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The Neurobiological Basis of Voluntary Exercise in Selectively-Bred High Runner Mice

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by

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DEDICATION

For the original Dr. Thompson, I am proud to share your name. For Lorraine and Jay, I am proud to be your daughter.

ABSTRACT OF THE DISSERTATION

The Neurobiological Basis of Voluntary Exercise in Selectively-Bred High Runner Mice

by

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University of California, Riverside, September 2017
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Exercise is a rewarding behavior with a multitude of positive effects on the body and brain, and animals vary greatly in terms of how much they choose to exercise. I examined neurobiological changes that have occurred in four replicate lines of mice selectively bred for high voluntary wheel running as compared with four non-selected control lines.

First, I followed up on a study which showed that high runner (HR) mice have larger midbrains than control (C) mice. I wanted to determine which midbrain regions have increased in size, and whether 10 weeks of wheel access would affect their size and/or cell density. I found a significant interaction between linetype (HR vs. C) and wheel access in the periaqueductal grey (PAG) region, with wheel access increasing PAG volume in C mice, while decreasing it in HR mice. HR mice also tended to have a larger ventral tegmental area, a region involved in processing rewards.

Next, I examined plasma levels of two endocannabinoids, 2-arachidonylglycerol (2-AG) and anandamide (AEA), in male and female HR and C mice with or without 6 days of wheel access (as used in the routine selection protocol). I found a significant interaction between sex, linetype, and wheel access, with female mice having lower levels of 2-AG and wheel access lowering 2-AG in some subgroups. The amount of running in the 30 minutes before sampling was a significant predictor of AEA levels in mice with wheel access, and HR had lower levels than C.

My third chapter examined reward substitution. Mice were housed with or without wheels and given access to artificial sweeteners as competing rewards. Building on data previously collected, we showed that all mice drank more of the artificial sweetener blends compared to water, although this increase was significantly smaller in HR mice, and only when they had access to wheels. This result suggests that HR mice prefer the reward of wheel running over that from sweeteners.

Overall, I show that HR mice have evolved several neurobiological differences that contribute to their ability to voluntarily exercise ~3 times as much as C mice on a daily basis.

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INTRODUCTION

Brain evolution is difficult to study, for several reasons. For one, the brain is hidden inside the skull, meaning that any histological or neurochemical inspection of the tissue itself cannot be done while the animal is alive. Second, although fossil records are available for the skeleton, records of how soft tissues have changed over time are harder to come by (Deacon 1990). Third, the brain is an extremely complicated set of structures and regions, which show a surprising amount of plasticity, control various aspects of behavior, and produce hormones and neurotransmitters. Any of these aspects might respond to natural or sexual selection acting on behavior, thus making it difficult, if not impossible, to cover all the bases when testing for aspects of brain function that have evolved (Garland, Jr. et al. 2016).

Selection experiments and experimental evolution are one way to address some of the questions regarding brain evolution. These types of experiments have some advantages over studying evolution in the wild, namely that many more aspects of the experiment can be controlled, the recent evolutionary history of the study organism can be known precisely, and the study can be replicated more easily (Garland and Rose 2009). Some of these studies fall under the umbrella of a “bottom-up” (e.g., see Dykhuizen and Dean 2009) – specific genes are mutated (either a specific one in question, or randomly), and this mutation causes a change in a protein's structure or expression level or pattern, which causes changes at the cell, tissue, and organ levels, and finally, perhaps, it

produces a change in behavior. Although this type of experiment may be necessary for elucidating the functions of individual genes, it is less relevant for understanding how populations respond to selection. In general, natural and sexual selection occur at the level of life history traits, behavior, and whole-organism performance, not at the level of individual gene function (Lande and Arnold 1983; Garland, Jr. et al. 2016; Orr and Garland 2017).

"Top-down" selection experiments are thus more useful for examining how natural or sexual selection might lead to evolutionary change. These types of experiments can impose a particular kind of selection on a behavior or other high-level, complex trait, and can then examine how organs, tissues, proteins, and finally, genes, change in response. Selection experiments can especially shed light on the evolution of exercise behavior. Although much is known about the proximate effects of exercise on the brain, the ultimate, evolutionary correlates and consequences of increased exercise behavior are poorly understood. Raichlen & Gordon (2011) show an inter-specific positive relationship in mammals between overall brain size and maximal metabolic rate. Raichlen & Polk (2012) provide evidence supporting a hypothesis that selection for increased exercise performance may have influenced the evolution of larger brains in humans. In both studies, the authors credit artificial selection experiments as key providers of evidence for how the evolution of exercise has affected the brain.

One artificial selection experiment was started in 1993 to examine voluntary exercise behavior (Garland Jr 2003; Garland, Jr. et al. 2011a; Wallace and Garland, Jr. 2016). An original group of 224 mice from the outbred Harlan Sprague Dawley: Institute for Cancer Research (Hsd:ICR) strain were divided randomly into two groups (each with 4 replicate lines) – one group destined to become the High Runner lines, and one destined to stay as the non-selected Control lines. Mice in all 8 lines were allowed to run on wheels for 6 days, as young adults, and the HR mice were chosen to breed based on how much they ran on days 5 and 6, while the C mice were bred randomly, without regard to amount of running (Swallow et al. 1998a). Over time, HR mice increased their daily wheel-running distance approximately three-fold. Besides the obvious, expected change in voluntary wheel running, a number of other changes have occurred (Garland Jr 2003; Garland, Jr. et al. 2011a, 2016; Wallace and Garland, Jr. 2016), including in other behaviors (Carter et al. 2000), organs (Swallow et al. 2005), tissues (Houle-Leroy et al. 2003), proteins (Thomson et al. 2002), and genes (Kelly et al. 2013).

Various aspects of the HR mouse neurobiology have evolved as well, and several studies have focused on the motivation for exercise, or the reward that results from exercise. For example, when prevented from running after 6 days of wheel access, HR mice showed greater neuronal activation in brain regions involved in locomotor activity and reward (Rhodes et al. 2003a). In addition, HR mice have alterations in their responses to pharmacological targeting of

dopamine, serotonin, and endocannabinoid receptors, two neurotransmitters involved in reward signaling (Rhodes et al. 2001a; Rhodes and Garland 2003a; Keeney et al. 2008, 2012; Claghorn et al. 2016a). When trained to press a lever to free the brake on a wheel, C mice performed similarly whether the duration of wheel running allowed was 90 seconds or 30 minutes, but HR mice only completed the training when the time allowed was 30 minutes (Belke and Garland 2007). This result suggests that the motivation for wheel running in HR mice may have evolved to emphasize longer periods of exercise, despite the fact that the increase daily running distances of HR mice have evolved mainly by increases in average running speeds. HR mice also have larger non-cerebellar brain size, and magnetic resonance imaging has shown that HR mice have an approximately ~13% larger midbrain than C mice (Kolb et al. 2013). The midbrain contains several regions involved in the control of locomotion, as well as pathways regulating motivation and reward. The cerebellum is also altered in the HR mice – although it is better known for its role in motor control, it also has a role in reward processing and goal-oriented behaviors (Caetano-Anollés et al. 2016).

