous studies have examined brain size in the HR mice. Based on dissections and with body mass as a covariate, Kolb et al. (2013) found that HR total and non-cerebellar brain mass of both sexes was significantly increased (as much as 7.0% for dry mass in males) when compared to C mice. Kolb et al. (2013) additionally used ex-vivo MRI imaging to reveal that HR mice had a 13% larger midbrain volume than C mice, with body mass as a covariate. The other regions examined by Kolb (forebrain, caudate, hippocampus, and cerebellum) were not statistically different, thus offering support for the mosaic model of brain evolution (Barton and Harvey 2000).

Thompson (2017) also reported that HR female mice tended to have heavier brains than C mice ($P = 0.0676$) and used histological methods for high resolution brain microscopy. In addition to comparing HR and C lines, separate groups of mice were provided access to running wheels or not for 10 weeks beginning at weaning. Mice with 10 weeks of wheel access had heavier brains than mice those that did not ($P = 0.0127$). Consistent with Kolb et al. (2013), HR mice tended to have larger whole midbrain (WM) volume ($P = 0.0713$). Substantia nigra (SN) volume was not different between linetypes, but the ventral tegmental area (VTA) tended to be larger in HR mice ($P = 0.0820$). Wheel access did not affect volume for the WM, SN or VTA. For the periaqueductal gray (PAG), linetype and wheel access had an interactive effect ($P = 0.0513$) with

wheel access decreasing PAG volume in HR mice but increasing it in C mice. Neither linetype nor wheel access had a significant effect on total cell count/area in any region.

Other studies have shown regional volumetric changes in rodents provided varying durations of voluntary exercise. For example, rats with 7 days of wheel running showed significant increases in volumes of motor, somatosensory, association, and visual cortices (Sumiyoshi et al. 2014). In another study, mice with 4 weeks of exercise had significantly increased volume of the hippocampus (Cahill et al. 2015). Biedermann et al. (2012) also found an increase in hippocampal volume of mice with wheels after 6–8 weeks.

The present study is a continuation of Thompson's work and investigates five brain regions associated with motor control, motivation, and/or reward (see references in Discussion). The red nucleus (RN) is a midbrain region that plays a prominent role in locomotion. Within the RN, the rubrospinal tract originates and projects axons to the brain stem, cerebellum, and spinal cord to function in the control of muscle tone and limb movement. The rubrospinal system receives somatosensory inputs with information processed by the cerebellum and the basal ganglia, and from cortical motor areas (Schieber and Baker 2013). To our knowledge, no previous study has examined the size of the RN in relation to any specific behavior, either among or within species.

Outside of the midbrain, limbic structures have also been reported to have size specializations (see Discussion for e.g., Makris et al. 2008; Gilman et al.

2014; Seifert et al. 2015; Rapuano et al. 2017) and/or to respond to chronic exercise (Biedermann et al. 2012; Cahill et al. 2015; Yamamoto et al. 2017). The additional structures studied here [hippocampus (HPC), basolateral amygdala (BLA), nucleus accumbens (NA), and ventral pallidum (VP)] have been implicated in not only affect, but also goal-directed and reward-seeking motivated behavior (Ambroggi et al. 2008; Stuber et al. 2011; Lee et al. 2016; Yang and Wang 2017; LeGates et al. 2018). The HR mice have a number of changes in their reward-associated dopaminergic neuromodulatory system, including decreased activity levels with dopamine transporter blocker methylphenidate (Ritalin) and decreased sensitivity to D1-type receptor antagonist SCH 23390 (Rhodes and Garland 2003; Rhodes et al. 2005). Moreover, previous studies of HR mice that used the HPC found evolved differences in gene expression (Bronikowski et al. 2004), increased hippocampal brain-derived neurotrophic factor (BDNF) following a week of wheel running compared to C mice (Johnson et al. 2003), and wheel-running activation of dentate gyrus cells by Fos-IR that reached a plateau for HR but not C mice (Rhodes et al. 2003a).

Materials & Methods

1. Ethical approval

All experimental procedures were approved by the UC Riverside Institutional Animal Care and Use Committee.

2. Experimental animals

Mice came from an artificial selection experiment with four replicate lines of High Runner (HR) mice selected for high voluntary wheel running and four replicate non-selected Control (C) lines (Swallow et al. 1998). The original progenitors were 224 outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain. After two generations of random mating, mice were randomly paired and assigned to the 8 closed lines. Each generation, beginning at six weeks of age, mice are housed individually with access to a running wheel for six days. In the HR lines, the highest-running male and female from each family are selected as breeders based on the total number of revolutions run on days five and six of the six-day test. In C lines, breeders are chosen without regard to wheel running.

The brain tissue of the same mice from Thompson (2017) was used in the present study. In brief, 100 females from generation 67, half HR and half C were weaned at 3 weeks of age and placed into individual cages. For 10 weeks, half of the mice from each linetype were provided 24/7 access to running wheels (1.12 m in diameter, as in the routine selection experiment: Swallow et al. 1998) and the other half had no access to wheels. Passive infrared sensors mounted within each cage measured home-cage activity (HCA) (Copes et al. 2015). During the final week of wheel access (or lack thereof) and into the 13th week, brains were removed following transcardial perfusion and subsequently preserved.

3. Tissue processing and imaging

Following removal, the brains were stored in 30% sucrose in 4% paraformaldehyde (PFA) for a minimum of 48 hours. Tissue was sectioned coronally at 40 micrometers thickness on a Leica CM1850 cryostat, with alternating slices placed on a slide designated for this experiment or a slide for a separate experiment. Following slicing, tissue was Nissl stained and digital images were taken for each region using a Zeiss Discovery V.12 stereo microscope and an attached Zeiss AxioCam. Magnification for each brain region was chosen for the best differentiation of region borders and zoom for clarity.

4. Region area and cell density measurements

a. Area measurements

ImageJ software (NIH) was used to import each photograph and the polygon-selection tool provided the means to free-hand outline each region of interest. The Franklin and Paxinos mouse brain atlas (Franklin and Paxinos 2008) and online Allen Mouse Brain Atlas (Allen Institute for Brain Science 2021) were used as guides. A photographed ruler for each magnification was used to set the scale of pixels/μm, which was used to calculate the area of the outlined brain region in each image. For the NA, some images centrally contained the anterior commissure, which was also traced, then later subtracted from the area, volume, and cell count measurements so as not to skew the results. Appendix B provides details about tracing procedures for individual regions.

b. Cell density

The Image-based Tool for Counting Nuclei (ITCN) was used in ImageJ to measure the number of cells within each photographed and outlined brain region of interest (Byun et al. 2006). Settings within the ITCN for each region included the average pixel width of cells, the minimum distance between cells, and dark peaks to be detected. The ITCN output for each mouse, including cell numbers and area measurements (μm^2), were merged and transformed from .txt into .xlsx format using Python 3.7.4 (see Appendix B). The total number of cells was then summed and divided by the total cross-sectional area, generating the measure of average cell count per unit area. For the NA, the total cell number and area of the anterior commissure were subtracted before calculating the cell count per unit area.

5. Volume calculations

Brain region volumes were calculated as in Thompson (2017) using the area (μm^2) output (from the ITCN) multiplied by 80µm (the distance between each section). Values for missing or damaged sections were replaced by taking the average of the previous and next section, or as follows for multiple sections:

> "E.g. if sections 8, 9 & 10 were missing, the volume of section 7 was subtracted from section 11 to get number *x*, and then *x* was divided by 4 to get number *y*, which was added to the volume of section 7 to get the volume of section 8, then added to the volume of section 8 to get the area of section 9, etc." (Thompson 2017)

The volumes from each section were then summed to get an overall region volume for each mouse. For the regions examined in the present work, the volumes were multiplied by two because images were taken unilaterally to produce higher magnified photographs for accurate cell counts. Mice that were judged to be missing sections at the anterior or posterior end(s) of a region, as well as mice with lost or particularly damaged tissue (sometimes region specific) from the slicing and staining processes, were excluded from the analyses. Thus, final sample sizes were considerably smaller than the starting number of 100.

6. Statistical analyses

Following numerous previous studies using mice from the selection experiment, mixed models were implemented in SAS Procedure Mixed, with wheel access and linetype as main effects, and replicate line nested within linetype (HR vs. C) as a random effect. The interaction between wheel access and linetype was tested relative to the wheel*line(linetype) random effect term. Additionally, a subset HR mice have a “mini-muscle” phenotype caused by a recessive allele which reduces hindlimb muscle mass by about half, and pleiotropically affects a variety of other traits (Garland et al. 2002; Kelly et al. 2013). Mini-muscle mice were discerned at dissection by examination of the triceps surae muscles, then included as an additional main effect in all analyses. Body mass, age, the amount of time brains spent in paraformaldehyde prior to sectioning, and how long the sections spent in a freezer ($\log_{10}$ transformed)

before Nissl staining were included as covariates for all volume analyses. All of these are viewed as nuisance variables, so they are presented in tables of statistical results but not otherwise discussed. Body mass was not a covariate for the cell density analyses. Mice were excluded from the final analyses that had residual values >3 standard deviations from the mean. P-values below 0.05 were treated as statistically significant.

Results

1. Red nucleus

The total volume of the red nucleus was significantly larger in HR compared to C mice (P = 0.0151, Figure 2.1, Table 2.1), with no statistically significant effect of wheel access for 10 weeks (P = 0.4324), no interaction between linetype and wheel access (P = 0.8930), and no effect of mini-muscle status (P = 0.8275). The number of cells per unit area in the RN was not significantly affected by linetype, wheel access, their interaction or mini-muscle status (Table 2.2).

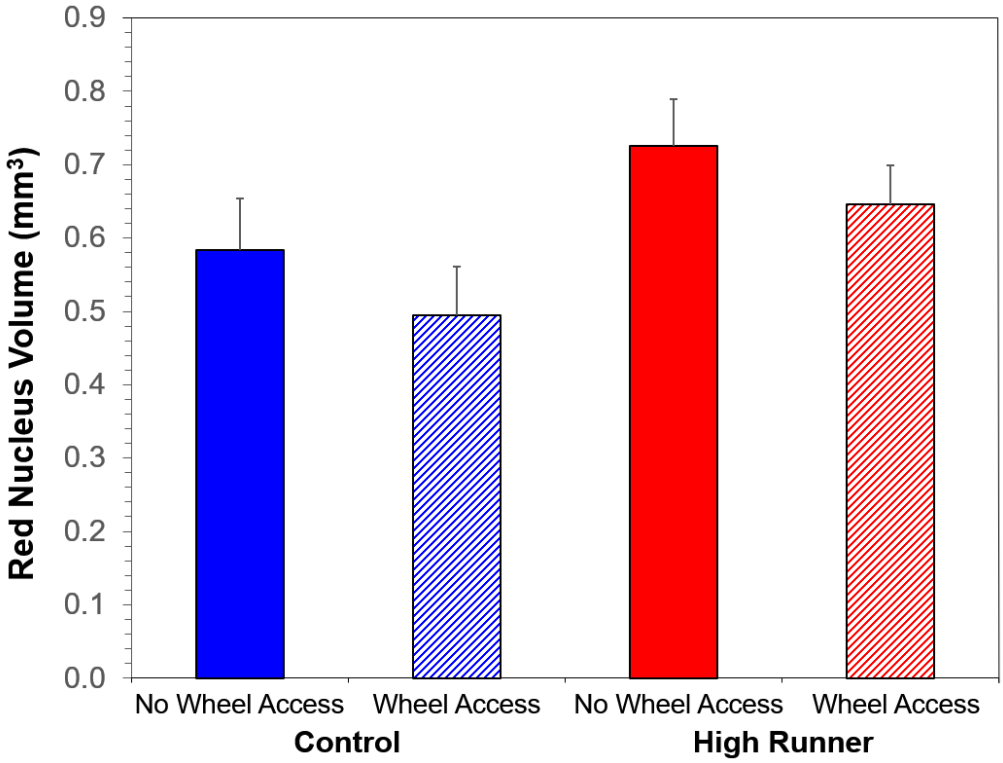

Figure 2.1. Red nucleus total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, age, time in PFA, and $\log_{10}$ time in the freezer included in the statistical model (Table 2.1). HR mice had a significantly larger red nucleus (P = 0.0151), with no significant effect of wheel access and no interaction.

2. Hippocampus

The total volume of the hippocampus was significantly larger in HR mice (P = 0.0138, Figure 2.2, Table 2.1), with no effect of wheel access (P = 0.9343), no interaction (P = 0.5779), and no effect of mini-muscle (P = 0.8304). The number of cells per unit area in the HPC was not significantly affected by linetype, wheel access, their interaction or mini-muscle status (Table 2.2).

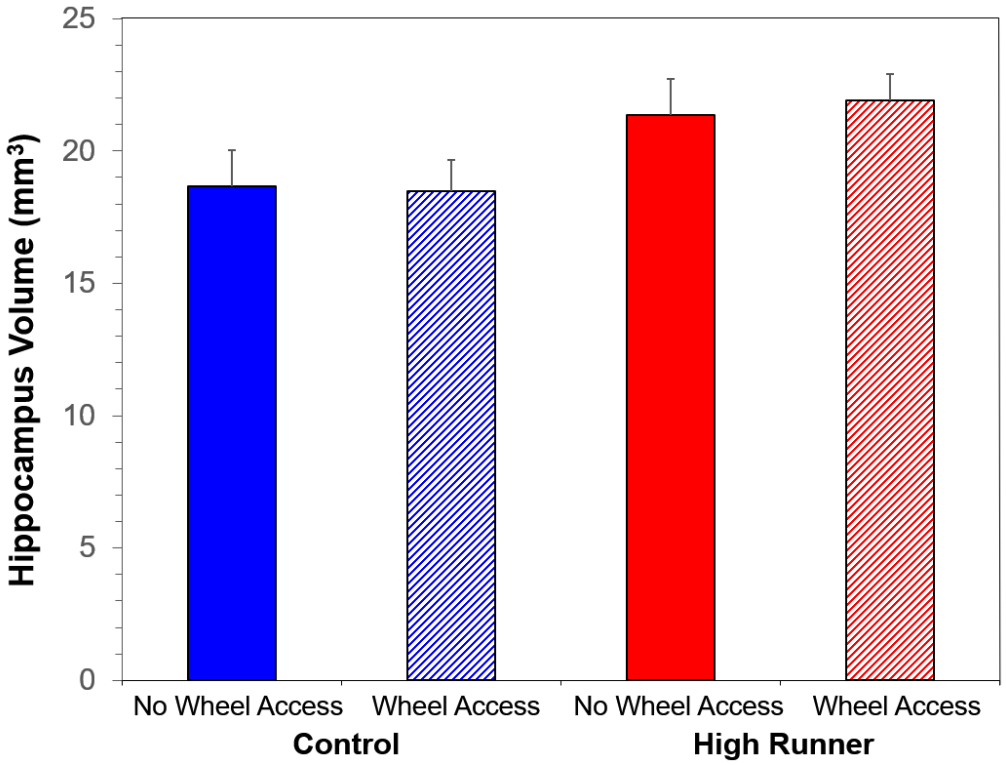

Figure 2.2. Hippocampus total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, age, time in PFA, and $\log_{10}$ time in the freezer included in the statistical model (Table 2.1). HR mice had a significantly larger hippocampus (P = 0.0138), with no significant effect of wheel access and no interaction.

3. Basolateral amygdala

The total volume of the basolateral amygdala was not affected by linetype (P = 0.1416), wheel access (P = 0.8071), their interaction (P = 0.3921) or mini-muscle status (P = 0.3818) (Figure 2.3, Table 2.1), and results were similar for the number of cells per unit area (Table 2.2).

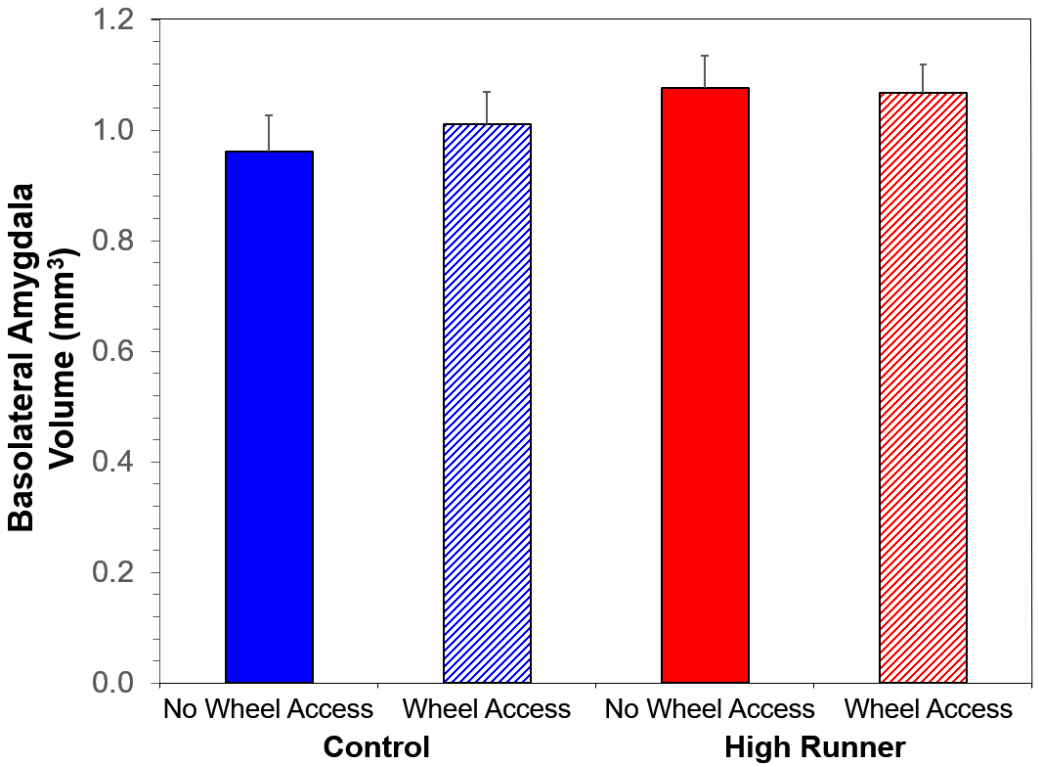

Figure 2.3. Basolateral amygdala total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, age, time in PFA, and $\log_{10}$ time in the freezer included in the statistical model (Table 2.1). There were no significant effects of linetype or wheel access, and no interaction.

4. Nucleus accumbens

The total volume of the nucleus accumbens was not statistically affected by linetype (P = 0.3773), wheel access (P = 0.1535), their interaction (P = 0.6053) or mini-muscle status (P = 0.5402) (Figure 2.4, Table 2.1). The number of cells per unit area (excluding the anterior commissure) was also not significantly affected by linetype, wheel access, their interaction or mini-muscle status (Table 2.2).

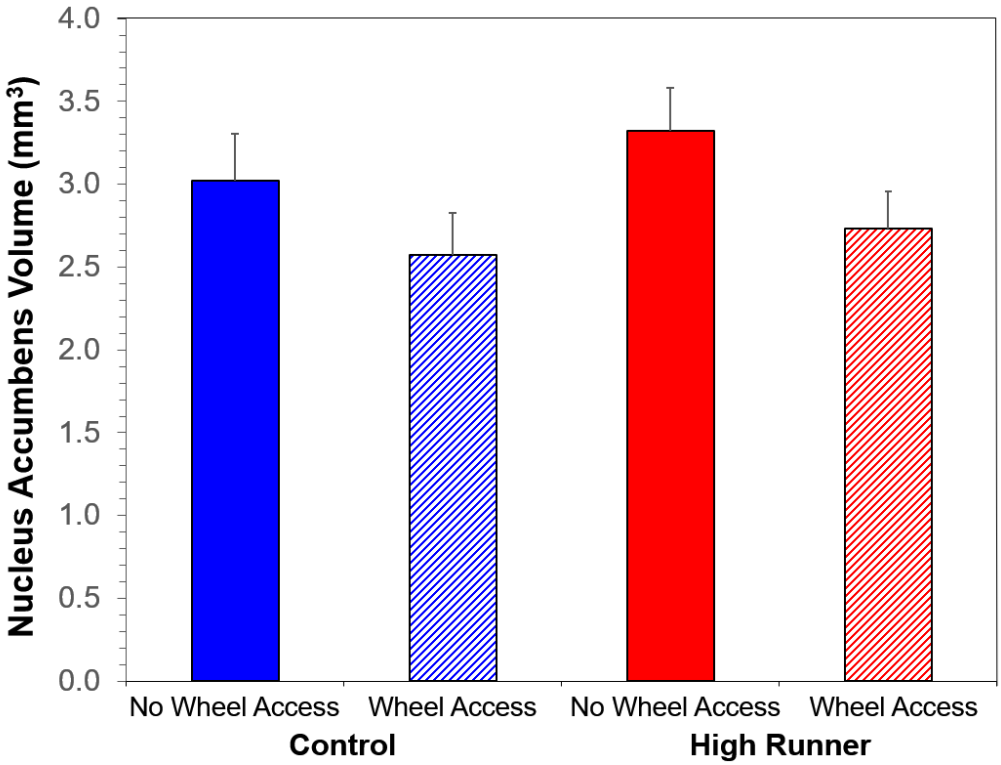

Figure 2.4. Nucleus accumbens total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, age, time in PFA, and $\log_{10}$ time in the freezer included in the statistical model (Table 2.1). There were no significant effects of linetype, wheel access, or their interaction on nucleus accumbens volume.

5. Ventral pallidum

The total volume of the ventral pallidum was not statistically affected by linetype (P = 0.1546), wheel access (P = 0.4397), their interaction (P = 0.7698) or mini-muscle status (P = 0.6902) (Figure 2.5, Table 2.1), and neither was the number of cells per unit area (Table 2.2).

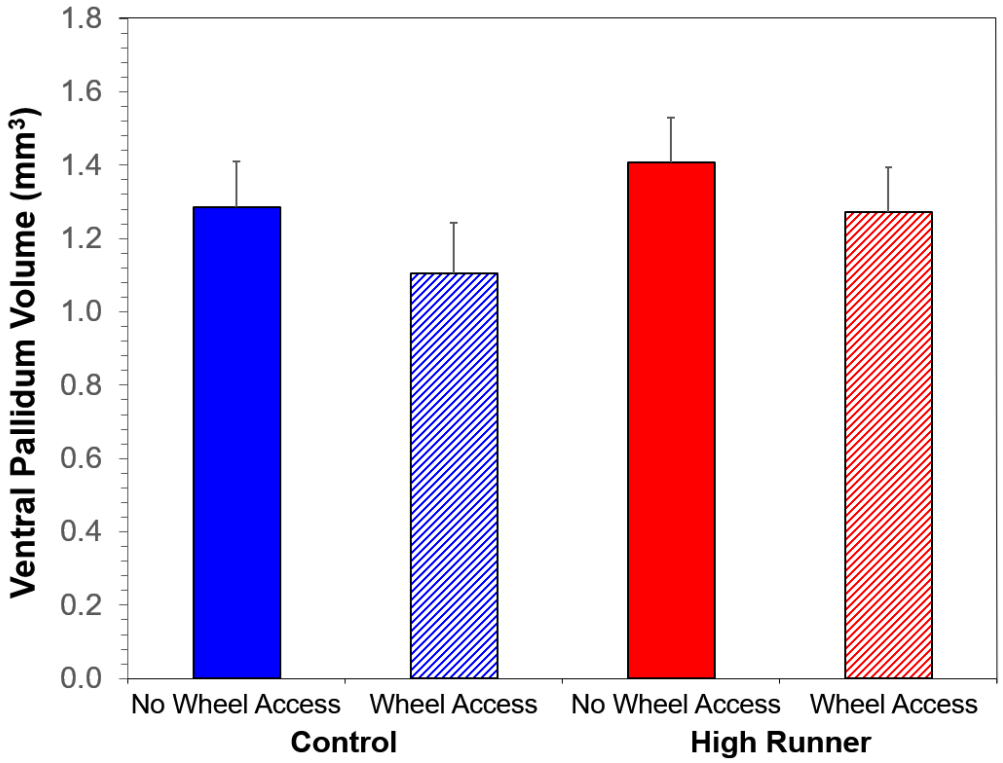

Figure 2.5. Ventral pallidum total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, age, time in PFA, and $\log_{10}$ time in the freezer included in the statistical model (Table 2.1). There were no significant effects of linetype or wheel access, and no interaction.

Discussion

The purpose of the present study was to test for differences in the size of key brain regions between lines of mice that have been bred for voluntary exercise and their non-selected control lines. In addition, we tested for effects of 10 weeks of wheel access, beginning at weaning, on the same brain regions. We also compared neuronal densities for each region. With body mass as a covariate, both the red nucleus of the midbrain and the hippocampus were significantly larger in HR than C mice (Fig. 2.1-2.2 and Table 2.1), thus

contributing to their overall brain size difference (Kolb et al. 2013; Thompson 2017). However, neither of these regions was affected by chronic wheel access. No effects of either genetic linetype of chronic exercise were observed for the basolateral amygdala, nucleus accumbens or ventral pallidum (Fig. 2.3-2.5 and Table 2.1). Finally, we found no statistical evidence that cell densities were affected by linetype or wheel access (Table 2.2).

Red nucleus

Given that the RN plays an important role in locomotion and that HR midbrains are enlarged (Kolb et al. 2013; Thompson 2017), we predicted larger red nuclei in HR mice. Plastic changes in response to exercise often improve organ function, therefore, we also expected that chronic locomotion on wheels might enlarge the RN, perhaps especially in HR mice because they run much more than C mice. To our knowledge, only one study has found volumetric differences in the RN: Kolpakwar et al. (2021) found that patients with early-onset Parkinson's disease (PD) had significantly larger volumes compared to patients in a late-onset disease group. Whether this volume change is related to the genetic basis of PD is unknown; however, the literature suggests the existence of susceptibility genes unique to either early- or late-onset PD (Hicks et al. 2002; Billingsley et al. 2018; Blauwendraat et al. 2020). Environmental risk factors for PD have also been identified (Kieburtz and Wunderle 2013; Delamarre and Meissner 2017), but only one study has shown that physically active

individuals have a decreased risk of PD (Thacker et al. 2008). Given that PD is characterized by the loss of midbrain dopamine, the presence of dopamine and dopamine receptor mRNA in the RN is noteworthy (Jellinger et al. 1981; Hurd et al. 2001). We predict that volume differences in this brain region contribute to the previously reported alterations in dopamine signaling in the HR mice (Rhodes and Garland 2003; Bronikowski et al. 2004; Rhodes et al. 2005; Mathes et al. 2010; Garland et al. 2011; see more regarding the HR "dopamine hypothesis" below).

The evolution and differentiation of the RN was primarily driven by quadrupedal locomotion, and the primary function of the RN involves the execution of voluntary movements by forelimbs and hindlimbs (Basile et al. 2021). Hence, a larger red nucleus in HR mice may indicate enhanced control of limb movement. Furthermore, the RN contains sensory neurons that respond to painful stimulation, and connectivity suggests that the RN may contribute to the body's analgesic response via the descending antinociceptive system (Prado et al. 1984; Basile et al. 2021). Interestingly, the PAG is part of the same system, and PAG volume is influenced by a linetype-by-wheel access interaction in HR and C mice (Thompson 2017). Wheel access decreased PAG volume in HR mice but increased PAG volume in C mice. Thompson (2017) speculated that a smaller PAG in HR brains might reduce the pain experienced during high levels of voluntary wheel running [only one study of pain sensitivity in HR mice has

been performed, and it failed to find differences in opioid-mediated pain sensitivity (Li et al. 2004)].

Hippocampus

The HPC is a critical limbic system structure that functions in spatial and episodic memory, as well as affective and motivated behaviors (Yang and Wang 2017). Unlike the RN, numerous studies have been devoted to volumetric differences of the hippocampal formation and its subregions. Maguire et al. (2000) is well recognized for their discovery that London taxi drivers (who undergo extensive navigational training) had increased volume of the HPC vs. control subjects, which was correlated with the amount of time spent as a taxi driver. Also in humans, a systematic review of the literature by Childress et al. (2013) revealed a significantly smaller hippocampal volume in veterans with chronic post-traumatic stress disorder vs. their control groups (9 of 12 studies).

In mice, hippocampal volume changes 2-3% in association with the female estrous cycle (Qiu et al. 2013). Additionally, mice that received daily lipopolysaccharide injections for 10 ten days as pups in a systemic inflammation paradigm had a 15-20% reduction in hippocampal volume compared to saline-treated controls (Malaeb et al. 2014). In a study considering genetic background, the CA1 subregion of the HPC was significantly larger in inbred C57Bl/6 mice compared to the DBA/2 inbred strain (Hikishima et al. 2017). Given the multitude of hippocampal changes observed in these studies and many others, an

evolutionary change in hippocampal volume in the HR is perhaps not surprising. Moreover, Bronikowski et al. (2004) found both increases and decreases in the expression of 53 genes in a comparison of female HR vs. C hippocampus, including an increase in expression of dopamine receptors D2 and D4. The authors argue for an association between D4 receptor expression and hyperactivity in the HR mice, as seen in attention deficit hyperactivity disorder (ADHD).

The majority of human and rodent volume studies of the HPC examine environmental effects, including voluntary exercise (Biedermann et al. 2012; Cahill et al. 2015; Scholz et al. 2015a; Li et al. 2017). As mentioned in the introduction, Biedermann et al. (2012) and Cahill et al. (2015) found increased hippocampal volumes in mice provided 4-8 weeks of wheel access. Additionally, two studies by Scholz (2015a,b) found an increase in whole HPC volume following environmental enrichment that included a running wheel for either 24 h (1.4% increase) or three weeks (3.8% increase) and a larger HPC after 80 5-min trials of rotarod training compared to mice that went without.

Previously, we found that wheel access for 40 days (beginning at 4 weeks of age) significantly increased dentate gyrus volume in both HR and C mice, in association with increased neurogenesis and BDNF concentration (Rhodes et al. 2003b). In the present study, 10 weeks of wheel access did not increase the volume of the whole HPC, but HR mice had larger hippocampal volumes, i.e., opposite to the effects observed for dentate gyrus by Rhodes et al. (2003b). This

discrepancy could be further investigated by using the HPC photographs and methods from our study to calculate the volumes of each hippocampal subregion (e.g., dorsal + ventral and/or CA1 + CA2 + dentate gyrus). Interestingly, in a mouse model of Alzheimer's disease, four months of wheel access can protect against myelin sheath degeneration within mouse CA1, which is associated with increased CA1 volume (Chao et al. 2018). Therefore, understanding the subregional differences for HR mice could provide important insights for some neurological diseases.

Basolateral amygdala, nucleus accumbens, and ventral pallidum

Considering all five regions examined, our results further support the mosaic model of brain evolution because the volumes of two regions varied between HR and C mice, but not the remaining three regions: the basolateral amygdala, nucleus accumbens, and ventral pallidum. Nonetheless, we predicted increased volumes in HR mice for these three limbic regions, possibly contributing to the greater HR whole brain size.

The BLA plays an integral role in anxiety, and excitatory projections from BLA to ventral HPC are sufficient to mediate anxiety (Yang and Wang 2017). HR mice of both sexes have resting corticosterone levels approximately twice those of C mice (Malisch et al. 2007) and males display increased anxiety-like behavior in an elevated plus maze (Singleton and Garland 2019). The BLA, as well as the NA and VP, are crucial to reward behaviors (Schultz 1998; Ambroggi et al. 2008;

Smith et al. 2009; Stuber et al. 2011; Berridge and Kringelbach 2015; Lee et al. 2016; Yang and Wang 2017), and, therefore, make prime targets for study because of the “dopamine hypothesis” (Rhodes et al. 2005) that describes changes in HR dopamine function associated with increased motivation to exercise.

Various lines of evidence suggest that HR mice are more highly motivated to run on wheels compared to C mice (Rhodes et al. 2005; Belke and Garland 2007). Exercise is a self-rewarding behavior (Sher 1998; Ekkekakis et al. 2005; Dishman et al. 2006; Brené et al. 2007; Garland et al. 2011; Novak et al. 2012) and one possible reason for HR increased motivation could be a modified sensitivity for exercise reward, supported by research showing HR mice have altered D1-type receptor signaling (Rhodes and Garland 2003). Alternatively, the HR drive to seek more reward (i.e., more running on wheels) may be increased, corroborated by Belke and Garland (2007) who used operant conditioning to show that shorter running durations have reduced reinforcing value in HR compared to C mice.

To our knowledge, no studies have correlated volume differences in the basolateral subregion of amygdala or VP with variations in reward or exercise reward. However, a reduction in the total reward-network volume, as well as right-side NA and left amygdala, is present in chronic alcoholic men (Makris et al. 2008). Also, young adult recreational marijuana users have greater gray matter density in left NA and left amygdala compared to non-using controls (Gilman et

al. 2014). Further studies reveal decreased NA volume in heroin-dependent patients (Seifert et al. 2015) and increased volume for children genetically at risk for obesity who show stronger reward-related responses to food cues (Rapuano et al. 2017). Lastly, in a study of elderly males (without cognitive impairment), self-reported exercise habits were associated with greater volume of the bilateral NA (Yamamoto et al. 2017).

We did not observe volumetric differences in BLA, NA, or VP in HR as compared with C mice, and therefore conclude that changes in the HR brain reward and motivational systems involving these particular regions are restricted to lower-level changes, e.g., neurotransmitter release and reception. Thompson (2017) found the HR ventral tegmental area tended to be larger in HR mice ($P = 0.0820$), and dopaminergic connections from this region may be influencing BLA, NA, and VP in a downstream fashion. Furthermore, mice with wheel access did not differ from sedentary mice for volumes of these regions. However, underlying effects of exercise on brain plasticity may be contributing to HR motivation in ways that do not affect volume but rather circuits and/or neuromodulation.

Additional conclusions and future directions

Overall, between the present study and Thompson (2017), no changes in regional neuronal density were uncovered. Therefore, neither wheel running (with the exception of dentate gyrus: see Rhodes et al. 2003b) nor selective

breeding appear to be driving neurogenesis in the nine investigated brain regions. Rather, the volumetric changes observed may be caused by hypertrophic (or hypotrophic in the case of HR PAG) effects on the neurons and/or glial cells of these regions. Future studies should test for differences in neuronal growth, glial activity, and microstructural changes in the HR brain, particularly in the red nucleus and hippocampus.

Considering all of the regions analyzed herein and by Thompson (2017), the VTA, NA, VP, and RN share a statistically non-significant pattern of wheel access decreasing the regional volume when compared to the groups without wheels (see Fig. 2.1, 2.4, 2.5, and Thompson's Fig 1.6). Given, the relationship between these four brain structures and dopamine transmission, this pattern is not easy to disregard. Running engages the brain's dopamine system for both locomotion and reward (Freed and Yamamoto 1985; Vargas-Pérez et al. 2004; Mathes et al. 2010; Greenwood et al. 2011; Novak et al. 2012; Zhu et al. 2016). Thus, further HR vs. C investigations of these four regions alongside dopamine release, reception, metabolism, and the genes regulating these processes is warranted.

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Table 2.1. Brain region volumes. Values for mixed models comparing linetype (HR vs. C), groups with ten weeks of wheel access (WhlAcc), and their interaction. Mini-muscle mice were identified at dissection (see Methods). Body mass, age, time spent in PFA prior to sectioning, and $\log_{10}$ time spent in the freezer before Nissl staining were included as covariates. HR mice had significantly larger red nucleus and hippocampus regions compared to C. See Fig. 2.1-2.5 for graphical representations of adjusted group means. P values < 0.05 are considered significant.