Although much is known about the neurobiology of the HR mice, much is still to be discovered. In my dissertation, I first further explore the regions within the midbrain that may have contributed to the larger volume seen in the HR mice (Chapter 1). In Chapter 2, I focus on the endocannabinoid system, based on previous studies which showed that male and female HR and C mice respond

differently to agonists and antagonists for cannabinoid receptor type 1 (Keeney et al. 2008, 2012). I show that the plasma levels of the two main endocannabinoids are affected by selective breeding, respond acutely to voluntary exercise, and differ between the sexes (Chapter 2). Third, I explore reward substitution in the HR mice with an experiment designed to measure if the incentive salience for exercise has been affected by selective breeding. Our results suggest that HR mice have reduced incentive salience for a sweet-taste reward, but only when they have access to wheels (Chapter 3).

REFERENCES

Belke, T. W., and T. Garland. 2007. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. J. Exp. Anal. Behav. 88:199–213.

Caetano-Anollés, K., J. S. Rhodes, T. Garland, Jr., S. D. Perez, A. G. Hernandez, B. R. Southey, and S. L. Rodriguez-Zas. 2016. Cerebellum transcriptome of mice bred for high voluntary activity offers insights into locomotor control and reward-dependent behaviors. PLOS ONE 11:e0167095.

Carter, P. A., J. G. Swallow, S. J. Davis, and T. Garland. 2000. Nesting behavior of house mice (Mus domesticus) selected for increased wheel-running activity. Behav. Genet. 30:85–94.

Claghorn, G. C., I. A. T. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol. Behav. 161:145–154.

Deacon, T. W. 1990. Rethinking mammalian brain evolution. Am. Zool. 30:629–705.

Dykhuizen, D. E., and A. M. Dean. 2009. Experimental evolution from the bottom up. Pp. 67–89 in T. Garland, Jr. and M. R. Rose, eds. Experimental Evolution: Concepts, Methods, and Applications of Selection Experiments.

Garland Jr, T. 2003. Selection experiments: an under-utilized tool in biomechanics and organismal biology. Vertebr. Biomech. Evol. 23–56.

Garland, Jr., T., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proc. R. Soc. B Biol. Sci. 278:574–581.

Garland, Jr., T., M. Zhao, and W. Saltzman. 2016. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integr. Comp. Biol. 56:207–224.

Garland, T., and M. R. Rose (eds). 2009. Experimental evolution: concepts, methods, and applications of selection experiments. University of California Press, Berkeley.

Houle-Leroy, P., H. Guderley, J. G. Swallow, and T. Garland, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. Am. J. Physiol.-Regul. Integr. Comp. Physiol. 284:R433–R443.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacol. Biochem. Behav. 101:528–537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland, Jr. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in

female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812–820.

Kelly, S. A., T. A. Bell, S. R. Selitsky, R. J. Buus, K. Hua, G. M. Weinstock, T. Garland, F. P.-M. de Villena, and D. Pomp. 2013. A novel intronic single nucleotide polymorphism in the myosin heavy polypeptide 4 gene is responsible for the mini-muscle phenotype characterized by major reduction in hind-limb muscle mass in mice. Genetics 195:1385–1395.

Kolb, E. M., E. L. Rezende, L. Holness, A. Radtke, S. K. Lee, A. Obenaus, and T. Garland. 2013. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. J. Exp. Biol. 216:515–523.

Lande, R., and S. J. Arnold. 1983. The Measurement of Selection on Correlated Characters. Evolution 37:1210.

Orr, T. J., and T. Garland. 2017. Complex Reproductive Traits and Whole-Organism Performance. Integr. Comp. Biol. 57:407–422.

Raichlen, D. A., and A. D. Gordon. 2011. Relationship between exercise capacity and brain size in mammals. PloS One 6:e20601.

Raichlen, D. A., and J. D. Polk. 2012. Linking brains and brawn: exercise and the evolution of human neurobiology. Proc. R. Soc. B Biol. Sci. 280:20122250–20122250.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. Mitchell, and T. Garland. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and

fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 158:120–131.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav. Neurosci. 117:1243.

Rhodes, J. S., and T. Garland. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 167:242–250.

Swallow, J. G., P. A. Carter, and T. Garland Jr. 1998. Artificial selection for increased wheel-running behavior in house mice. Behav. Genet. 28:227–237.

Swallow, J. G., J. S. Rhodes, and T. Garland, Jr. 2005. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. Integr. Comp. Biol. 45:426–437.

Thomson, S. L., T. Garland Jr, J. G. Swallow, and P. A. Carter. 2002. Response of Sod-2 enzyme activity to selection for high voluntary wheel running. Heredity 88:52.

Wallace, I. J., and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evol. Anthropol. Issues News Rev. 25:98–104.

CHAPTER 1

The volume of the midbrain is affected by selective breeding and voluntary exercise

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Abstract

Artificial selection experiments offer a unique view on brain evolution. One such experiment has selected for high voluntary wheel running behavior for over 80 generations, producing 4 replicate lines of high runner (HR) mice, which run ~3-fold more on a daily basis than 4 replicate lines of non-selected control (C) mice. A recent study of these mice showed that the HR mice had larger non-cerebellar brain mass and a larger midbrain volume when compared with C mice. We chose to further investigate the effects of selective breeding and long-term wheel access on overall brain size and characteristics of specific regions within the midbrain, including the periaqueductal grey (PAG), substantia nigra (SN), and ventral tegmental area (VTA). Mice from both HR and C lines (total N = 100) were allowed access to wheels, or kept without access to wheels, for 10 weeks, after which aspects of neuroanatomy, such as cell count, area, and volume of particular regions were measured. Both linetype (HR vs C) and wheel access for 10 weeks increased brain mass with no significant interaction between the two factors. The volume of the whole midbrain tended to be larger in HR mice ($p = 0.0713$), with no effect of wheel access and no interaction. Linetype and wheel access had an interactive effect on the volume of the periaqueductal grey, such that wheel access increased the volume in C mice, but decreased it in HR mice. The volume of the VTA tended to be larger in HR mice, with no effect of wheel access and no interaction. Neither linetype nor wheel access had an effect on the volume of the substantia nigra. We did not find any differences in numbers of

cells per unit area for any of the regions examined. The PAG is involved in pain transmission, as well as control of vocalizations. The VTA is involved in reward signaling, and has recently been shown to have some amount of control over voluntary wheel running via the endocannabinoid system, which is known to be altered in HR mice. Further investigation of these regions, and perhaps other regions within the neocortex, may help us to better understand the increased exercise phenotype observed in the HR mice.