	Red Nucleus *(n = 67)*			Hippocampus *(n = 68)*			Basolateral Amygdala *(n = 68)*			Nucleus Accumbens *(n = 57)*			Ventral Pallidum *(n = 63)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	11.34	0.0151	1,6	11.83	0.0138	1,6	2.86	0.1417	1,6	0.91	0.3773	1,6	2.65	0.1546
Wheel Access	1,6	0.71	0.4324	1,6	0.01	0.9343	1,6	0.07	0.8072	1,6	2.67	0.1535	1,6	0.68	0.4398
Linetype*WhlAcc	1,6	0.02	0.8930	1,6	0.35	0.5779	1,6	0.85	0.3921	1,6	0.30	0.6054	1,6	0.09	0.7699
Mini-muscle	1,46	0.05	0.8276	1,47	0.05	0.8304	1,47	0.78	0.3819	1,36	0.38	0.5402	1,42	0.16	0.6902
Body Mass	1,46	2.49	0.1212	1,47	1.21	0.2779	1,47	2.91	0.0946	1,36	0.09	0.7604	1,42	0.44	0.5087
Age	1,46	9.69	0.0032	1,47	20.74	<.0001	1,47	13.86	0.0005	1,36	17.35	0.0002	1,42	2.13	0.1519
PFA Time	1,46	5.72	0.0209	1,47	13.46	0.0006	1,47	4.30	0.0435	1,36	9.11	0.0047	1,42	4.80	0.0340
log Freezer Time	1,46	0.13	0.7235	1,47	0.85	0.3620	1,47	7.55	0.0085	1,36	0.55	0.4645	1,42	0.24	0.6264

Table 2.2. Cell count/area. Values for mixed models comparing linetype (HR vs. C), groups with ten weeks of wheel access (WhlAcc), and their interaction. Mini-muscle mice were identified at dissection (see Methods). Age, time spent in PFA prior to sectioning, and $\log_{10}$ time spent in the freezer before Nissl staining were included as covariates. For all five brain regions, cell count/unit area did not differ between groups. P values < 0.05 are considered significant.

	Red Nucleus *(n = 68)*			Hippocampus *(n = 68)*			Basolateral Amygdala *(n = 68)*			Nucleus Accumbens *(n = 54)*			Ventral Pallidum *(n = 63)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	2.11	0.1963	1,6	0.07	0.7950	1,6	0.06	0.8124	1,6	0.09	0.7694	1,6	0.02	0.8806
Wheel Access	1,6	1.13	0.3293	1,6	0.22	0.6589	1,6	0.27	0.6188	1,6	0.34	0.5802	1,6	0.00	0.9538
Linetype*WhlAcc	1,6	1.00	0.3549	1,6	1.45	0.2736	1,6	1.73	0.2369	1,6	3.97	0.0935	1,6	2.21	0.1878
Mini-muscle	1,48	0.64	0.4265	1,48	0.72	0.3988	1,48	1.14	0.2912	1,34	0.48	0.4912	1,43	0.80	0.3756
Age	1,48	0.19	0.6643	1,48	0.05	0.8170	1,48	0.13	0.7236	1,34	12.92	0.0010	1,43	8.37	0.0060
PFA Time	1,48	0.02	0.8818	1,48	1.05	0.3107	1,48	0.93	0.3384	1,34	6.30	0.0170	1,43	2.36	0.1315
log Freezer Time	1,48	0.01	0.9049	1,48	2.69	0.1074	1,48	4.30	0.0435	1,34	0.26	0.6168	1,43	6.78	0.0126

APPENDIX B

Chapter 2 brain region tracing notes

General notes

Images were of coronally sectioned tissue. Placeholder images were taken when a section was missing or damaged, then later accounted for in Excel prior to analyses. Magnifications were determined based upon the highest level possible to distinguish the entire region of interest (often using other structural markers), but also have decent clarity for the ITCN.

Red nucleus (RN)

The bilateral RN was imaged at 40x magnification. Tracing of the RN was of the left nucleus in the images (I used the right side in rare instances of tissue damage so that I had at least an estimate of volume and cell count, however, if there seemed to be a lateralization imbalance, I would use the average of prior and subsequent left-side traces). The region traced included the parvicellular and magnocellular RN, while avoiding the more rostral prerubral field. Franklin & Paxinos (3rd edition) mouse brain atlas bregma -3.08 mm to -4.04 mm.

Hippocampus (HPC)

The left-side whole HPC was imaged at 20x magnification and traced. Franklin & Paxinos (3rd edition) mouse brain atlas bregma -0.94 mm to

approximately -3.88 mm. The (caudally located) ventral subiculum was excluded from the traces.

Basolateral amygdala (BLA)

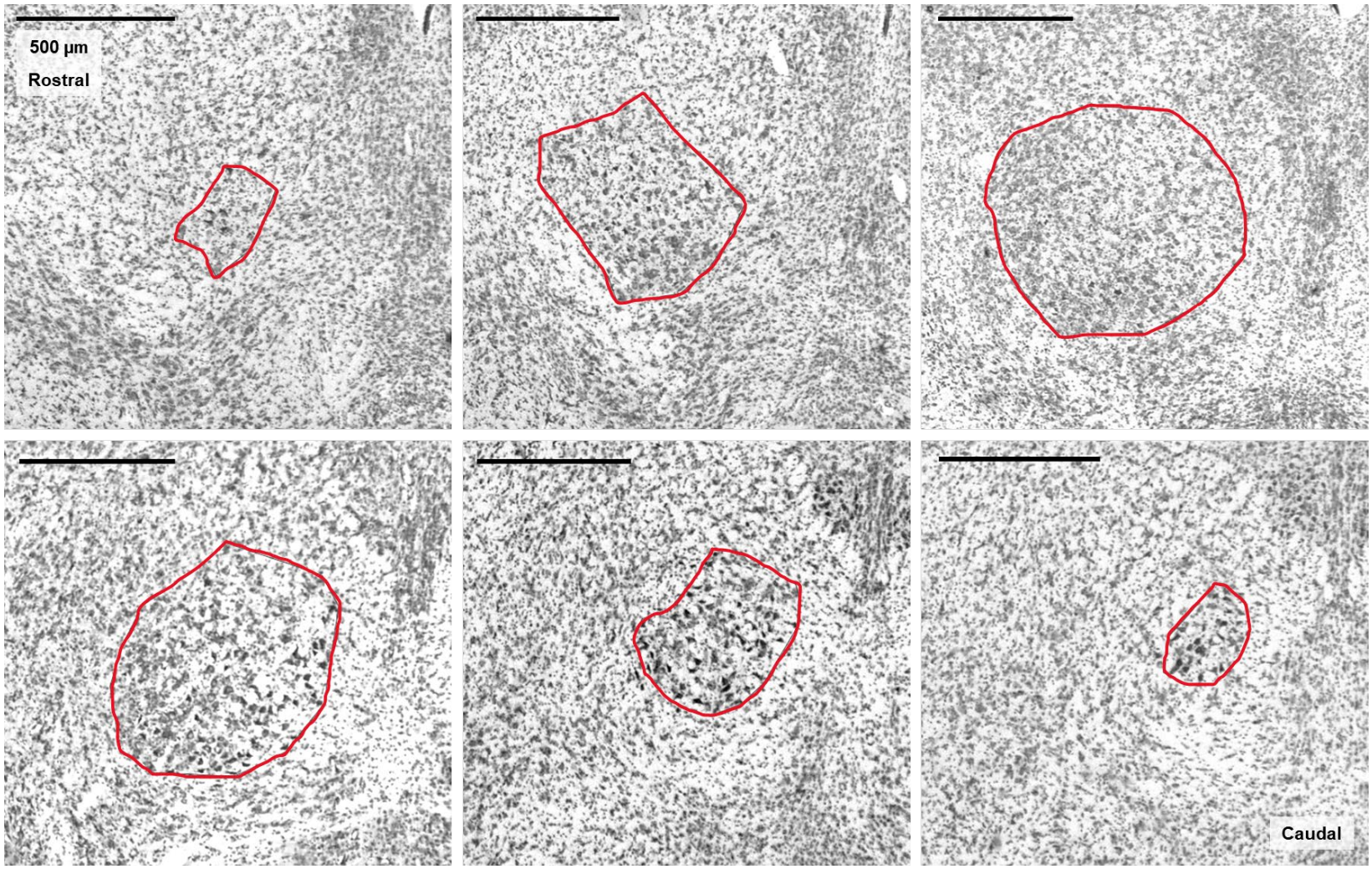
The left-side BLA was imaged at 24x magnification and traced, while trying to avoid the more dorsolateral amygdala and basomedial amygdala. Franklin & Paxinos (3rd edition) mouse brain atlas bregma -0.58 mm to approximately -2.80 mm.

Nucleus accumbens (NA)

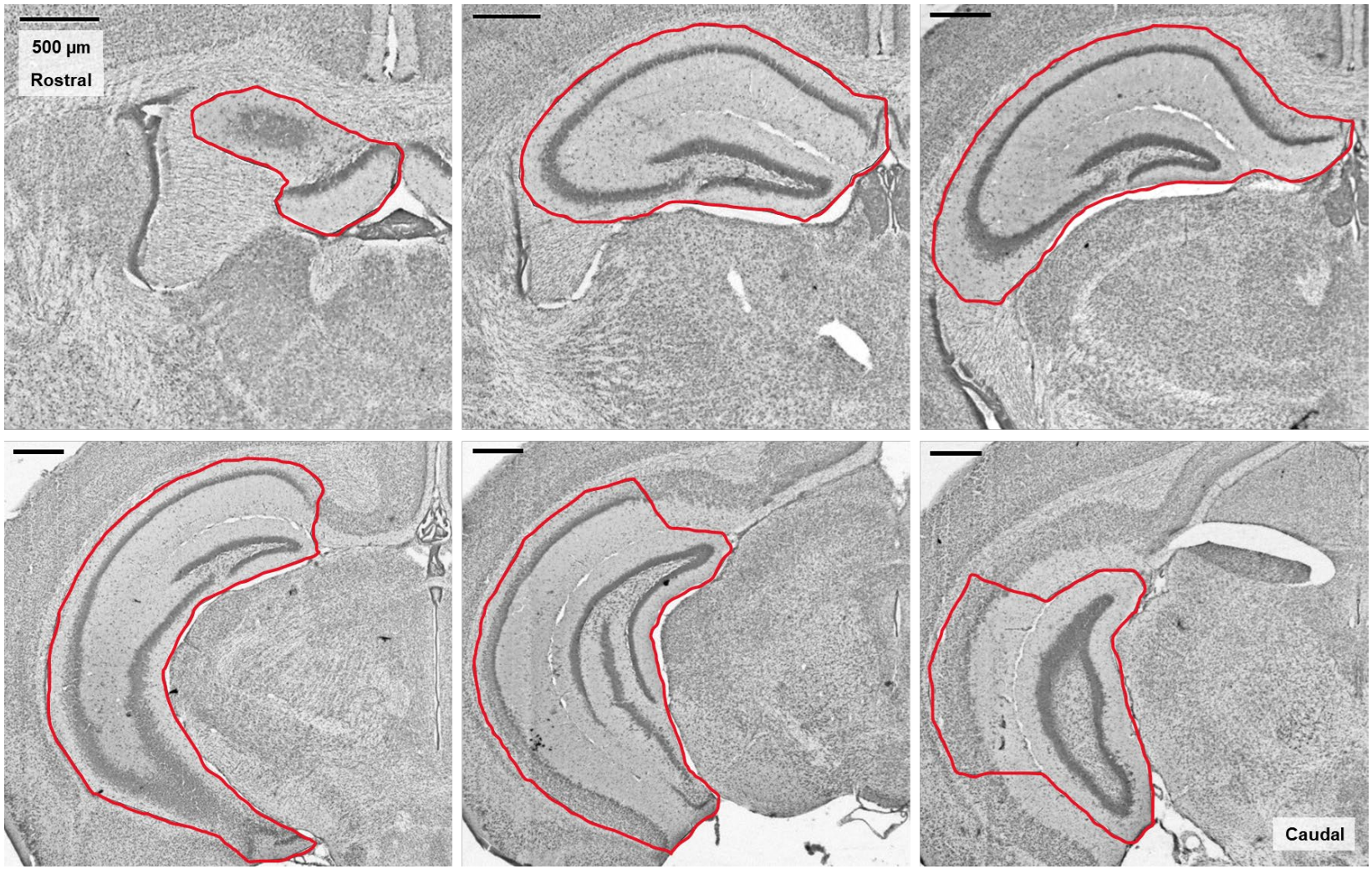
The left-side NA was imaged at 31x magnification and traced. Both NA core and shell were included. Franklin & Paxinos (3rd edition) mouse brain atlas bregma 1.94 mm to approximately 0.74 mm.

Ventral pallidum (VP)

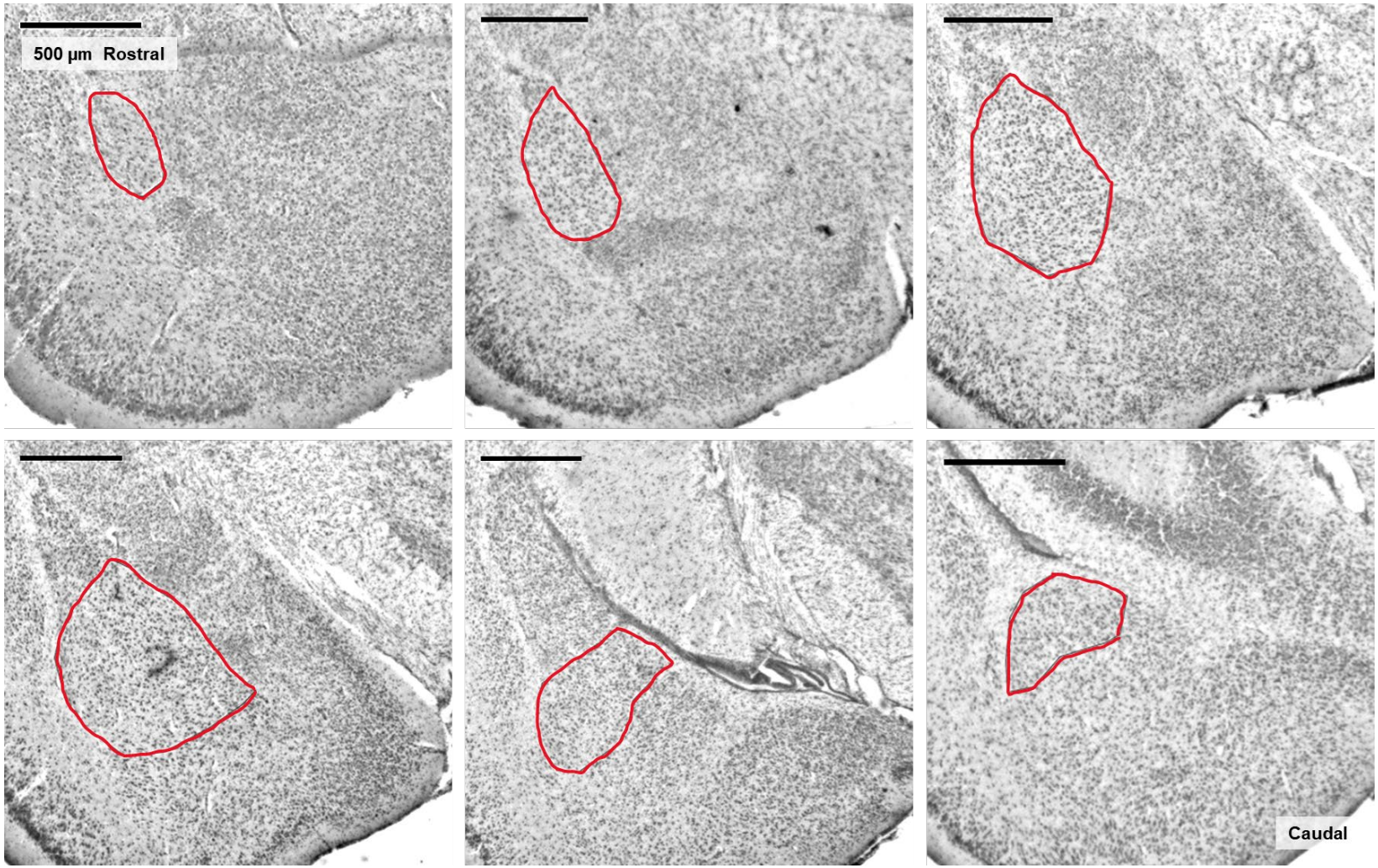
The left-side NA was imaged at 31x magnification and traced. The rostral VP that is composed of often several small strips or pieces was excluded, as this would not have been possible to trace with consistency. Franklin & Paxinos (3rd edition) mouse brain atlas bregma 0.74 mm to approximately -0.46 mm.



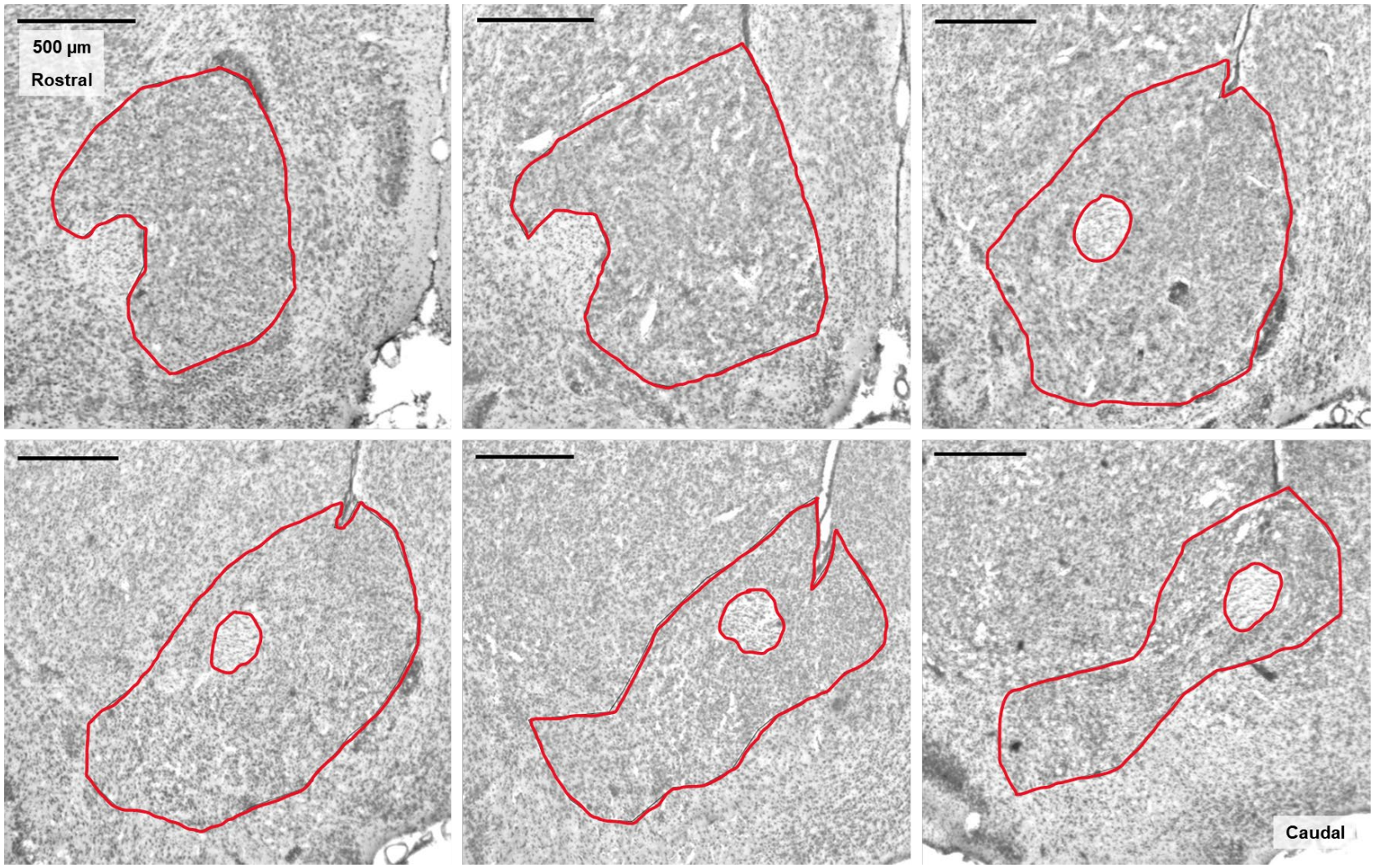
Appendix B Figure 1. Representative traces of left-side red nucleus at 40x magnification. Each row is rostral to caudal, with the top row rostral to the bottom row. The black line in each image is equal to 500µm.



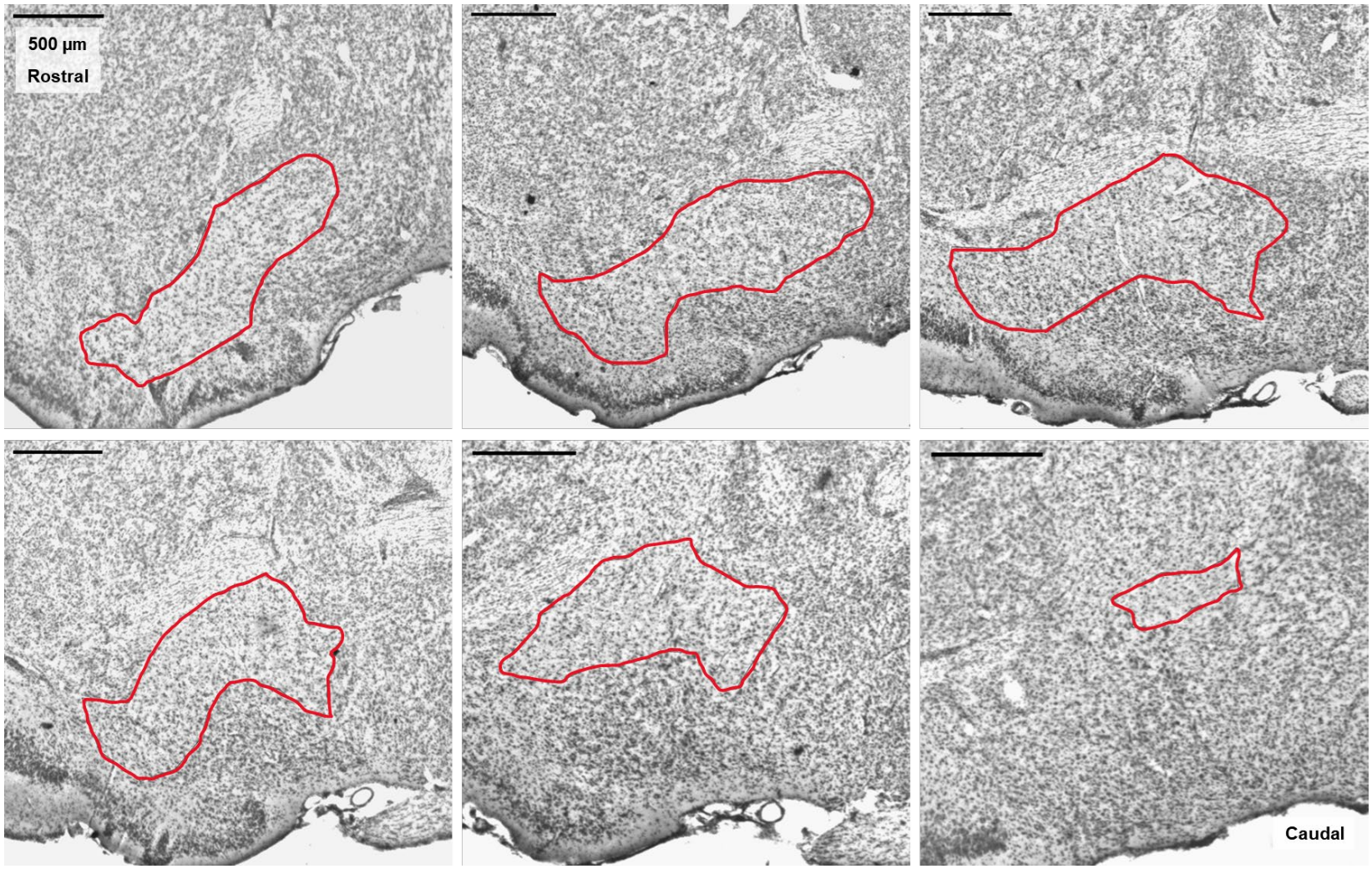
Appendix B Figure 2. Representative traces of left-side hippocampus at 20x magnification. Each row is rostral to caudal, with the top row rostral to the bottom row. The black line in each image is equal to 500µm.



Appendix B Figure 3. Representative traces of left-side basolateral amygdala at 24x magnification. Each row is rostral to caudal, with the top row rostral to the bottom row. The black line in each image is equal to 500µm.



Appendix B Figure 4. Representative traces of left-side nucleus accumbens at 31x magnification. Each row is rostral to caudal, with the top row rostral to the bottom row. The black line in each image is equal to 500µm.



Appendix B Figure 5. Representative traces of left-side ventral pallidum at 31x magnification. Each row is rostral to caudal, with the top row rostral to the bottom row. The black line in each image is equal to 500µm.

Chapter 2 Python 3.7.4 script for merging and transforming data output from ImageJ ITCN into .xlsx format.

In sum, ImageJ ITCN data was saved as separate .txt files for each mouse ID, then for each brain region, all of the .txt files were copied into the Input Directory folder. A separate folder was designated for the merged Python .txt file output. The output file was then imported into Excel for data organization prior to SAS analyses. Script is as follows:

```
from tkinter import filedialog
from tkinter import *
import os
import time
import sys


def browse_button_input():
    # Allow user to select a directory and store it in global var
    # called folder_path
    global input_path
    inputFolderName = filedialog.askdirectory()
    input_path.set(inputFolderName)
    print("Input Directory: " + inputFolderName)

def browse_button_output():
    # Allow user to select a directory and store it in global var
    # called folder_path
    global output_path
    outputFolderName = filedialog.askdirectory()
    output_path.set(outputFolderName)
    print("Output Directory: " + outputFolderName)

def formatData(file):
    fileName = input_path.get() + "/" + file

    inputLines=open(fileName, "r").readlines()

    DataEntryNumber = len(inputLines)/4

    NumEntries = int(DataEntryNumber)



    for i in range(0, NumEntries):
```

```
        f=','

        c=inputLines[4*i].split("_")
        if len(c)!=5:
            if len(c)!=6:
                print()
                print("Parse Error: file " + fileName)
                print("Input File Line " + str(4*i + 1) +" is incorrect. Check source file for
integrity")
                print()
                print("Expected Format: Image:
MouseID_XN_X.X_Region_L/R_Tracer.tif")
                print("or")
                print("Expected Format: Image: MouseID_XN_X.X_Region_Tracer.tif")
                print("Actual Format: " + inputLines[4*i])
                print()

        d=c[0].split(": ")

        c[0]=d[1]

        e=inputLines[4*i+1].split()

        #c[5]=c[5].replace('.tif\n', '')

        #if c[4] != 'L':
            #if c[4] != 'R':
                #c.insert(4,'N/A')

        #c.pop(5)


        print(len(c))
        if len(c)==5:
            c[4]=c[4].replace('.tif\n', '')
            c.insert(4, 'N/A')
        elif len(c)==6:
            c[5]=c[5].replace('.tif\n', '')

        c.extend([e[3],e[5]]) #e[3] and e[5] represent cell count and area of each
data entry


        g=f.join(c)
```

```
        finalOutput.append(g)
    return finalOutput

def saveData(dataString, saveName):
    savePath = output_path.get()+"/"+saveName+" Formatted Data.txt"
    of=open(savePath, "w+")

    for i in range(0, len(dataString)):
        of.write(dataString[i])
        of.write('\n')

def numberFromFile(file):
    fileName = input_path.get() + "/" + file

    inputLines=open(fileName, "r").readlines()
    c=inputLines[0].split("_")
    d=c[0].split(": ")
    return d[1]



print("Garland Lab Tools: ITCN Data Formatter v1.20")
print("Written by Shaarang Mitra")
print()
print()

print("enter Input Directory: ")

root = Tk()
input_path = StringVar()
lbl1 = Label(master=root,textvariable=input_path)
lbl1.grid(row=0, column=1)
button2 = Button(text="Enter Input Directory", command=browse_button_input)
button2.grid(row=0, column=3)

mainloop()

print("enter Output Directory: ")

root = Tk()
output_path = StringVar()
lbl1 = Label(master=root,textvariable=output_path)
```

```
lbl1.grid(row=0, column=1)
button2 = Button(text="Enter Output Directory",
command=browse_button_output)
button2.grid(row=0, column=3)

mainloop()

print("Individual output files, or combined?")
print("1. Individual")
print("2. Combined")
outputType = input("Enter Choice: ")

dirs = os.listdir(path=input_path.get())

finalOutput = []
dataToSave = []
mouseID = 0


if outputType == "1":
    for file in dirs:
        finalOutput = []
        mouseID = "" + numberFromFile(file)
        dataToSave = formatData(file)
        saveData(dataToSave, mouseID)
elif outputType == "2":
    for file in dirs:
        finalOutput = []
        mouseID = "" + numberFromFile(file)
        dataToSave.extend(formatData(file))
        saveData(dataToSave, "")

print()
print()
print("Operations complete, check for Parse Errors above")
time.sleep(200)
```

CHAPTER 3

Conditioned place preference for cocaine and methylphenidate in female mice from lines selectively bred for high voluntary wheel-running behavior

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Abstract

Behavioral addictions can come in many forms, including over-eating, gambling, and over-exercising. All addictions share a common mechanism involving activation of the natural reward circuit and reinforcement learning, but the extent to which motivation for natural and drug rewards share similar neurogenetic mechanisms remains unknown. A unique mouse genetic model in which four replicate lines of female mice were selectively bred (>76 generations) for high voluntary wheel running (High Runner or HR lines) alongside four non-selected control (C) lines were used to test the hypothesis that high motivation for exercise is associated with greater reward for cocaine (20 mg/kg) and methylphenidate (10 mg/kg) using the conditioned place preference (CPP) test. HR mice run ~3 times as many revolutions/day as C mice, but the extent to which they have increased motivation for other rewards is unknown. Both HR and C mice displayed significant CPP for cocaine and methylphenidate, but with no statistical difference between linetypes for either drug. Taken together, results suggest that selective breeding for increased voluntary running has modified the reward circuit in the brain in a way that increases motivation for running without affecting cocaine or methylphenidate reward.

Introduction

Predisposition for addiction to drugs and other natural rewards has a strong genetic component, and a great deal of effort has been devoted to trying to find the genes, molecular mechanisms, and specific neurological circuitry involved (Crabbe 2002; Brené et al. 2007; Foroud et al. 2010; Lüscher 2016; Koskela et al. 2017; Uhl et al. 2019). One of the central hypotheses in recent literature is that genes that increase motivation for one reward also increase motivation for other rewards, i.e., that the neurobiological pathways are not reward-specific, but rather generalize across rewards of varying types. This hypothesis is related to the concept of the "addictive personality," which posits that the particular reward is less important than the general tendency to become addicted to whatever reward is available. Support for this hypothesis can be found in human twin studies where genetic risk for addiction to one drug is strongly correlated with risk for addiction to other drugs (Kendler et al. 2003). Additional support comes from studies comparing different inbred strains of mice for behavioral responses to various drugs and natural reinforcers; these studies show strong genetic correlations (McGlacken et al. 1995; Belknap et al. 2008). The hypothesis that propensity for addiction generalizes across rewards is also supported by the idea that there is only one reward circuit in the brain, and both drugs of abuse and natural reinforcers, such as wheel running and feeding, activate the same circuit, which results in similar neuroadaptations (Kelley and Berridge 2002).

On the other hand, individuals vary in their emotional reactions to different types of rewards. For example, some people like the feeling of being high on cocaine or methylphenidate (Ritalin), while others do not (Volkow et al. 1999). Likewise, some people and animals derive pleasure from running (Sher 1998; Ekkekakis et al. 2005; Dishman et al. 2006; Brené et al. 2007; Garland et al. 2011; Novak et al. 2012) whereas others do not. In fact, exercise is even proposed to have addictive properties, as humans and rodents have shown signs of "withdrawal," including anxiety and depression, after being denied exercise (Baekeland 1970; Wichmann and Martin 1992; Aidman and Woollard 2003; Brené et al. 2007; Kanarek et al. 2009; Kolb et al. 2013; Jee 2016). The generality of neurogenetic predisposition for addiction across multiple types of drugs has been established for humans (Kendler et al. 2003), and for non-human animals (Ellenbroek et al. 2005; Belknap et al. 2008; Self and Staley Gottschalk 2010), and an extensive literature relates motivational circuits involved in drug and food rewards (McGlacken et al. 1995; Kelley and Berridge 2002). However, to the best of our knowledge, no studies have evaluated the extent to which genetic predisposition for exercise reward is associated with increased drug reward. Hence, the main purpose of the present study was to use a novel mouse model to test the hypothesis that selective breeding for a genetic predisposition for exercise results in a correlated response with respect to cocaine and methylphenidate rewards.

Over the past 26 years, we have maintained 4 replicate High Runner (HR) lines that have now been selectively bred for >90 generations for voluntary exercise on wheels, as compared with 4 non-selected Control (C) lines. Given a wheel attached to their home cage, HR mice run ~3x as many revolutions per day as C mice (Swallow et al. 1998; Careau et al. 2013). This type of artificial selection experiment offers a way to reliably alter phenotypes and provide results more consistent with the polygenic nature of complex traits (e.g., physical activity levels) than transgenic approaches targeting a single or a few genes (Swallow and Garland 2005; Garland and Rose 2009). Further, the 4-fold replication of both the HR and C lines reduces the chance of the experimental results being a consequence of random mutation and/or genetic drift (Henderson 1989). Human ethnic and racial diversity in physical activity is also modeled to some extent by the use of multiple lines (Svenson et al. 2007; Belcher et al. 2010).

The wheel-running literature suggests that exercise is a motivated or rewarding behavior for rodents and other animals, even in the wild (Sherwin 1998; Novak et al. 2012; Meijer and Robbers 2014). Voluntary exercise by rodents on wheels may also serve as a preclinical model of human voluntary exercise (Eikelboom 1999; Knab and Lightfoot 2010; Garland et al. 2011; Rosenfeld 2017; Lightfoot et al. 2018). In addition to changes in the physical ability for wheel running, several lines of evidence suggest that the HR mice have increased motivation to run on wheels compared to C mice (Rhodes and Garland 2003; Rhodes et al. 2003, 2005; Rhodes and Kawecki 2009; Garland et al.

2011). Evidence that the reward circuit has been altered in HR mice relative to C mice includes differential sensitivity to the locomotor-activating effects of dopamine re-uptake transporter blockers, cocaine, methylphenidate (Rhodes et al. 2001, 2005; Rhodes and Garland 2003), endocannabinoid agonist WIN 55,212-2, and antagonist rimonabant (Keeney et al. 2008, 2012), linetype differences in circulating endocannabinoid levels (Thompson et al. 2017), and notably, differential activation of the reward circuit during withdrawal from wheels (Rhodes et al. 2003; Saul et al. 2017). However, changes in dopamine signaling and activation of the reward circuit are shared across all forms of motivation and reward. For the HR mice, the extent to which the reward circuit has been altered in such a way as to specifically increase motivation for running and not motivation for other potentially rewarding stimuli remains unknown.