Introduction

Behavioral capabilities are known to correlate with sizes of associated brain structures. This correlation occurs both via coadaptation of behavior and brain over evolutionary time, leading to differences among species and populations, and because of neural plasticity in response to the expression of a particular behavior within the lifetime of an individual. Inter-specific comparisons are more common, and, in birds, have shown a positive relationship between the ability to remember food storage locations, the complexity of song repertoire, the number of behavioral innovations, and the volume of the brain regions responsible (Krebs et al. 1989; Devoogd et al. 1993; Lefebvre et al. 1997). The sum of the volumes of the neocortex and striatum is also positively correlated with behavioral innovation in primates (Reader and Laland 2002). Additionally, in cartilaginous fishes, species that live closer to the top of the ocean and rely more on vision for catching prey have a larger optic tectum (Yopak and Lisney 2012). However, species comparisons cannot help us parcel out whether a larger brain region is caused by the lifetime experience of an animal or its evolutionary history, or both.

In terms of comparisons of individuals within a single species, perhaps most famously, London taxi drivers had larger hippocampal volume when compared with control subjects, and the amount of time spent as a taxi driver was positively correlated with volume of the posterior region of the hippocampus (Maguire et al. 2000).

However, it is unclear whether their hippocampi increased in size due to their frequent use of that region, or whether some individuals were born with larger hippocampi and were therefore better at a job that requires frequent spatial navigation. The former option is certainly possible because brain plasticity, even increases in volume, can occur rapidly. For example, environmental enrichment, rotarod training, and voluntary exercise on wheels have all been shown to cause volume changes in the brains of rodents in as little as 7 days (Sumiyoshi et al. 2014; Cahill et al. 2015; Scholz et al. 2015a,b).

An approach that can elucidate both brain evolution and plasticity involves replicated selection experiments with model organisms, such as laboratory rats or mice, that target a particular behavior (Rhodes and Kawecki 2009). One long-term artificial selection experiment for voluntary exercise behavior was started in 1993 from an original population of outbred Hsd:ICR mice (Swallow et al. 1998a). Eight lines of mice were created – four high-runner (HR) lines, with the top runners chosen to breed in the next generation, and four control (C) lines, bred randomly with regard to wheel-running (sib-mating is disallowed in all lines). Total wheel revolutions on days 5 and 6 of a 6-day period of wheel access are used as the basis for selection in the HR lines. Mice from the HR lines currently run almost 3 times as far as C mice on a daily basis, primarily because they run faster. The HR mice have lower lean body mass (Swallow et al. 2001), increased maximal oxygen consumption during forced exercise (VO_2max) (Swallow et al. 1998b), increased heart ventricle mass (Kelly et al. 2017), and

several other adaptations beneficial to sustained, endurance-type locomotor activity (Garland, Jr. et al. 2011, 2016; Wallace and Garland, Jr. 2016).

In addition, many neurobiological changes have occurred as a result of selective breeding: the HR mice are hyperactive in home cages when they do not have wheel access, have an altered dopaminergic system (Rhodes et al. 2001; Rhodes and Garland 2003), altered hippocampal neurogenesis with long-term wheel access (Rhodes et al. 2003), an altered endocannabinoid system (Keeney et al. 2008, 2012; Thompson et al. 2017), and greater brain-derived neurotrophic factor acutely after running (Johnson et al. 2003). Only one study has examined their neuroanatomy, and found that, after 6 days of running, HR mice had an increased non-cerebellar brain mass when compared to C mice (Kolb et al. 2013). Moreover, volume measurements from MRI of *ex vivo* brains showed a statistically significant increase in midbrain volume (~13%) in the HR mice.

Because all the mice in the Kolb et al. (2013) study received 6 days of wheel access, it is conceivable that some or even all of the increased brain size of HR mice was caused by plasticity in response to the exercise. Therefore, in the present study, we housed mice both with and without wheels for over 10 weeks, and then compared brain volumes and cell densities of key regions.

Following from the previous observation of increased midbrain size in HR mice (Kolb et al. 2013), three regions of interest within the midbrain were chosen for histological analysis, based on multiple working hypotheses (e.g., see Elliott and Brook 2007) about the source of the increase in voluntary exercise seen in

the HR mice. For example, one hypothesis is that the HR mice are less sensitive to pain, which might allow them to exercise more before stopping due to muscle or joint soreness (exercise-induced pain). The periaqueductal gray (PAG) plays a major role in both ascending and descending pain transmission (Behbehani 1995). Although a previous study (Li et al. 2004), found no difference in the response of HR and C mice to opioid antagonists (naloxone, naltrexone), i.e., they both had an equal decrease in tail-flick latency and a proportionally equal decrease in running, it is possible that changes in the periaqueductal gray have occurred without a change in opioid-mediated pain sensitivity (other neurotransmitters are found in the PAG, including gamma-aminobutyric acid and glutamate). Although much of it is modulated by opioids, many of the neurons in the PAG release gamma-amino-butyric-acid (GABA), glutamate and aspartic acid, all of which can be involved in pain neurotransmission (Behbehani 1995).

Several previous studies of these mice have found changes in the dopaminergic system (Rhodes et al. 2001; Rhodes and Garland 2003), which is involved in both the processing of rewards and motivation for behavior, as well as movement itself (Schultz 1998; Roeper 2013). As an important part of both dopamine pathways, the substantia nigra (SN) might be expected to be involved whether the HR mice had evolved changes in their locomotor abilities or the way in which they process rewards (e.g., see Belke and Garland 2007).

The ventral tegmental area is another critical region involved in the processing of rewards (Mercuri et al. 1992; Ilango et al. 2014). In addition, a

recent study has specifically implicated this region in the motivation for voluntary wheel running via the endocannabinoid system (Dubreucq et al. 2013), and several studies have shown that the endocannabinoid system is altered in the HR mice (Keeney et al. 2008, 2012; Thompson et al. 2017).