A widely used method to measure the rewarding (i.e., the attractive and motivational) value of a stimulus in animals is the conditioned place preference test (CPP). For reviews of the method, see Schechter and Calcagnetti (1993), Tzschentke (1998, 2007), Cunningham et al. (2006), Prus et al. (2009). In brief, CPP is a form of classical conditioning that involves an animal receiving repeated access to an appetitive (or aversive) stimulus in a particular context (Bardo and Bevins 2000; Cunningham et al. 2006). Within the same experiment, animals are exposed to a second context but without the stimulus of interest. Following repeated conditioning trials, a choice test is administered in which animals receive unrestricted access to both contexts in the absence of the stimulus. An

increase in time spent in the paired context relative to a control value is taken as evidence that the stimulus under investigation was rewarding (Bardo and Bevins 2000; Prus et al. 2009; Huston et al. 2013).

The goal of the present study was to determine whether HR mice display greater CPP than C mice to cocaine (Experiment 1) and methylphenidate (Experiment 2; see Appendix C for Experiment 3 wheel CPP). Based on the hypothesis that selection for voluntary wheel running generally increased motivation for reward, we hypothesized that HR mice would display greater CPP for both cocaine and methylphenidate.

Materials & Methods

1. Ethical approval

All procedures were approved by the University of California, Riverside, Institutional Animal Care and Use Committee, which follows the National Research Council Guide for the Care and Use of Laboratory Animals (revised 2011).

2. Experimental animals

We used 64 adult female mice for each of three experiments (n = 8 per each of 8 mouse lines in each experiment; each mouse was from a different family). Females were used because they generally run more than males (Swallow et al. 1998; Koteja et al. 1999; Rezende et al. 2006; Careau et al.

2013). Mice were from generations 77 and 82 of an ongoing, replicated, selective breeding experiment for high voluntary wheel-running behavior, as previously described (Swallow et al. 1998; Careau et al. 2013). The original progenitors were 224 outbred, genetically variable laboratory house mice (*Mus domesticus*) of the Hsd:ICR strain. After two generations of random mating, mice were randomly paired and assigned to 8 closed lines (10 pairs in each), with 4 replicated high-runner (HR) lines and 4 replicated control (C) lines. Beginning at ~6 weeks of age, each generation of mice are housed individually with access to a running wheel for 6 days. In the HR lines, the highest-running male and female from each family are selected as breeders based on the total number of revolutions run on days 5 and 6 of the 6-day test. In C lines, breeders are chosen without regard to wheel running. Within each line, the chosen breeders are randomly paired, avoiding sibling pairings.

3. General methods

The overall experimental timeline is shown in Figure 3.1. Mice were weaned at postnatal day 21 and housed 4 per cage, separated by sex and line, in standard mouse cages (27 x 17 x 12.5 cm) with *ad libitum* food and water. Two weeks prior to experimental procedures (~10 weeks of age), mice were transferred to a reverse photoperiod. Rooms were controlled for temperature (21±1 °C) and photoperiod (12:12 L:D) with lights on at 20:00 h and off at 08:00 h Pacific Standard Time. Red incandescent lamps were utilized so that

investigators could handle mice during the dark phase (Zombeck et al. 2008). After a week of acclimation to the reversed photoperiod, mice were switched to individual housing in standard home cages. At ~12 weeks of age, all mice underwent a 5-day preconditioning phase (as in Zombeck et al. 2008) followed by conditioning to a particular texture (conditioned stimulus, or CS) with cocaine (20 mg/kg; experiment 1; generation 77) or methylphenidate (10 mg/kg; experiment 2; generation 82) as the unconditioned stimuli (US). None of the mice had any access to wheels prior to or during these experiments.

We used the CPP method established by Christopher Cunningham's group for laboratory mice (Cunningham et al. 2006). The method uses a single chamber, with two different floor textures, referred to hereafter as GRID and HOLE to serve as the conditioned stimuli. No visual or olfactory cues were used. Mice are trained and tested during the dark phase of the light-dark cycle and in a dark room, as mice are nocturnal and more comfortable behaving in the dark. On the test day, the subjects are placed in the same size arena but with half the floor HOLE and half GRID texture. CPP is established by comparing percent time on the HOLE side (or GRID side, the math is equivalent since they sum to 1) between the HOLE-paired and GRID-paired mice. Exactly half the mice are conditioned to HOLE and half to GRID. Hence, it is a balanced, between-subjects design. Importantly, HOLE-paired and GRID-paired mice are treated identically with the only exception being which texture (HOLE or GRID) they received cocaine or saline on. Hence, whatever bias might occur at the time of testing, it

cannot confound interpretation of CPP since both groups would experience that same bias. Although not necessary for interpretation of CPP, in the current study we also administered a pretest before conditioning, so that we could refine the CPP measure by adjusting for individual differences in pre-test preferences. However, it is important to note that this does not correct for possible biases that develop after the pre-test, which again illustrates the advantage of the between-subjects method in which possible biases that develop after the pre-test do not confound interpretation of CPP.

The place conditioning chambers are black acrylic boxes (33 x 18 x 16 cm) with removable clear plastic tops to allow video-taping from above, following the general protocol of Zombeck et al. (2008), who also studied outbred Hsd:ICR mice. The floors are interchangeable and consist of three types of distinct textures: stainless steel sheets with 6.4 mm round holes (HOLE), grids composed of parallel stainless steel rods mounted 6.4 mm apart (GRID), and combination half-hole/half-grid (HOLE/GRID) floors (Appendix C Figure 1).

The CPP apparatuses were cleaned with warm, soapy water and dried between every usage. To absorb urine and collect feces, clean sheets of disposable paper or washable mats were placed underneath the chambers during each new test. Video recording was accomplished with overhead Logitech HD C525 Webcams, 1.6 meters above the ground, with 720p resolution at 30 frames per second, and later analyzed with automated software (see below).

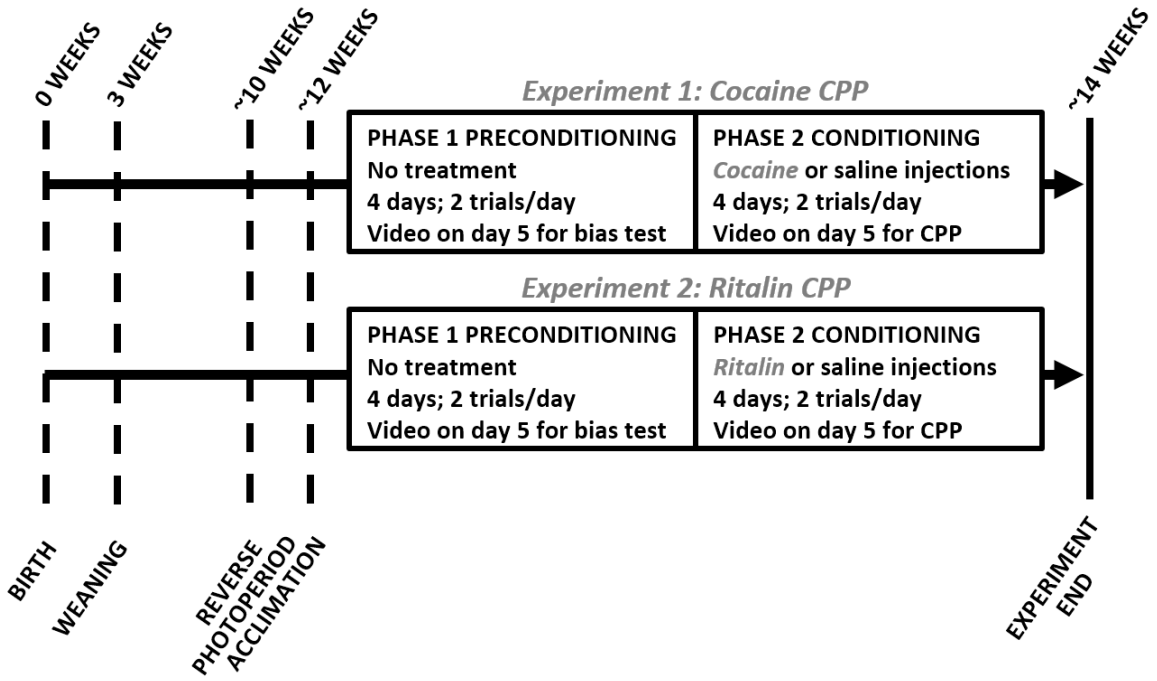

Figure 3.1. Simplified experimental timeline. Each experiment used 64 mice sampled from a distinct generation. For further detail, see text and Appendix C Figures 2 and 3.

4. CPP experiment 1 with cocaine reward

a. Preconditioning phase

We first determined individual preferences for the floor textures before giving the mice any reward. Body mass was recorded just prior to the first preconditioning exposure. On preconditioning days, animals were individually placed in the combination HOLE/GRID chambers for 30 minutes twice daily. The first trial of the day took place at ~09:00 h and ended before 13:00 h, ~1-4 h after lights off, when mice are most active (Girard et al. 2001; Malisch et al. 2009). The second trial of the day began at ~15:00 h and ended before 19:00 h, ~1 hour before lights came back on. This was done for 5 consecutive days without any

treatment, always on combination HOLE/GRID chambers. For the last two trials on preconditioning day 5, animals were video recorded to test for preexisting bias for HOLE versus GRID, i.e., in which side the animal spent more time (Appendix C Figure 2, left half).

b. Conditioning phase with cocaine as US

After two days of rest, the conditioning phase began. Mice were weighed prior to the first conditioning trial. Twice daily for 4 days, each animal was removed from its home cage, given a saline or cocaine intraperitoneal (i.p.) injection, and individually placed into its assigned CS, a CPP chamber for 30 minutes with a HOLE- or GRID-textured floor. Cocaine hydrochloride (Sigma Aldrich, St. Louis, MO) was administered via i.p injection at a dose of 20 mg/kg in an injection volume of 5 ml/kg (as in Zombeck et al. 2008). Each mouse received conditioning to only one texture: either HOLE (n=32) or GRID (n=32). If mice were placed in a HOLE chamber in the morning, then they were placed in a GRID chamber in the afternoon. This order was reversed for the next day, in order to counterbalance the time of day in which each mouse experienced each condition. Thus, during the four days of conditioning, each mouse experienced each condition twice in the morning and twice in the afternoon. For the last two trials on conditioning day 5, all animals were injected with saline, placed into the combination HOLE/GRID chambers, and video recorded (Appendix C Figure 2, right half).

5. CPP experiment 2 with methylphenidate reward

a. Preconditioning phase

Preconditioning in experiment 2 followed the protocol from experiment 1, except that mice alternated mornings and afternoons in either the HOLE or the GRID chambers for the first 4 days instead of using the combination HOLE/GRID chambers. This change was made because it allowed more rapid testing of the fairly large number of animals involved. We counterbalanced these conditions so that each mouse experienced each condition twice in the morning and twice in the afternoon. On day 5, both trials used the combination HOLE/GRID chambers to test for preexisting bias (Appendix C Figure 3, left half).

b. Conditioning phase with methylphenidate as US

Conditioning in experiment 2 was similar to experiment 1, except methylphenidate took the place of cocaine and was injected as 5 ml/kg methylphenidate hydrochloride (Sigma Aldrich, St. Louis, MO) dissolved in 0.9% saline, administered at a dose of 10 mg/kg (as in Tilley and Gu 2008; Appendix C Figure 3, right half).

6. Statistical analysis

We analyzed all recorded preconditioning and CPP videos in a semi-automated fashion with TopScan LITE video tracking software (Clever Sys, Inc.). Whenever the tracking software failed to accurately follow the animal, the videos

were manually analyzed in real-time with a stopwatch and tally system of the time spent in the HOLE versus GRID sides of the chamber. The video analyst was blind to conditioning treatment and linetype (HR vs. C).

Following numerous previous studies on these lines of mice, data were analyzed using nested analysis of covariance (ANCOVA) in SAS Procedure Mixed with replicate line nested within linetype (C or HR) as a random effect. In such a model, the effect of linetype is tested relative to the variation among replicate lines with 1 and 6 degrees of freedom. The effects of texture and the texture * linetype interaction are also tested with 1 and 6 d.f. The CPP data were statistically analyzed following the general strategy of Mustroph et al. (2016). First the average proportion time spent on the GRID side (or HOLE side; statistical results are equivalent because values sum to 1) during the CPP test was corrected for bias established during the pretest. Specifically, the proportion of time on the GRID side during the pretest (average of two trials) was subtracted from the proportion time spent on the GRID side during the CPP test (average of two trials). This adjusted proportion time on GRID (after subtracting pre-existing bias) was used as the outcome variable for CPP. This outcome was compared between mice that received drug on GRID versus drug on HOLE. A significant difference between the GRID-paired group vs. the HOLE-paired group ("Texture" in Figure 3.2) for corrected proportion time spent on GRID (or HOLE) establishes CPP. This was implemented in a linear model that included the texture the mice were conditioned on along with linetype (and line nested within linetype). Hence,

a main effect of texture indicates CPP collapsed across the two linetypes. A main effect of linetype indicates a difference in preference for GRID after correcting for pretest bias, collapsed across texture (HOLE and GRID). Hence, this effect is difficult to interpret because half the mice are conditioned to GRID and half to HOLE, so collapsing across them will display a large variance. Nevertheless, if the linetype effect is significant (in practice it never was), that means the linetypes differed in their bias for one texture that developed after the pretest and was unrelated to the drug conditioning. A significant interaction between linetype and texture is the key term that indicates whether one linetype conditioned more strongly to the reward than the other. Age and time of day had no effect when included as covariates, and thus were not included in the final models.

Previous authors have found that CPP is can be negatively related to distance traveled in the apparatus (Gremel and Cunningham 2007). Because of the potential that the HR mice would move more in the apparatus (which did in fact occur, as shown in Results section *3.4*), we wanted to evaluate whether there was a difference in CPP between HR and C after controlling for variation attributed to distance traveled. Therefore, we also analyzed models that included distance traveled and the interaction between distance and texture in the model to account for the possibility that CPP magnitude decreases with distance. These additional terms were never statistically significant (results not shown) and so the final models presented do not include them.

Data points with residual values >3 standard deviations above or below the mean were re-examined and excluded if deemed appropriate. For main effects and interactions, P-values below 0.05 were treated as statistically significant. The data that support the findings of this study are available from the corresponding author upon reasonable request.

Results

1. CPP experiment 1 with cocaine reward

a. Preconditioning bias test

Mice from generation 77 (N = 63) had a preexisting bias to spend more time on the GRID texture in the conditioning chambers as compared with the HOLE texture in both the selectively-bred HR lines (P = 0.0482) and the non-selected C lines (P = 0.0231) (Fig. 3.2A left panel). Animals spent between 55-60% of their time on GRID and 40-45% of their time on HOLE.

b. Conditioned place preference

Mice from both the HR and C lines conditioned to the reward-paired floor texture with cocaine (P = 0.0006), with no interaction between linetype and texture (P = 0.2521) (Fig. 3.2A center panel). Figure 3.2A (right panel) shows the majority of individuals falling above the 1:1 line and thus are considered to have developed a preference for their reward-paired texture.

2. CPP experiment 2 with methylphenidate reward

a. Preconditioning bias test

Mice from generation 82 (N = 61) from the non-selected C lines had a preexisting bias for GRID (P = 0.0025), but those from selectively-bred HR lines did not (P = 0.4972), resulting in a significant difference between the two linetypes (P = 0.0236) (Fig. 3.2B left panel). On average, C animals had a preference for GRID, spending approximately 65% of their time on GRID and 35% of their time on HOLE, while HR animals did not have this GRID preference (52% of time of GRID vs. 48% on HOLE).

b. Conditioned place preference

Both HR and C lines of mice conditioned to the reward-paired floor texture with methylphenidate (P = 0.0006), with stronger conditioning for mice paired with the HOLE texture, and no interaction between linetype and texture (P = 0.8494) (Fig. 3.2B center panel). Figure 3.2B (right panel) shows the majority of individuals falling above the 1:1 line and thus are considered to have developed a preference for their reward-paired texture.

3. Movement distances during the conditioned preference trials

During both studies, mice from the HR lines tended to move greater distances than C mice (P = 0.0773 and P = 0.0887 for cocaine and methylphenidate, respectively). Combining P values by Fisher's (1925) method,

the combined P = 0.0410. During the cocaine experiment, average distances moved per recorded CPP trial (log_{10} mm) were 4.695 $\pm$ 0.044 and 4.564 $\pm$ 0.044 for HR and C mice, respectively (SAS LS Means and standard errors). Values during the Ritalin trials were 4.622 $\pm$ 0.035 and 4.525 $\pm$ 0.033.

Discussion

The main finding of the study is that genetic predisposition for increased voluntary wheel running behavior in HR mice is not associated with increased cocaine or methylphenidate CPP. This implies that selective breeding resulted in specific changes in the natural reward circuit to cause increased motivation for wheel running, or reward received from running, rather than general addictive tendencies for multiple types of rewards. The exact molecular genetic changes in the reward circuit are still being worked out. The cumulative data suggest that some aspect of dopamine signaling has been altered (Rhodes and Garland 2003; Rhodes et al. 2003, 2005; Bronikowski et al. 2004; Mathes et al. 2010; Waters et al. 2013), but dopamine is involved in all salient behaviors (Berridge and Robinson 1998; Berridge and Kringelbach 2015). Future research is needed to disentangle the details of the changes in the dopamine reward circuit and other interacting circuits that have evolved in HR mice to produce specific increases in motivation for running without altering drug reward.

Cocaine and methylphenidate CPP

Both cocaine and methylphenidate produced robust CPP in the HR and C lines, consistent with a large and well-tested experimental foundation, particularly in mice (e.g., see Tzschentke 1998). We used a relatively high dosage of cocaine (20 mg/kg), following a previous study that used the same outbred strain of mice as was used to begin the HR selection experiment (Hsd:ICR) (Zombeck et al. 2008). This dose was also chosen because it produced differing locomotor responses in HR and C mice, suggesting the psychoactive effects may have been perceived differently (Rhodes et al. 2001). Similarly, at doses of 15 mg/kg and 30 mg/kg, methylphenidate reduced running of HR mice but increased running of C mice (Rhodes and Garland 2003). Moreover, 30 mg/kg methylphenidate caused a significantly higher activation of the c-fos gene in the medial frontal and sensory cortex of HR mice, indicating a greater amount of recent neuronal activity, as compared with C mice (Rhodes et al. 2005). We used a lower dose of methylphenidate (10 mg/kg) herein, but we hypothesized these behavioral and neuronal activation patterns indicated HR mice perceived methylphenidate differently from C within a broad dose range. Nonetheless, we found no statistically significant difference in cocaine or methylphenidate CPP between HR and C mice, suggesting that they do not perceive the rewarding effects of these drugs differently, and that the locomotor sensitivity and neuronal activity differences are not related to the behavioral reward response.