We also examined the whole midbrain as a fourth "region" of interest. This gave us a dataset that was more directly comparable to the previous study that identified the midbrain as being larger in the HR mice, via MRI imaging of ex vivo brains (Kolb et al. 2013). In addition, it is possible that, if each of the regions in the midbrain got slightly, but not significantly, larger, that we would see an increase in volume only at the level of the whole midbrain.

Materials & Methods

1. Animals

Fifty HR females and 50 C females from generation 67 were weaned at 3 weeks of age and placed directly into individual cages. Half of the mice were allowed access to wheels (1.12 m in diameter, as in the routine selection experiment: Swallow et al. 1998b) and the other half were placed in cages without access to wheels. All mice had home-cage activity (HCA) measured with passive infrared sensors (Copes et al. 2015). During the 10th week of wheel access or no wheel access, mice were transcardially perfused and their brains were dissected and weighed, a process that lasted into the 13th week. Therefore, age was used as a covariate in all statistical analyses (see below).

2. Tissue processing and imaging

After dissection, brains were placed into 30% sucrose in 4% paraformaldehyde for at least 48 hours. Brains were then mounted and sectioned on a Leica CM1850 cryostat at 40 micrometers thickness. Every other section was placed on a slide and used for Nissl staining (the remaining sections were kept for a different experiment). Digital images were taken at a set magnification for each region using a Zeiss Discovery V.12 stereo microscope and an attached Zeiss AxioCam.

3. Area and cell density measurements & volume calculations

a. Area measurements

Images were imported into ImageJ software (NIH). The polygon-selection tool was used to free-hand outline regions of interest, using either the Franklin and Paxinos mouse brain atlas (Franklin and Paxinos 1997) or the Allen Mouse Brain Atlas (Allen Institute for Brain Science 2004) as a guide. A photograph of a ruler at the same magnification was used to set the scale of how many pixels in the image were equal to one micrometer. This scale was used in order for ImageJ to calculate the area, in micrometers, of the traced region in each photograph. For the whole midbrain and PAG traces, the cerebral aqueduct was removed for ~ the last third of each region, as its large size may have significantly affected the area measurement of the region and it often contained large amounts of debris which may have affected the cell counts.

b. Cell numbers

A free plugin for ImageJ, the Image-based Tool for Counting Nuclei (ITCN) was used to measure the numbers of cells within the regions of interest (Byun et al. 2006). The ITCN requires inputs of the average pixel width of cells, the minimum distance between cells, and a threshold value. The total number of cells counted was summed, and then divided by the total cross-sectional area of all sections used to yield a measure of the average number of cells per unit area.

c. Volume calculations

Volume calculations were done using the area measurements output by ImageJ, and multiplying each value by 80 micrometers (the width between each section). Values for missing sections were interpolated as follows: 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. The volumes of each section in a region of interest were summed to get one total volume for that region per mouse. Values for the substantia nigra and ventral tegmental area are half of the total volume, as traces were magnified as much as possible for accurate cell counts, meaning only half of the brain was visible at a time. Mice that were judged to be missing sections at either end of a region were excluded

from the analyses, so final sample sizes were sometimes considerably smaller than 100.

4. Statistical Analyses

As with numerous previous studies of these lines of mice, data were analyzed using nested analysis of covariance (ANCOVA) in SAS Proc Mixed, with line nested within linetype (HR vs C) as a random effect. A subset of HR mice have a reduced hindlimb muscle-mass phenotype called "mini-muscle." This phenotype is caused by a Mendelian recessive autosomal small nucleotide polymorphism, and it has shown a variety of pleiotropic effects (Garland, Jr. et al. 2002; Swallow et al. 2005; Hannon et al. 2008). Mini-muscle status was determined for individual mice by dissection of the triceps surae muscles and included as a cofactor in all analyses. Covariates included in analyses when appropriate were body mass, age, amount of time that brains spent in paraformaldehyde before they were sectioned, and amount of time that sections spent in the freezer before they were stained. When needed, dependent variables were transformed to improve normality of residuals. Data points with residual values >3 standard deviations from the mean were excluded. For main effects, p-values below 0.05 were treated as statistically significant. For interactions, p-values below 0.1 were treated as significant, as the power to detect interactions is generally lower than that for detecting main effects (Wahlsten 1990, 1991).

Results

1. Body mass

HR mice tended to weigh less than C mice (25.14 grams vs. 27.81 grams, $p = 0.0912$, Table 1.1). Wheel access did not have a significant effect ($p = 0.1533$), and the linetype by wheel access interaction was not significant ($p = 0.2825$). Age at time of dissection had a significant effect ($p = 0.0447$), but mini-muscle status did not ($p = 0.6895$).

2. Wheel running

As expected, HR always ran more than C mice (Figure 1.1), with that difference reaching statistical significance in weeks 2-10 (Table 1.1). The average ratio of wheel running over all 10 weeks for HR/C was 3.27 (LS Means from SAS).

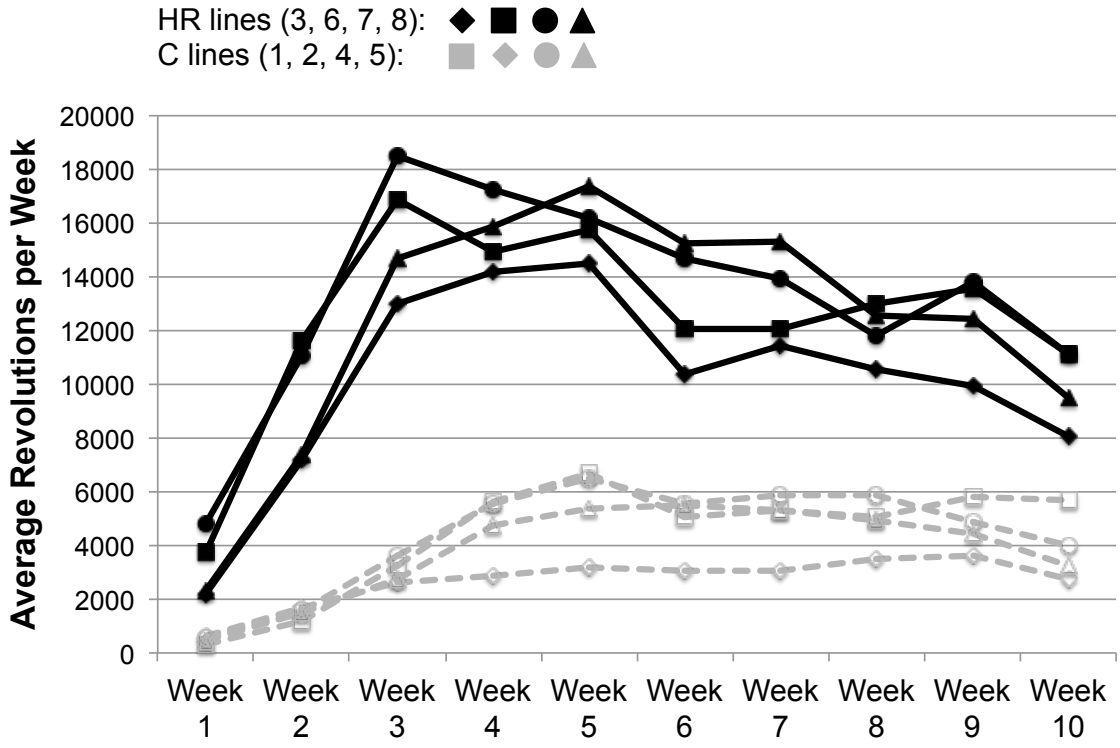

Figure 1.1. Average wheel revolutions run per week (simple means) during the course of the experiment. Mini-muscle status was included as a covariate, but only had a significant effect in Week 2 (Table 1.1).

3. Home-cage activity

The linetype-by-wheel access interaction was significant for home-cage activity in all weeks (Table 1.1). HR mice without wheel access always had the highest HCA, and wheel access decreased HCA in both HR and C mice, with a much larger decrease for HR mice (Figure 1.2).

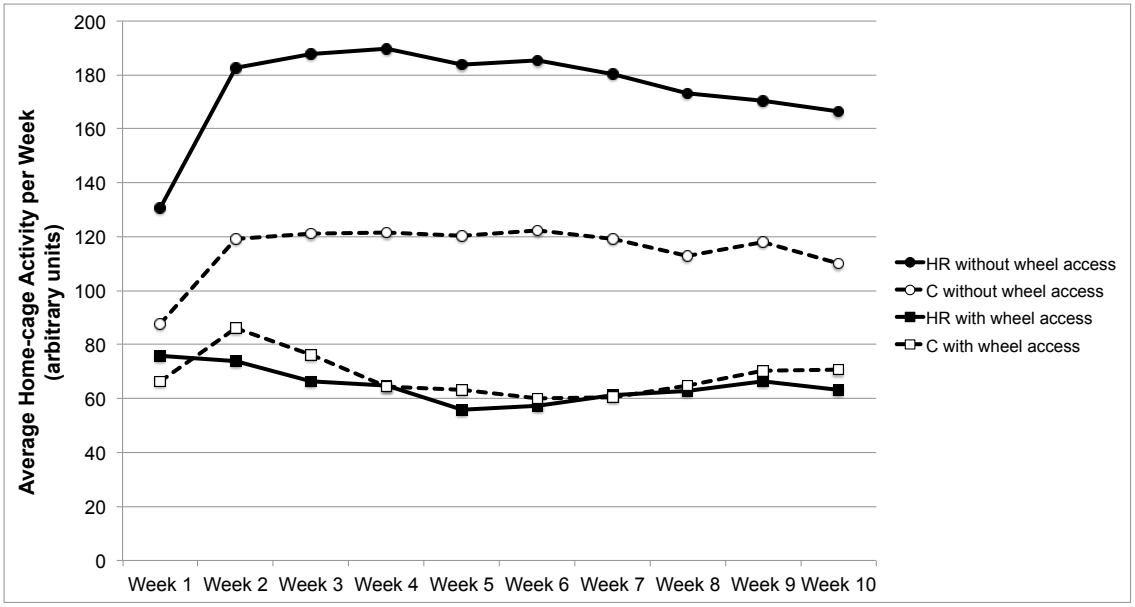

Figure 1.2. Average home-cage activity per week (LS means from SAS) over the course of the experiment. HR mice are shown in bold lines, dark circles without wheel access, and dark squares with wheel access. C mice are shown in dashed lines, open circles without wheel access, and open squares with wheel access. Mini-muscle status was included as a covariate, but never had a significant effect.

4. Brain mass

Adjusting for body mass, HR mice tended to have heavier brains than C mice (LS means: 0.494 grams vs. 0.471 grams, p = 0.0676, Figure 1.3). In addition, mice with wheel access for 10 weeks had heavier brains than mice that did not have wheel access (0.492 grams vs. 0.473 grams, p = 0.0127), with no significant interaction between the wheel access and linetype factors (p = 0.4535, Table 1.1). Mini-muscle status, body mass, and age at time of dissection were all

included in the analysis, but only body mass and age had significant effects (p = 0.2410, p = 0.0056, and p = 0.0009, respectively).

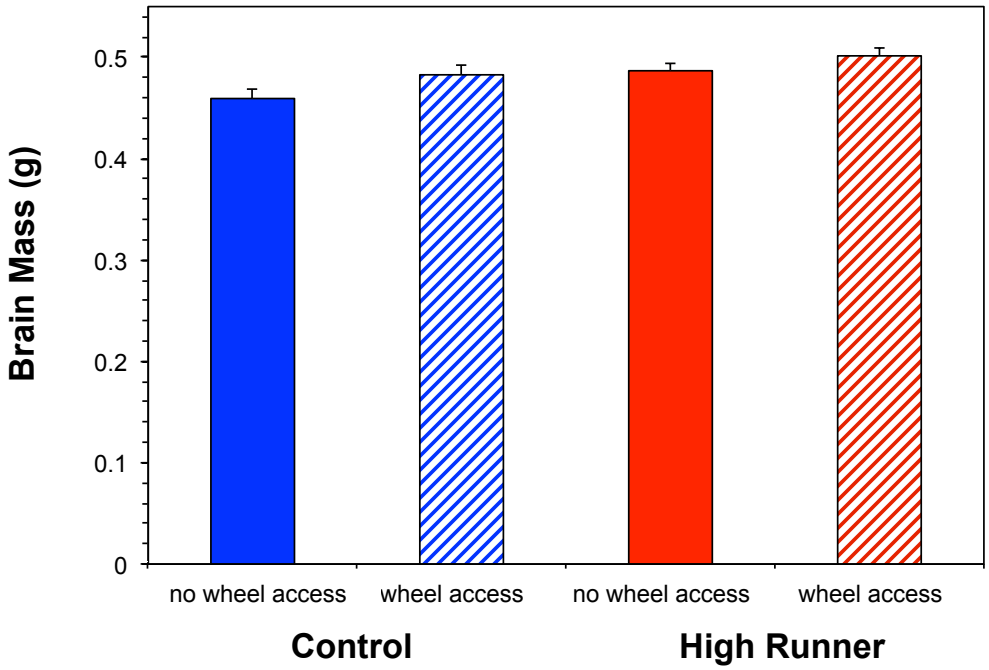

Figure 1.3. Average brain mass in grams (LS means from SAS). HR mice tended to have heavier brains than C mice (p = 0.0676), and mice with wheel access for 10 weeks had heavier brains than mice that did not have wheel access (p = 0.0127), with no interaction (p = 0.4535). Mini-muscle status, body mass, and age were included as covariates (Table 1.1).