Strengths and limitations

One strength of our study is the CPP method we used, in which interpretation of CPP is not confounded by pre-existing biases in preference because of the balanced, between-subjects design (see Methods). Another major strength is the selective breeding model, which includes a total of 8 reproductively isolated lines (or strains), 4 of which were bred for increased running, while 4 unselected lines serve as multiple controls, maintained at the same time for more than 77 generations. The replicate lines allow us to empirically test the extent to which selection as opposed to random genetic drift contributes to phenotypic variation (Garland and Rose 2009).

The limitations of our study include that we only examined one dose of cocaine and one for methylphenidate. It is possible that we could have observed a different result had we explored other doses. On the other hand, cocaine CPP does not display a strong dose-response (Bardo et al. 1995); hence, it is unlikely we would have seen different results had we used other doses. Moreover, we explored two different drugs with similar pharmacological actions, and saw the same result -- no difference between genetic linetypes -- which increases the likelihood it would generalize to other doses. Another limitation is the possibility that a ceiling effect for CPP prevented us from observing genetic differences. This seems unlikely given that stronger CPP has been observed in inbred strains of mice for doses similar to those used here (Cunningham et al. 1999).

An additional limitation is our use of only one sex. Given the large number of animals to be tested (because we needed to measure mice from eight separate lines), we chose only one sex. We chose females because they generally run more than males in both the HR and control lines (Swallow et al. 1998; Koteja et al. 1999; Rezende et al. 2006; Careau et al. 2013). Use of females raises the possibility that the estrus cycle may have affected results. However, we note that mice from both HR and C lines did indeed condition to both drugs. We are aware of only one study that has specifically addressed estrus-cycle effects on CPP. In rats, Walker et al. (2002) found that vaginal lavage performed immediately prior to the conditioning session induced a significant preference. Given that vaginal lavage is generally required to score estrus stage, as we have done in previous studies (e.g. see Gomes et al. 2009), we were concerned that doing so in the present studies could affect results. Additionally, Korol et al. (2004) reported that learning strategy (place vs. response) varied across the estrous cycle in female rats. In any case, it would be of interest to include males in future studies of CPP in these lines of mice.

Conclusions

Overall, we conclude that selective breeding for increased voluntary wheel-running behavior does not alter perception of cocaine or methylphenidate reward in mice. This suggests that the neurogenetic underpinnings of high motivation for exercise are somewhat specific to exercise because they do not

transfer to these drug rewards. Of course, it may be the case that the HR mice find other drugs more rewarding than C mice, such as opioid, serotoninergic, or cannabinoid drugs. Future studies are needed to evaluate HR and C perceptions of other drug rewards as well as using gold standard operant conditioning methods in addition to CPP for measuring drug reward and motivation.

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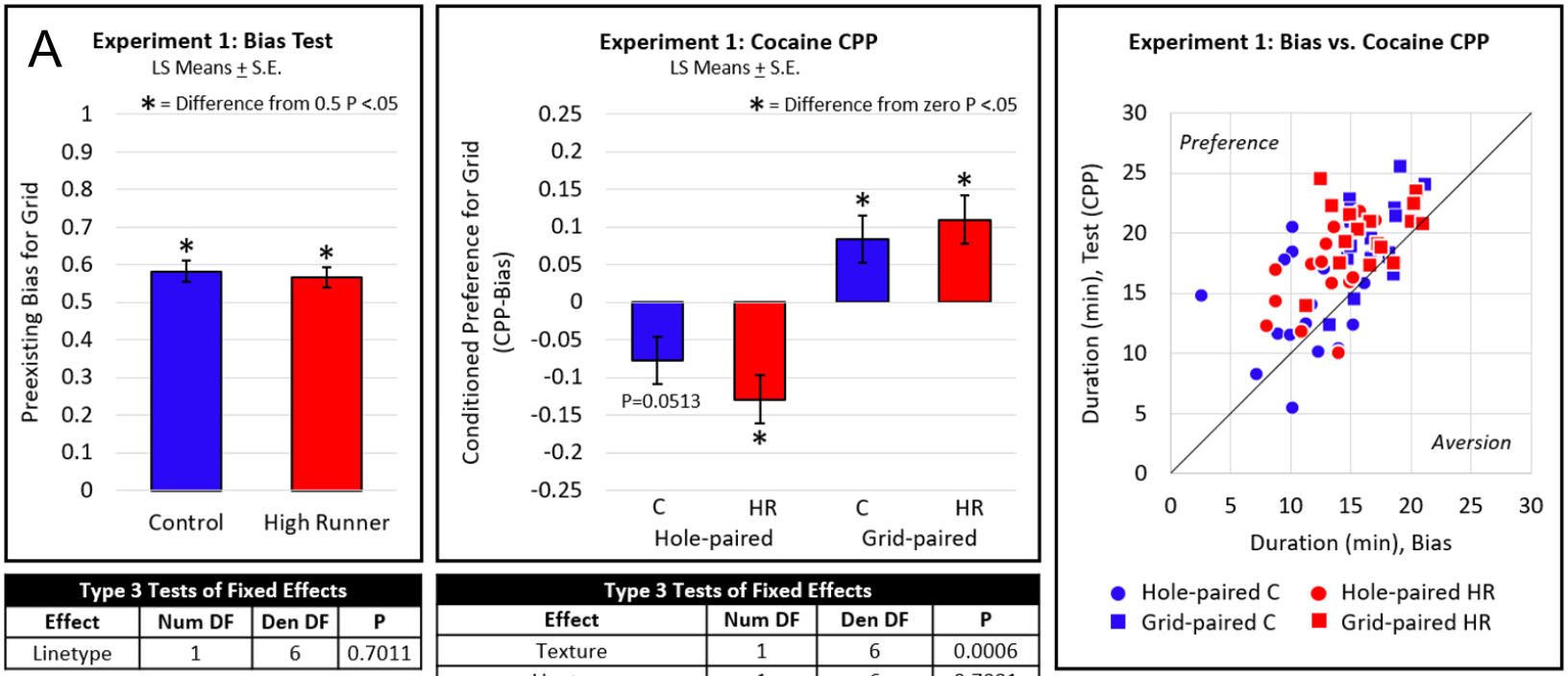
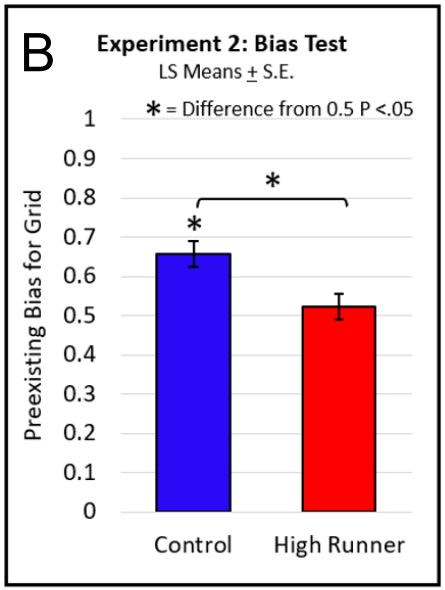


Type 3 Tests of Fixed Effects			
Effect	Num DF	Den DF	P
Linetype	1	6	0.7011

Type 3 Tests of Fixed Effects			
Effect	Num DF	Den DF	P
Texture	1	6	0.0006
Linetype	1	6	0.7081
Texture x Linetype	1	6	0.2521

Figure 3.2. Preexisting bias and conditioned place preferences for Experiment 1, cocaine. A) (Left panel) Mice (generation 77, N = 63, 1 outlier removed) from both the selectively bred High Runner lines (P = 0.0482) and the non-selected Control lines (P = 0.0231) had a preexisting bias to spend more time on the GRID as compared with the HOLE. (Center panel) Mice from both HR and C lines conditioned to the reward-paired floor texture with cocaine (P = 0.0006), with no effect of linetype (P = 0.7081) and no interaction between linetype and texture (P = 0.2521). (Right panel) Duration (min) spent on HOLE floor (for HOLE-paired mice) or GRID floor (for GRID-paired mice) during cocaine CPP plotted against baseline duration (min) spent on those floor types during bias testing. The one-to-one line is shown.



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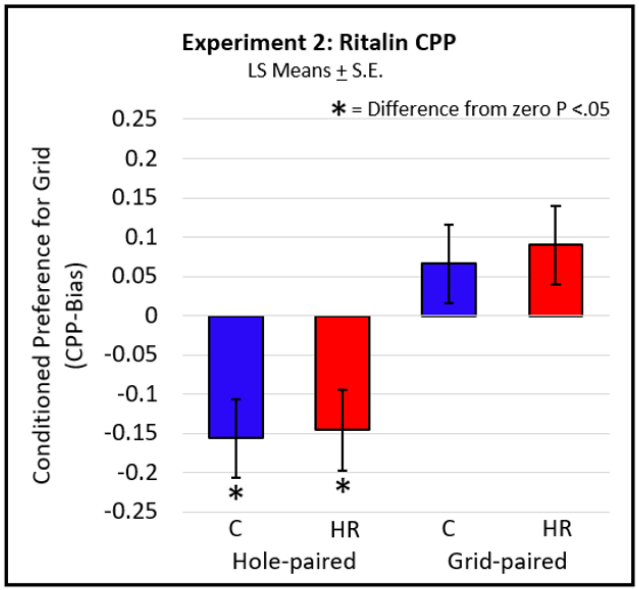

Type 3 Tests of Fixed Effects			
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Linetype	1	6	0.7984
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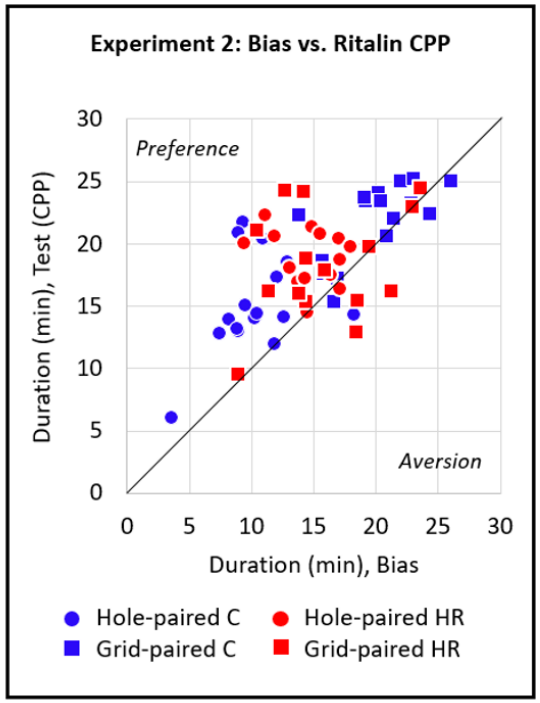

Figure 3.2. Cont. Preexisting bias and conditioned place preferences for Experiment 2, methylphenidate. B) (Left panel) In this experiment (generation 82, N = 61), individuals from the C lines had a preexisting bias for GRID (P = 0.0025), but those from HR lines did not (P = 0.4972), resulting in a significant difference between the two linetypes (P 0.0236). (Center panel) Mice from both HR and C lines conditioned to the reward-paired floor texture with methylphenidate (P = 0.0006), with stronger conditioning for the HOLE-paired texture, but no effect of linetype (P = 0.7984) and no interaction between linetype and texture (P = 0.8494). (Right panel) Same as A for the methylphenidate experiment.

APPENDIX C

The content herein contains an extended description of biased, unbiased, and mixed conditioned place preference (CPP) methodology, followed by the Materials & Methods, Results, and Discussion for my third CPP experiment using wheel access for a reward, which was not included in the publication (Schmill et al. 2021). Author contribution is the same as Schmill et al. (2021).

Materials & Methods

A "biased" protocol uses a baseline preference test to determine the least-preferred context, and then pairs that context with the reward. Thus, to be considered as having formed a CPP, an animal must prefer the reward-paired context enough to overcome their innate dislike for that context. In an "unbiased" protocol, preexisting bias is not measured, but instead subjects are conditioned to two contexts using a balanced design of reward/drug and vehicle between each context (Schechter and Calcagnetti 1993; Tzschentke 1998; Cunningham et al. 2003). A "mixed" protocol uses both a baseline preference test and a balanced design to account for any bias toward either context (Schechter and Calcagnetti 1993). Our experiments used the "mixed" method: each mouse was well habituated to the conditioning chambers prior to a preconditioning bias test, followed by a balanced assignment of contexts. In addition, the bias determined for each mouse was subtracted from their final preference score prior to statistical analyses.

CPP experiment 3 with wheel access reward

Experiment 3 followed the general design of experiments 1 (cocaine CPP) and 2 (methylphenidate CPP) but used wheel access as the unconditioned stimulus (US) and thus prompted some changes to the methodology. A detailed experimental timeline is given in Appendix C Figure 4.

a. Preconditioning phase

Preconditioning occurred for 5 days just as in experiment 2. No wheel access was provided at this time. On day 5, both trials used the combination HOLE/GRID chambers to test for preexisting bias (Appendix C Figure 4, left half).

b. Conditioning phase with wheel access as US

After two days of rest, before lights-on at 20:00 h mice were weighed, and moved into individual home cages that were connected via a short tunnel to Standard Wahman-type wheels (1.12 m circumference; Lafayette Instruments, Lafayette, IN; Swallow et al. 1998). Wheel revolution data were recorded every minute for ~23 hours and analyzed as total revolutions, number of 1-minute intervals with at least one revolution, mean running speed (total revolutions/active intervals), and maximum speed (highest 1-minute interval).

For the first 12 h of the conditioning phase, all mice were provided access to the wheels. At 20:00 h on following days, we alternatively restricted or granted wheel access for every mouse. Peak wheel running lasts for only a few hours,

which necessitated a design that spread conditioning over an 8-day period, rather than the 4 days used in experiments 1 and 2.

Time of peak wheel-running begins ~1 h after lights off (Girard et al. 2001; Malisch et al. 2008), and we presume that peak running coincides with the maximum experienced reward. Mice were taken from their home cages (with or without wheel access provided) during the time of peak activity to condition them most effectively to the floor textures in the CPP chambers. Conditioning trials occurred once daily, with the first mice placed in CPP chambers at 09:00 h, 1 h after lights off. Conditioning trials ended ~4 h later.

During each conditioning trial, mice were taken from their cages in the wheel room, transferred (in darkness) to the experimental room, placed in CPP chambers with either HOLE or GRID for 30 minutes, then immediately returned to their cages. Thus, if any rewarding effects, i.e., hedonic impact or development of incentive salience (Robinson and Berridge 1993), occurred during wheel running, they could be experienced both immediately before and after the 30 minutes in the conditioning chamber. Each mouse received conditioning to only one texture: either HOLE (n=32) or GRID (n=32). On days 9 and 10 of the conditioning phase, wheel access was restricted, and mice were placed into the combination HOLE/GRID chambers, where video recording began ~1 h after lights off (Appendix C Figure 4, right half).

Results

CPP experiment 3 with wheel access reward

a. Preconditioning bias test

Mice from generation 79 (N = 63) had a preexisting bias for spending more time on the GRID texture in the conditioning chambers as compared with the HOLE texture (HR P = 0.0231, C P = 0.0087) (Appendix C Figure 5, left panel). Similar to experiment 1 (cocaine CPP), animals spent between 55-60% of their time on GRID and 40-45% on HOLE.

b. Conditioned place preference

Mice from both HR and C lines did not condition to the reward-paired floor texture when alternating days of wheel access was provided as the US (P = 0.7999) (Appendix C Figure 5, center panel), with no interaction between linetype and texture (P = 0.8530). Appendix C Figure 5 (right panel) shows that about half of the mice fall above and half below the 1:1 line, signifying that the mice developed neither a preference nor an aversion to their paired texture.

Discussion

Negative results for wheel running CPP

The lack of success at demonstrating CPP to running does not imply that running is not rewarding in mice. There is abundant evidence that wheel running is rewarding for rodents (Kagan and Berkun 1954; Belke and Wagner 2005;

Brené et al. 2007; Novak et al. 2012; Raichlen et al. 2012; Heyse et al. 2015). Often, CPP is weak or difficult to establish for natural reinforcers such as food and running (Zombeck et al. 2008). Obtaining CPP to running is challenging because of the difficulty in temporally pairing the running reward with the standard CPP contextual stimulus (i.e., access to a wheel within a CPP chamber). The effects of drug rewards are experienced differently in the CPP apparatus: with the onset of the psychoactive effects of drugs coming while the animal is in the specific context, the animal learns that the context is predictive of the reward. Hence, during the testing phase, when the animal is placed in the context after receiving a saline (vehicle) injection, it expects it will experience the rewarding effects of the drug. This contrasts with natural rewards such as food and wheel running, in which the animal may know that if the food or wheel is not there, they will not experience the reward, even if they are hungry or in exercise withdrawal.

Nevertheless, several studies have shown CPP for exercise on wheels using similar procedures as used herein (Antoniadis et al. 2000; Lett et al. 2000, 2001b,a, 2002; Ralph et al. 2002; Belke and Wagner 2005; Greenwood et al. 2011; Trost and Hauber 2014; Basso and Morrell 2015; Fernandes et al. 2015; Herrera et al. 2016; see Appendix C Table 1 for a summary of these studies). The explanation(s) for the discrepancies are not clear. As compared with previous studies (Appendix C Table 1), we chose to use a longer period of wheel access (~23 hours) for each conditioning trial to better match our selective

breeding procedure. Placement into the chambers occurred approximately 12-15 hours after the beginning of wheel access, timed to coincide with the nightly window of peak wheel-running activity (Girard et al. 2001; Malisch et al. 2008).