5. Whole midbrain

a. Volume

As seen in one previous study (Kolb et al. 2013), the total volume of the midbrain tended to be larger in the HR mice ($p = 0.0713$, Figure 1.4). Ten weeks of wheel access did not have a significant effect ($p = 0.6070$), with no interaction between linetype and wheel access ($p = 0.8002$, Table 1.1). Mini-muscle status and body mass did not have a significant effect on the volume of the whole midbrain, but age tended to decrease midbrain volume ($p = 0.0855$).

b. Cell count/area

The number of cells per unit area in the whole midbrain was not significantly affected by linetype ($p = 0.7122$) or wheel access ($p = 0.3165$), with no interaction ($p = 0.4454$, Table 1.1).

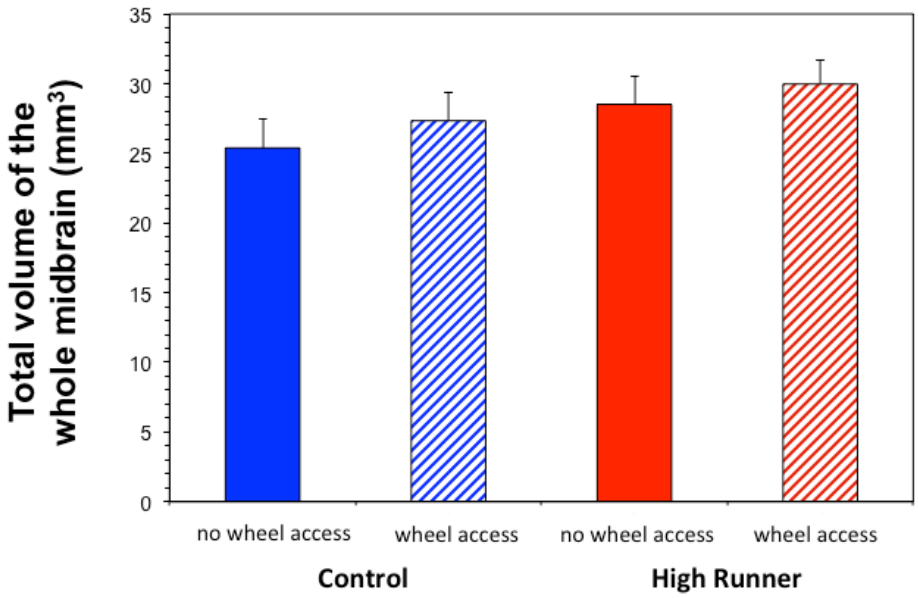

Figure 1.4. Whole midbrain total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, and age included as covariates (Table 1.1). The volume of the midbrain tended to be larger in HR mice ($p = 0.0713$), with no effect of wheel access and no interaction.

6. Periaqueductal grey

a. Volume

Linetype and wheel access had an interactive effect on the volume of the periaqueductal grey (interaction $p = 0.0513$, Figure 1.5): wheel access increased the volume of the PAG in C mice, while decreasing it in HR mice. Age had a significant negative effect ($p = 0.0004$, Table 1.1).

b. Cell count/area

Neither linetype (p = 0.6278, Table 1.1), nor wheel access (p = 0.1127) affected the number of cells per unit area in the PAG, with no significant interaction (p = 0.4282).

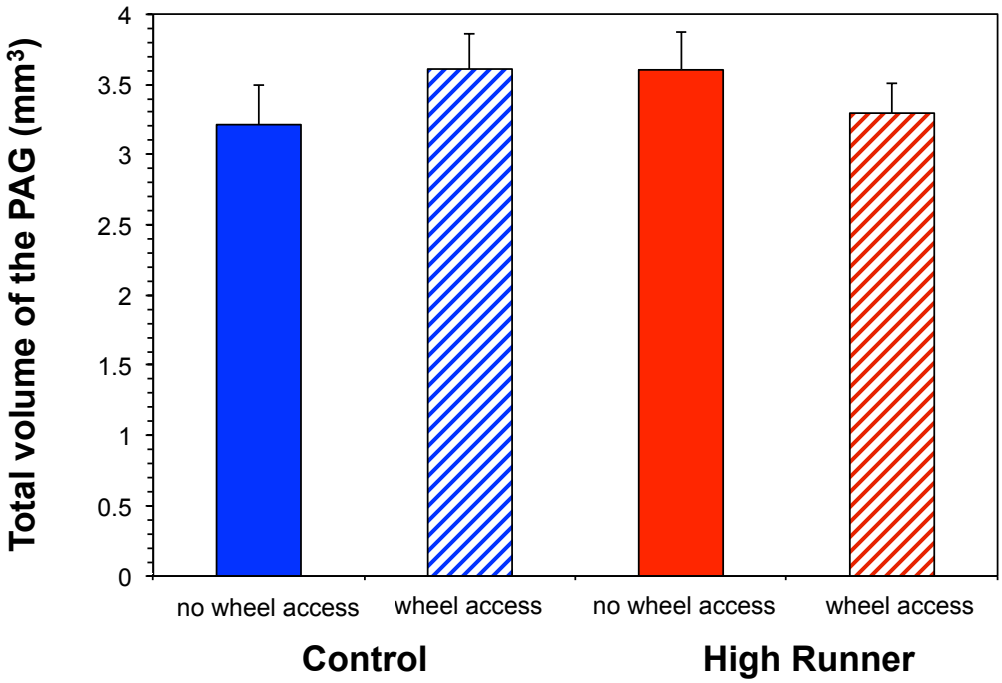

Figure 1.5. Periaqueductal grey total volume in cubic millimeters (LS means from SAS), with mini-muscle status, body mass, and age included as covariates (Table 1.1). Linetype and wheel access interacted in their effect on the volume of the periaqueductal grey (p = 0.0513). Wheel access increased the volume of the PAG in C mice, while decreasing it in HR mice.