To our knowledge, 12 published studies and one thesis project have used wheel access as a rewarding stimulus during CPP studies using rats, mice, or hamsters (Appendix C Table 1). We deemed a "protocol" as any unique CPP test done with a specific cohort of animals with wheel access included and a "success" as any of these protocols that yielded a significant CPP. With these criteria, successful conditioning was reported for at least one protocol in all 12 published studies listed in Appendix C Table 1, although not all attempted protocols produced conditioning. Tallying across these previous studies yielded 46 total protocols, of which ~72% were successful. We wished to know if any of the unsuccessful CPP protocols had factors in common with our study or with each other.

Eight of the previous studies in Appendix C Table 1 reported at least one protocol that did not result in CPP formation. The 13 unsuccessful protocols included experiments that involved fragmented circadian rhythms (Antoniadis et al. 2000), administration of high or low dose naloxone (mu-opioid receptor antagonist; Lett et al. 2001a), a 2- or 22-hour wheel confinement followed by a 30-min delay before placement in a conditioning chamber (Lett et al. 2002), testing for preference at a different circadian time than when animals were conditioned (Ralph et al. 2002), only two weeks versus six weeks of wheel

conditioning (Greenwood et al. 2011), intra-VTA leptin treatment during the probe trial (Fernandes et al. 2015), two hours of voluntary running for conditioning over 10 days (see Figure 5 C in Herrera et al. 2016), and both control and activity anorexic mice conditioned to a chamber holding a running wheel (Plaksiy 2017).

Several points emerge from consideration of the studies in Appendix C Table 1. First, the way in which preexisting bias was considered did not seem to correlate with likelihood of protocol success. Second, two of the unsuccessful protocols used overnight access to wheels like us, but so did multiple successful protocols. Third, eight days (and mice only had access to wheels on four of those days) might be too few days to form a consistent preference for a wheel context: 10 of the 13 unsuccessful protocols used eight-day durations (as used here) whereas successful protocols ranged from eight days to six weeks. Greenwood et al. (2011) also highlight this potential need for longer experimental duration. Their study used overnight access to wheels, as in our study, and found a CPP after six weeks, but not two weeks, of conditioning.

Fourth, sex might also affect the likelihood of forming a CPP (Becker and Koob 2016), although no sex difference was reported by Basso and Morrell (2015) with rats in their wheel access CPP study, or by Cunningham and Shields (2018) in an alcohol CPP study using two strains of inbred mice. Plaksiy (2017) was the only other wheel study to use female mice, and they did not obtain a CPP for wheels. Their study used three experimental groups: control, food restricted, and activity anorexia mice. Animals from each group were either

conditioned to a chamber holding a running wheel or dish of food. A preference was not formed for the wheel context in either their control or activity anorexia groups. More studies are needed to determine if female mice are inherently less likely to form a CPP with wheels, as compared with males. The lack of wheel CPP studies with females is noteworthy, given that female rodents generally run more on wheels than do males (Schnur and Barela 1984; Konhilas et al. 2004; Lightfoot et al. 2004; Lightfoot 2008; Meek et al. 2009; McMullan et al. 2016; Rosenfeld 2017) and female mice often have higher exercise capacity than males (Konhilas et al. 2004; Oydanich et al. 2019).

Fifth, and an item that stands out in our study and Plaksiy (2017) is the probe trial duration of 30 minutes. All other experiments listed in Table 1 used 10 or 15 minute trials that were analyzed to determine CPP, with the exception of Basso and Morrell (2015), who used 60 minutes and did achieve a CPP. A sixth key difference is that seven of the Table 1 studies provided wheel access for days or weeks before the rodents experienced a conditioning chamber. Such a design is aimed at habituation; however, prior exposure to exercise may confound CPP formation with exercise training effects (physical conditioning). Those seven studies applied the early wheel treatment to all animals, with no control groups lacking prior wheel access (Lett et al. 2001b,a, 2002; Belke and Wagner 2005; Trost and Hauber 2014; Fernandes et al. 2015; Herrera et al. 2016). Finally, no other wheel conditioning study has used mice of similar origin to the ones used here, i.e., ICR or CD1. General differences in reward systems

might be attributable to lineage differences that have little to do with the experimental design.

In summary, we cannot with certainty identify an obvious methodological feature of experiment 3 that would account for a failure to achieve a CPP with wheel access as the rewarding stimulus. In future studies it would be of interest to use both sexes, conditioning chambers with more sensory cues, a longer period of wheel access, as with the six weeks of Greenwood et al. (2011), a shorter probe trial duration, or a 2-hour aftereffect paradigm (rather than overnight).

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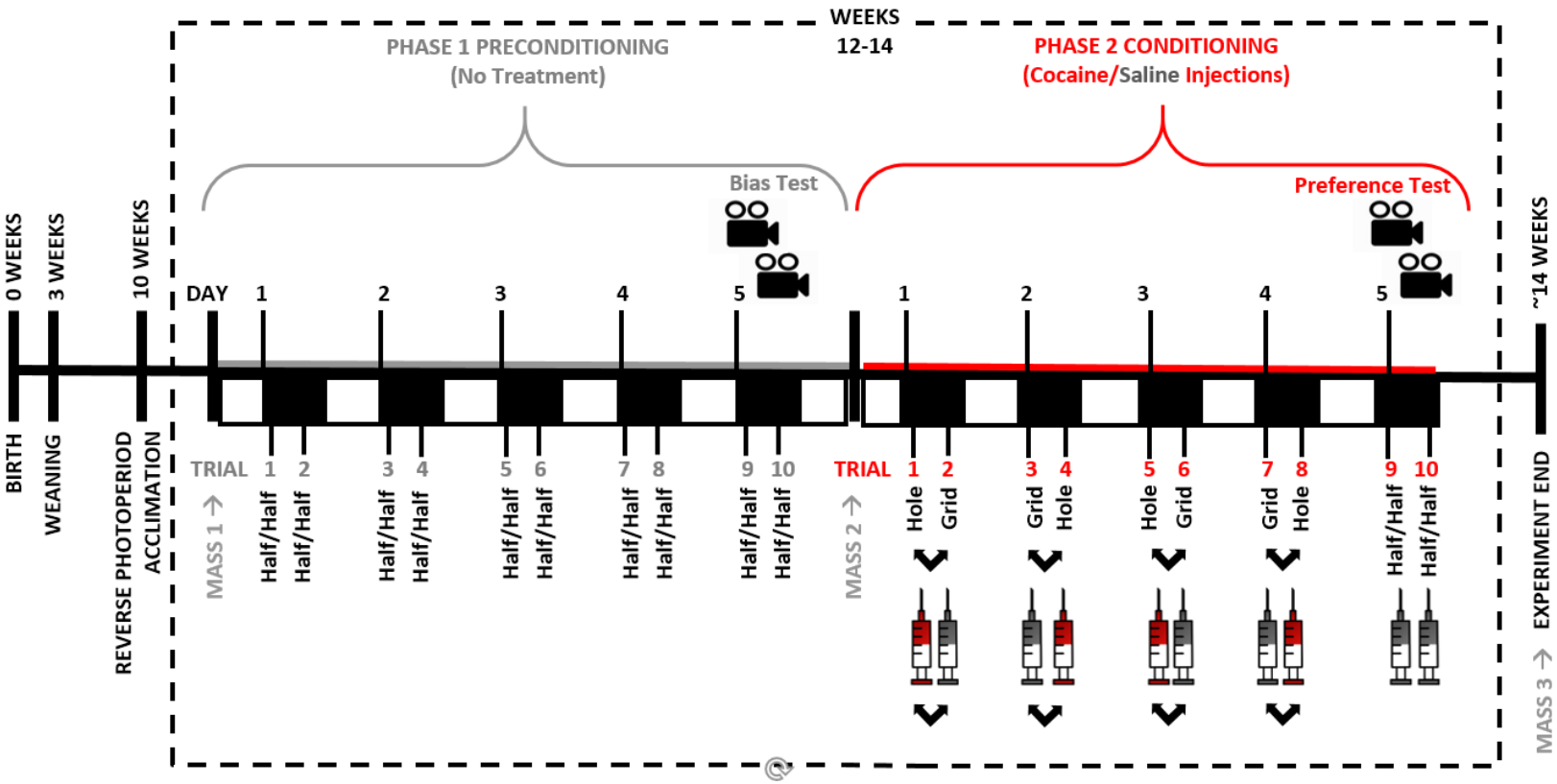
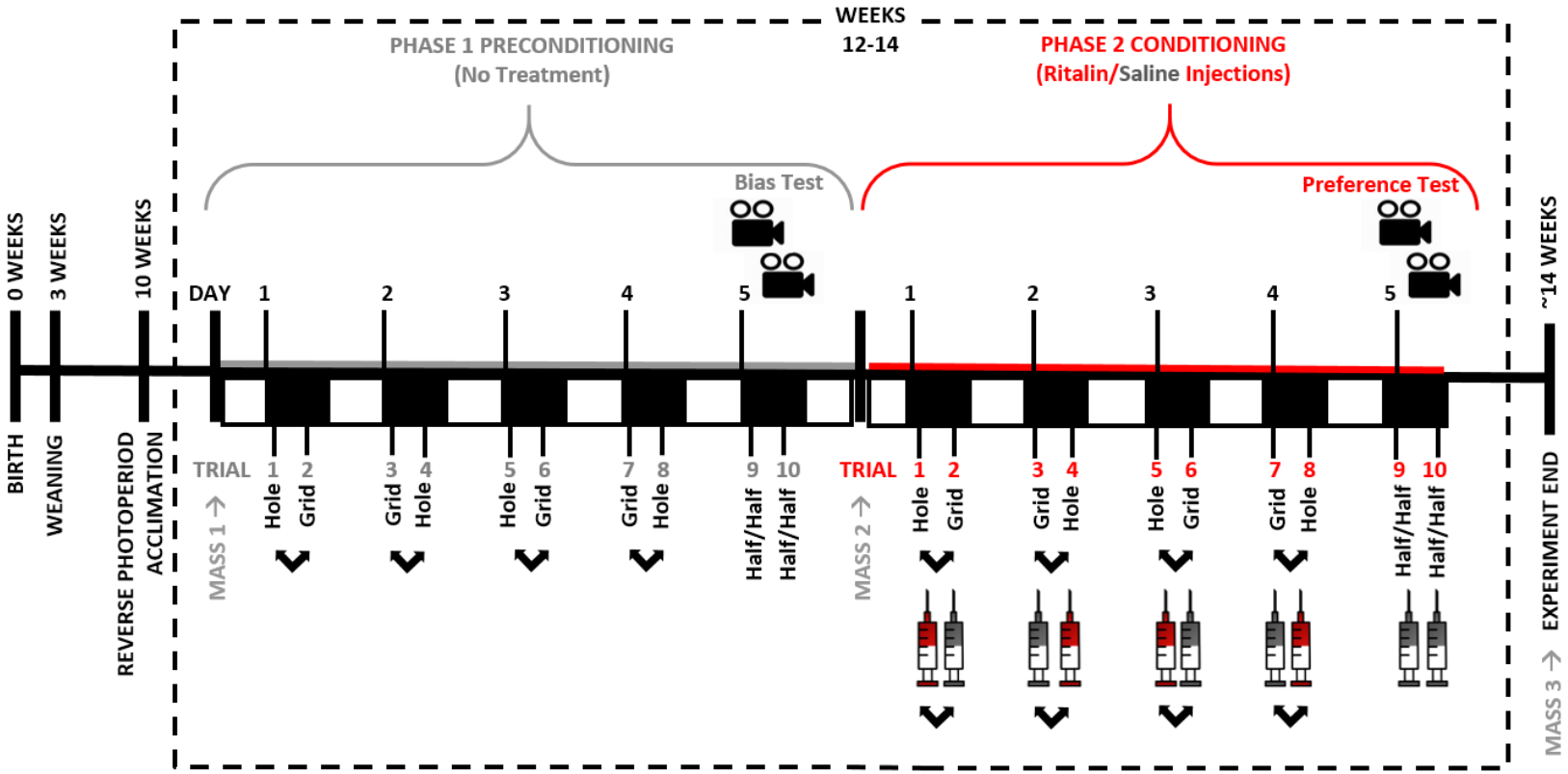
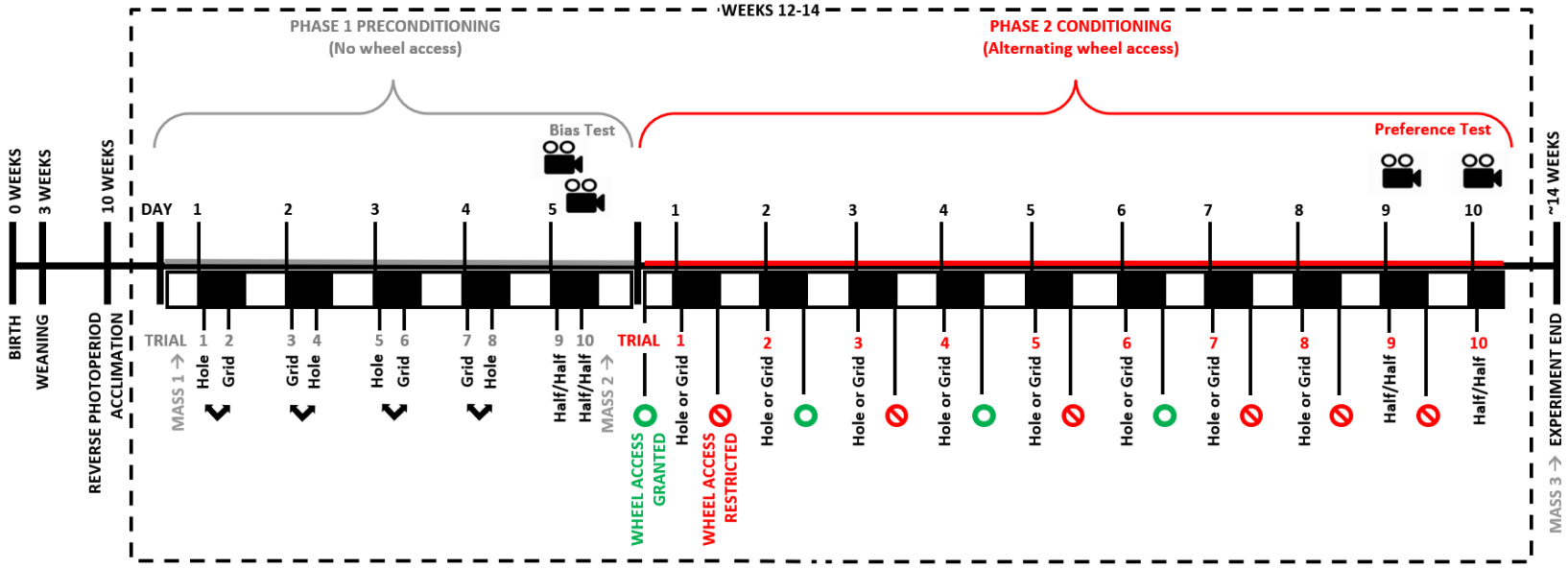
Appendix C Figure 1. Conditioned place preference (CPP) chambers used in all experiments. Chambers are same from Zombeck (2008). The interchangeable stainless-steel bottoms have holes or a grid of rods for conditioning, or a combination of hole/grid for preference testing. The walls are black, and the lids are clear plexiglass to allow for videotaping from above.



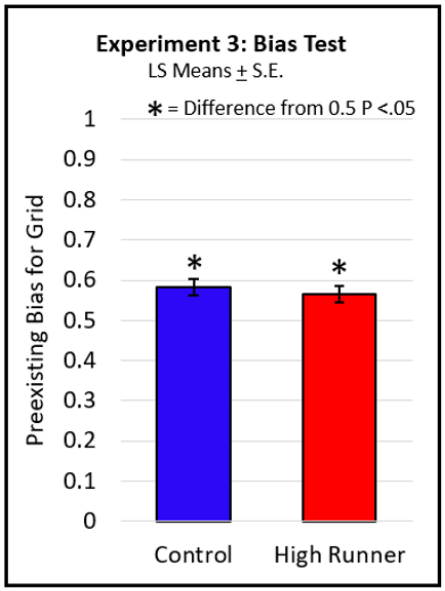
Appendix C Figure 2. Timeline for conditioned place preference (CPP) with cocaine as the US. Phase 1 began at approximately postnatal week 12 and lasted 5 days. Mice were placed individually into combination hole/grid (Half/Half) CPP chambers twice per day for 30 min, for 4 days and video recorded in combination hole/grid chambers twice on day 5. Next, Phase 2 conditioning lasted 4 days. Mice were placed individually into CPP chambers twice per day for 30 min, with half of the subjects injected with cocaine and paired with a hole or grid floor, followed by the opposite for the next trial and injected with saline. Mice were given saline and video recorded twice on combination hole/grid floors on day 5.



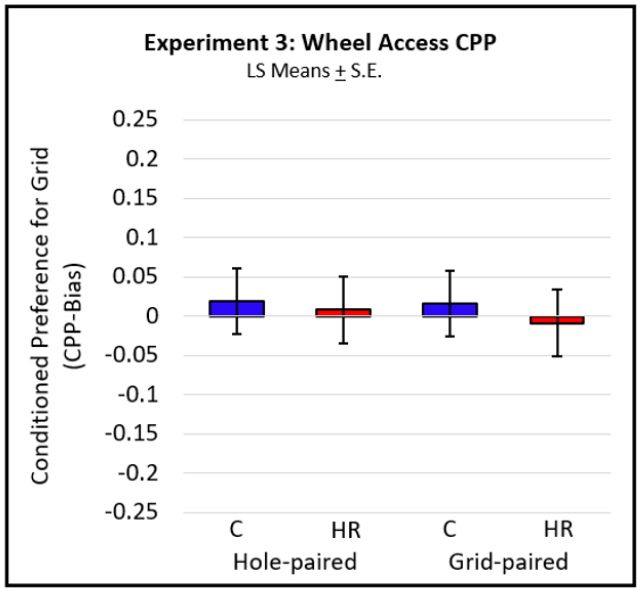
Appendix C Figure 3. Timeline for conditioned place preference (CPP) with Ritalin as the US. Phase 1 began at approximately postnatal week 12 and lasted 5 days. Mice were placed individually into combination hole or grid CPP chambers twice per day for 30 min, for 4 days and video recorded in combination hole/grid chambers twice on day 5. Next, Phase 2 conditioning lasted 4 days. Mice were placed individually into CPP chambers twice per day for 30 min, with half of the subjects injected with Ritalin and paired with a hole or grid floor, followed by the opposite for the next trial and injected with saline. Mice were given saline and video recorded twice on combination hole/grid floors on day 5.