7. Substantia nigra

a. Volume

Neither linetype nor wheel access had a significant effect on the volume of the substantia nigra ($p = 0.9321$ and $p = 0.8080$, with no interaction between the two factors ($p = 0.3563$, Table 1.1).

b. Cell count/area

Neither linetype ($p = 0.8270$) nor wheel access ($p = 0.3997$) affected the number of cells per unit area in the SN, with no significant interaction ($p = 0.2868$, Table 1.1).

8. Ventral tegmental area

a. Volume

HR mice tended to have a larger ventral tegmental area ($p = 0.0820$, Figure 1.6), with no effect of wheel access ($p = 0.4180$, Table 1.1), and no interaction ($p = 0.8184$). Body mass was not included as a covariate for this analysis as it showed the opposite trend (negative) as it did in all other analyses, which did not seem biologically reasonable. Age had a significant negative effect on volume of the VTA ($p = 0.0061$).

b. Cell count/area

Neither linetype ($p = 0.8525$, Table 1.1) nor wheel access ($p = 0.4421$) had a statistically significant on the number of cells per unit area in the VTA, with no significant interaction ($p = 0.7056$).

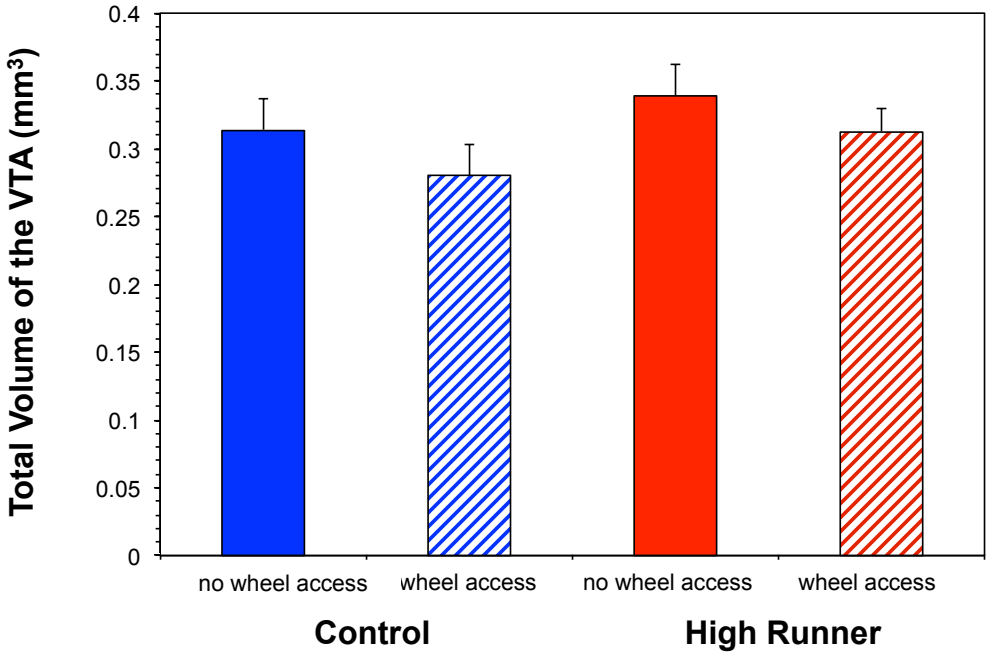

Figure 1.6. Ventral tegmental area total volume in cubic millimeters (LS means from SAS), with mini-muscle status and age included as covariates, but not body mass (Table 1.1). HR mice tended to have a larger ventral tegmental area ($p = 0.0820$), with no effect of wheel access and no interaction.

Discussion

As expected, HR mice tended to weigh less than C mice at the conclusion of the experiment (Table 1.1). However, 10 weeks of wheel access did not significantly affect body mass, with no significant interaction between linetype

and wheel access. Female mice from these lines do tend to have smaller changes in body mass than males when given access to a wheel, as has generally been reported for females versus males in laboratory mice and rats (Swallow et al. 1999, 2005, references therein). In any case, the use of body mass as a covariate in analyses of brain size (see also Fig. 3 in Martin and Harvey 1985) is important because linetype differences are somewhat confounded with body mass differences in the HR and C mice (Kolb et al. 2013).

Wheel-running and home-cage activity results were largely as previously reported, with HR mice running ~3-fold more than C over the course of the experiment (Garland, Jr. et al. 2011; Copes et al. 2015). HR mice have elevated levels of activity in their home cages when they do not have access to wheels, but appear to transfer that increased activity into wheel running when given the opportunity (Rhodes et al. 2005; Copes et al. 2015; Acosta et al. 2017).

We were able to confirm the results of a previous study (Kolb et al. 2013), which found that HR mice had larger brains than C mice. Although they found a statistically significant difference only in non-cerebellar brain mass, we found a difference in total brain mass between HR and C ($p = 0.0676$). In addition, we found that access to a wheel for 10 weeks increased brain mass in both HR and C mice, a result not been reported previously. Chronic exercise upregulates growth factors in the brain, including brain-derived neurotrophic factor and nerve growth factor (Dishman et al. 2006; Sumiyoshi et al. 2014). The increase in brain mass seen here with exercising mice may also be partially due to prevention of

age-related decline, as brain mass generally decreases with age (see Results, and Colcombe et al. 2006).

We were also able to confirm one other major result of the Kolb et al. (2013) study, which found that HR mice had larger midbrains than C mice, although our result here ($p = 0.0713$) did not reach the level of statistical significance in the previous study. Although our sample size here was double that in the previous study, we tested an additional factor (wheel access) and used a different method of estimating volume. Wheel access did not have a significant effect on midbrain volume in the present study, which suggests that the increase seen in HR mice is due primarily to their selective breeding. However, as discussed above, HR mice have increased home-cage activity when they do not have access to wheels (Fig 1.2), and it is possible that this increased activity, though not at the same level as the voluntary wheel running, has a positive effect on midbrain volume.

We found volumetric changes in two specific midbrain regions – the PAG and VTA. The PAG is involved in ascending and descending pain transmission. Interestingly, wheel access increased the size of the PAG in C mice, but decreased it in HR mice, suggesting that pain sensitivity may have changed differentially. One possibility is that the decrease in PAG volume in HR mice reduces any pain they may feel during "excessive" wheel running and hence helps permit those high amounts of running. Although HR mice had decreased pain sensitivity while exercising in a previous study (Li et al. 2004), C mice

showed the same response. The PAG does have other functions besides pain transmission, such as the processing of fear and anxiety, and the production of vocalizations (Behbehani 1995). An increase in anxious behavior has been seen in one HR line in the elevated-plus maze test (Jónás et al. 2010), but not when all four HR lines were tested together (Hiramatsu et al. 2017). In any case, our results suggest that either pain sensitivity, fear processing or vocalizations may be altered in the HR mice.