Appendix C Figure 4. Timeline for conditioned place preference (CPP) with wheel access as the conditioning agent**.** Phase 1 began at approximately postnatal week 12 and lasted 5 days. Mice were placed individually into CPP chambers twice per day ~1 h after lights shut off, for 30 min, switching between a hole or grid floor for 4 days and video recorded on combination hole/grid floors twice on day 5. Phase 2 lasted 10 days. Mice were placed individually into CPP chambers once per day ~ 1 h after lights shut off, for 30 min, with each half of the subjects receiving a hole or grid floor, followed by the opposite the next day. Wheel access was granted or restricted on alternating days (1-8) and mice were video recorded on combination hole/grid floors on days 9 and 10.



Type 3 Tests of Fixed Effects			
Effect	Num DF	Den DF	P
Linetype	1	6	0.5930



Type 3 Tests of Fixed Effects			
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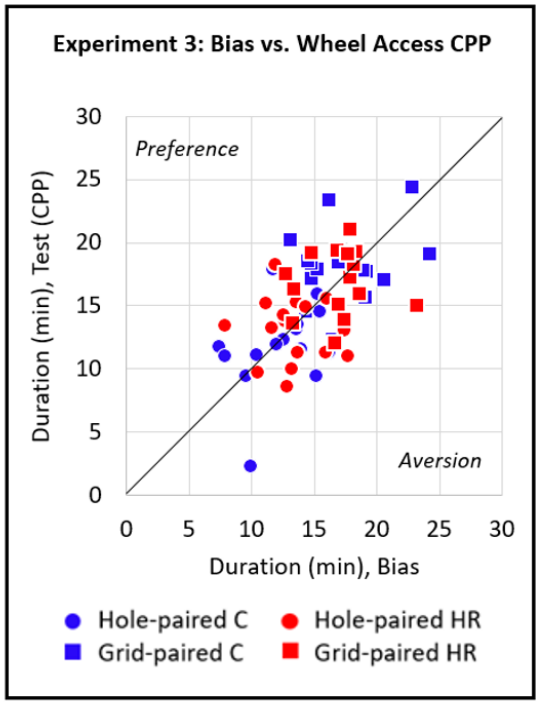
Linetype	1	6	0.7080
Texture x Linetype	1	6	0.8530



Appendix C Figure 5. Experiment 3, running wheels. (Left panel) Mice (generation 79, N = 63, 1 outlier deleted) from both the selectively bred High Runner lines (P = 0.0231) and the non-selected Control lines (P = 0.0087) had a preexisting bias to spend more time on the GRID as compared with the HOLE. (Center panel) Neither HR nor C mice conditioned to the reward-paired floor texture (P = 0.7999), with no effect of linetype (P = 0.7080) and no interaction between linetype and texture (P = 0.8530). (Right panel) Duration (min) spent on HOLE floor (for HOLE-paired mice) or GRID floor (for GRID-paired mice) during wheel CPP plotted against baseline duration (min) spent on those floor types during bias testing. The one-to-one line is shown.

Appendix C Table 1. Summary of previous studies that have employed wheel running as a rewarding stimulus in a CPP paradigm.

Publication	Animal Model	Bias Protocol[a]	Food	Total Conditioning Duration[b]	Conditioning Trial Duration	Probe Trial Duration
	Experimental Design (Conditioning Stimuli; Wheel Access)					
	Result(s)					
Antoniadis et al. 2000	♂ Golden Hamsters	Mixed	*Ad lib.*	8 days	30 min	15 min
	CPP apparatus color, shape, and odor; Wheel located inside apparatus					
	Young and aged hamsters with consolidated locomotor circadian rhythms preferred the wheel context, whereas aged hamsters with fragmented circadian rhythms did **not** develop a preference.					
Lett et al. 2000	♂ Sprague-Dawley Rats	Biased	Restricted	12 days	30 min	10 min
	Horizontal wall stripes with diamond lattice texture flooring or vertical stripes with parallel bars; 2 hr or 22.5 hr with wheels, then placed into conditioning chambers					
	CPP for wheel context was formed by both 2 hr and 22.5 hr duration experiments.					
Lett et al. 2001a	♂ Sprague-Dawley Rats	Biased	Restricted	8 days	30 min	10 min
	Same stimuli as Lett et al. 2000; 2 hrs confined to wheel → injection (i.p.) of saline or naloxone → 10 min in home-cage → conditioning chamber					
	Saline + wheel formed CPP but **not** naloxone + wheels (indicates the rewarding effect of wheel running is mediated by endogenous opioids in rats).					
Lett et al. 2001b	♂ Sprague-Dawley Rats	Biased	*Ad lib.*	8 days	15 min	10 min
	Same stimuli as Lett et al. 2000; 10 min drinking solution (salt or saccharin water) → 30 min on wheel → conditioning chamber					
	Wheel running simultaneously formed CPP and conditioned taste aversion to either solution.					
Lett et al. 2002	♂ Sprague-Dawley Rats	Biased	Restricted	8 days	30 min	10 min
	Same stimuli as Lett et al. 2000; *Experiment 1)* 2 hr or 22 hr confined to wheel → 10 min home-cage delay → 30 min paired CPP chamber; *Experiment 2)* Same as first, but delay was either 0 min, 10 min, or 30 min					
	Experiment 1) CPP formed for both 2 hr and 22 hr wheel groups. *Experiment 2)* CPP formed for 0 min or 10 min delay groups but **not** the 30 min delay group for both 2 hr and 22 hr wheel groups.					

Appendix C Table 1 Continued

Publication	Animal Model	Bias Protocol[a]	Food	Total Conditioning Duration[b]	Conditioning Trial Duration	Probe Trial Duration
	Experimental Design (Conditioning Stimuli; Wheel Access)					
	Result(s)					
Ralph et al. 2002	♂ Golden Hamsters	Mixed	*Ad lib.*	8 days	30 min	15 min
	3-sided chamber with horizontal stripes + isoamyl acetate odorant or 8-sided black chamber with eucalyptus odorant; Home cages had free access to wheels and a wheel was placed in one of the two contexts during conditioning for each hamster; *Experiment 1)* 14 hr light/10 hr dark cycle (Zeitgeber Time), conditioning and probe trials either 1 hr into dark phase (ZT13) or mid-light phase (ZT04); *Experiment 2)* Constant light (Circadian Time), conditioning and probe trials at equivalent times to experiment 1 (CT13 or CT04)					
	For both experiments, CPP was formed for a reward-paired context only at the time that training had taken place but **not** when tested for preference at a different time.					
Belke and Wagner 2005	♂ Wistar Rats	Unbiased	Measured	12 days	30 min	10 min
	Same stimuli as Lett et al. 2000; Operant lever-press training followed by wheel reinforcement sessions (using the opportunity to run for 45 sec followed by place conditioning					
	60% of total animal time was spent in wheel-paired context (i.e. CPP formed), but 4 of 12 individuals did **not** form a CPP.					
Greenwood et al. 2011	♂ Fischer 344 Rats	Neither	*Ad lib.*	2 weeks & 6 weeks	30 min	10 min
	Wall stripes & texture flooring with slight variations from Lett et al. 2000; Overnight access to wheels with morning conditioning					
	CPP formed in the 6-week group but **not** the 2-week group.					
Trost and Hauber 2014	♂ Sprague-Dawley Rats	Unbiased	Restricted	12 days	Unknown	10 min
	Black and white vertical striped walls + floor with square holes or gray walls + floor with circular holes; 2 hr in enclosed wheel, then placed into conditioning chambers; A repeat experiment with the same animals injected (i.p.) with Flupenthixol (D1/D2 receptor antagonist) or saline prior to conditioning					
	CPP formed for the context experienced under the aftereffect of wheel running in both experiments.					
Basso and Morrell 2015	♂♀ Sprague-Dawley Rats	Biased	*Ad lib.*	14 days	23 hr	60 min
	Wall stripes & texture flooring using small paper squares or ¼" Bed-o'Cobs®; 23 hr in alternating conditioning chambers with or without wheel access					
	CPP formed for wheel context in ~2/3 of individuals with no difference between sexes.					

Appendix C Table 1 Continued

Publication	Animal Model	Bias Protocol[a]	Food	Total Conditioning Duration[b]	Conditioning Trial Duration	Probe Trial Duration
	Experimental Design (Conditioning Stimuli; Wheel Access)					
	Result(s)					
Fernandes et al. 2015	♂ $STAT3^{DAT\ KO}$ & Control Mice (C57Bl/6 background)	Mixed	*Ad lib.*	14 days	30 min	15 min
	Black compartment with grid floor or white compartment with mesh floor; 2 hr in home cage with wheel or no wheel, then placed into conditioning chambers					
	CPP formed for mice provided wheels and STAT3 deletion increased this effect as compared with controls. Intra-VTA leptin inhibited the formation of CPP in controls, but not in $STAT3^{DAT\ KO}$ mice provided wheels.					
Herrera et al. 2016	♂ Fischer 344 Rats	Biased	*Ad lib.*	10/20/30 days	20 min	10 min
	Hole/Grid texture flooring; Voluntary or forced 2 hr run, then placed into conditioning chambers					
	Increasing CPP formed over time (10 days → 20 days → 30 days) for both groups, no differences between voluntary or forced running conditions. Note: Fig. 5c P1 (probe 1; 10 days) voluntary run group appears to have **not** formed a significant CPP.					
Plaksiy 2017 (Thesis)[c]	♀ Adolescent C57Bl/6 Mice	Mixed	Varies	8 days	30 min	30 min
	3 white walls and white floor, 1 wall with vertical or horizontal stripes, and food or a running wheel paired with each side; Mice separated into control, food restricted (FR), or activity-based anorexia (ABA) treatment groups					
	After treatment, ABA and control mice did **not** form a CPP to either context except when analyzed in subsets (some ABA preferred food context, some ABA preferred wheel context).					
Schmill et al. 2020	♀ Hsd:ICR Mice (HR and C)	Mixed	Varies	8 days	30 min	30 min
	Hole/Grid texture flooring; 23 hr in home-cage with alternating wheel access or blocked wheel access and placed in conditioning chambers near peak activity time					
	CPP was **not** formed after accounting for individual preexisting biases.					

[a] See Appendix C Materials and Methods for the differences among biased, unbiased, and mixed studies. Note: Greenwood et al. 2011 found no preexisting bias and therefore used "Neither" a biased nor an unbiased method.

[b] This refers to the total duration of the experimental procedure. In all of the cited studies, animals received wheel access for half of the total days.

[c] This thesis refined its CPP methodology in a pilot study and first experiment, therefore only "Experiment 2" was used for this table and the tally referred to in the Discussion text, excluding the cohort of food conditioned mice.

CONCLUSION

Exercise is extensively beneficial for physical and mental health, as well as vital for reducing the risk of numerous socially and economically important diseases and disorders, including obesity, metabolic syndrome, cardiovascular disease, certain cancers, cognitive impairment, and dementia (Dishman et al. 2006; Booth Frank W. et al. 2012; Phillips and Kennedy 2012; Fulghum and Hill 2018; Ruegsegger and Booth 2018; U.S. Department of Health and Human Services 2018). Unfortunately, the vast majority of adults in the United States fail to meet public health recommendations for physical activity. In 2015, only approximately 20% of young adults and adults reported getting enough physical activity to meet basic guidelines (U.S. Department of Health and Human Services 2018). In other countries, a parallel situation exists. For instance, using accelerometry, Luzak et al. (2017) found that only 14 percent of German adults adhered to WHO recommendations of 2.5 hours/week of moderate-to-vigorous physical activity, with over a third of subjects not achieving a single 10-minute bout. Increasing our understanding of the determinants of exercise, along with the short- and long-term effects of physical activity, is necessary for improving individual and overall human health.

As a whole, exercise is a complex behavior responsible for a multitude of integrative underlying physiological factors and processes that may help to improve health outcomes. Such mechanisms take place at lower levels (e.g., expression of specific genes or gene systems), at intermediate levels (e.g., cell

growth or organ function), and at higher levels (e.g., increased aerobic capacity or even novel behavior) of biological organization (Garland and Kelly 2006). We build our collective knowledge in the field of exercise physiology by conducting research across these various levels, and with a myriad of animal models.

The animals studied in this dissertation came from an artificial selection experiment that breeds lines of mice for high amounts of voluntary running on wheels (Swallow et al. 1998). At present, the selection experiment has continued >90 mouse generations. Mice from the four replicate High Runner (HR) lines run approximately three times as much on a daily basis as their non-selected Control (C) counterparts, indicative of increased ability and motivation to exercise. Potentially related to both ability and motivation, Kolb et al. (2013b) found that, when controlling for body mass, HR mice have heavier whole brains, in addition to midbrains that are 13% larger than C mice. Hence, the HR mouse model allows for investigation of increased physical activity as the primary trait of interest, but also the many underlying evolved changes in the central nervous system, integrated with those in the periphery.

This dissertation research targeted three separate components of the HR phenotype: an altered endocannabinoid (eCB) system (Keeney et al. 2008, 2012; Thompson et al. 2017), a mosaic neuroanatomy (Kolb et al. 2013b; Thompson 2017), and behavior associated with rewarding drugs or exercise (Rhodes et al. 2001; Rhodes and Garland 2003). Proximately, I aimed to discover specific anatomical or physiological changes conducive to increased voluntary exercise.

Ultimately, I sought to further understand the biomedical and evolutionary significance of mammalian physical activity.

In Chapter 1, I found interesting complexities of the eCB system in the upper small-intestinal epithelium of HR compared to C mice. The eCB system has become more well-characterized in recent years, particularly in the small intestine, as it relates to energy intake and gut-brain signaling (DiPatrizio 2021). However, no studies have previously examined the effects of exercise or a genetic predisposition for increased levels of exercise within the proximal jejunum, a key location for eCB homeostatic activity. Using ultra-performance liquid chromatography coupled with tandem mass-spectrometry, I evaluated levels of the primary eCBs, the monoacylglycerol 2-arachidonoyl-*sn*-glycerol (2-AG), and the fatty acid ethanolamide, arachidonoyl ethanolamide (AEA, anandamide), as well as their lipid analogs docosahexaenoyl glycerol (DHG), docosahexaenoylethanolamide (DHEA), and oleoylethanolamide (OEA) in the jejunum epithelium. Results showed that HR mice have significantly less 2-AG than C mice in the jejunum. 2-AG and DHG both showed a significant 3-way interaction of linetype, sex, and six days of wheel access. In addition, we found an across-tissue correlation for 2-AG between jejunum and plasma (Thompson et al. 2017), that suggests 2-AG signaling in different systems may be modulated by a similar interaction of genetic background, biological sex, and voluntary exercise. Future studies should investigate eCB metabolism, as well as receptor

expression and binding, in relation to exercise and monoacylglycerols in the small intestine.

In Chapter 2, I tested hypotheses that specific subregions of the brain may have altered neuroanatomy (volume or cell density), and that some of these subregions could contribute to the larger HR brain size described by Kolb et al. (2013b). I also examined whether 10 weeks of chronic wheel access could affect the anatomy of each region of interest. Remarkably, I found that the red nucleus of the midbrain and the whole hippocampus had significantly greater volume in HR mice when compared to C brains, with body mass as a covariate. Also, neither the HR (with their propensity for a great deal of running) nor C brain subregions changed with chronic wheel access. A larger red nucleus in HR lines has novel implications for voluntary locomotion, including dopamine neurotransmission and perhaps exercise-related pain modulation. A larger hippocampus could be an especially important contributor to increased HR motivation and ability for exercise. No volume differences in basolateral amygdala, nucleus accumbens, and ventral pallidum suggests that the HR brain reward and motivational systems involving these regions are restricted to lower-level changes, e.g., neurotransmitter release and reception.

In Chapter 3, I conducted three separate behavioral experiments of conditioned place preference (CPP) using either cocaine, Ritalin or wheel access (see Appendix C) as reward stimuli. The generality of predisposition for addiction across varying types of drugs has been established for humans and animals

(Kendler et al. 2003; Ellenbroek et al. 2005; Belknap et al. 2008; Self and Staley Gottschalk 2010), and an extensive literature relates motivational circuits involved in drug and food rewards (McGlacken et al. 1995; Kelley and Berridge 2002). Both HR and C mice showed significant CPP when paired with cocaine or Ritalin. Surprisingly, we found that HR mice, who display withdrawal-like symptoms when deprived of wheel access (Rhodes et al. 2003; Malisch et al. 2009; Kolb et al. 2013a; Saul et al. 2017), do not respond differentially to cocaine or Ritalin rewards as compared with C mice. In other words, a genetic predisposition for exercise "addiction" does not result in a correlated response with respect to some drug rewards. In our wheel reward experiment, neither HR nor C lines formed place preferences, and we speculate this finding could have been caused by various aspects of methodology (see review of wheel-running CPP studies in Appendix C). Future investigations should assess CPP in response to other types of drugs in HR mice and C mice, such as opioid, serotoninergic or cannabinoid drugs.

Taken as a whole, the research findings from this dissertation make important contributions to our understanding of exercise motivation and/or ability in an evolutionary context. With the addition of this research to the considerable prior knowledge about voluntary exercise discovered through use of the HR mouse model, I anticipate important strides in the understanding of adaptations for physical fitness in association with human health outcomes.

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