The volume of the VTA tended to be larger in HR mice, with no effect of wheel access. The VTA is involved in reward processing and has also been specifically implicated in the control of voluntary wheel running via the endocannabinoid system (Dubreucq et al. 2013), which is altered in HR mice (Keeney et al. 2008, 2012; Thompson et al. 2017).

Because we did not observe any significant differences in the number of cells per unit area in any of the regions investigated, it is difficult to conclude from this study what might be causing the increases in volume. Correlates of brain plasticity can also include changes in dendritic structure (Hickmott and Steen 2005) or neuronal remodeling. For example, Lerch et al. (2011) trained mice to navigate a maze and observed an increase in volume of the hippocampus as measured by MRI of *ex vivo* brains. They measured number of cells stained in the hippocampus with antibodies for four proteins, associated with changes in neuron number, changes in astrocyte number, increased neurogenesis, and increased neuronal remodeling. On an individual mouse basis, only the levels of

the neuronal remodeling protein were positively correlated with the increase in volume. Further exploration of the VTA may help us understand more about the changes seen in the midbrain of the HR mice.

Because we found a trend towards significance for the linetype effect in the volume of both the whole midbrain and VTA (Table 1.1), further analysis of these results should be done with the amount of wheel running and/or home-cage activity included as a covariate (see also Copes et al. [2015] regarding how both aspects of physical activity are significant positive predictors of food consumption). The larger total brain mass or larger region volume in the HR mice may be caused directly by changes in genes which govern the development of these regions, or these changes may come about through the genetic differences which cause the higher amounts of physical activity seen in the HR mice (Figure 1.7). In Kelly et al. (2017), for example, the larger heart ventricle size in HR mice was statistically explainable by their greater amount of running over the course of several weeks, as compared with C mice. A similar effect may be happening here, and including measures of physical activity as covariates may help us determine whether the differences between HR and C mice are best explained by the amount of activity an individual mouse experiences during its lifetime (i.e., phenotypic plasticity) or inherent genetic differences that directly affect the trait in question, or both. Also of interest is whether HR mice have evolved changes in brain plasticity in response to wheel running that might be

adaptive in their own right, which has been termed self-induced adaptive plasticity (Swallow and Garland Jr 2005; Garland, Jr. and Kelly 2006)

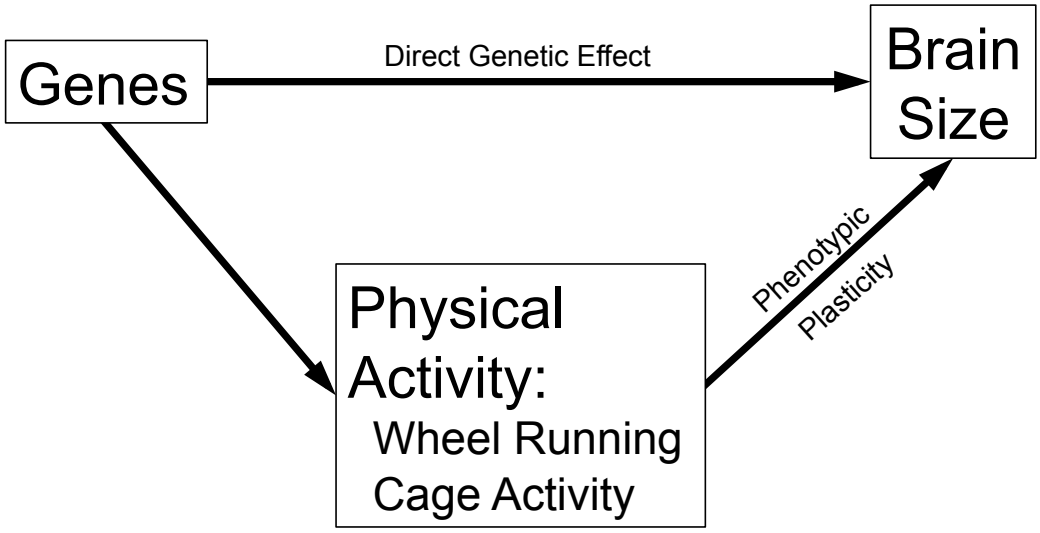

Figure 1.7. Direct vs. Indirect Genetic Effects on Brain Size. The larger brain size, or differences in brain region volumes, in HR mice as compared with C mice may be attributable to direct genetic effects and/or indirect genetic effects mediated through the higher amounts of physical activity by HR mice (Figures 1.1, 1.2).

Table 1.1. Values for ANCOVAs comparing linetype (HR vs. C), wheel-access groups (wheel access vs. sedentary), and their interaction. Mini indicates effects of having the mini-muscle phenotype (see text). Body mass and age were included as covariates where appropriate. For the brain volumes and cell counts, the amount of time that brains spent in paraformaldehyde before they were sectioned and the amount of time that sections spent in the freezer before they were stained were also included as covariates (results not shown). Bolded values are significant ($p<0.05$, except for interactions which are considered significant at $p<0.1$, see Materials and Methods). Signs after p-values indicate the direction of the effect from the model, + is HR > C, or wheel access > no wheel access, or mini-muscle mice > non-mini-muscle mice.

Trait	N	$P_{linetype}$	$P_{wheelaccess}$	$P_{interaction}$	P_{mini}	$P_{bodymass}$	P_{age}
Body mass	100	0.0912-	0.1533-	0.2825	0.6895+		**0.0447+**
Brain mass	93	0.0676+	**0.0127+**	0.4535	0.2410+	**0.0056+**	**0.0009-**
Week 1 wheel running	50	0.0509+			0.2614+		
Week 2 wheel running	49	**0.0120+**			**0.0427+**		
Week 3 wheel running	50	**0.0002+**			0.6905+		
Week 4 wheel running	49	**<0.0001+**			0.2229-		
Week 5 wheel running	49	**<0.0001+**			0.4946-		
Week 6 wheel running	49	**0.0001+**			0.1441-		
Week 7 wheel running	49	**0.0030+**			0.7025+		
Week 8 wheel running	50	**0.0002+**			0.4917-		
Week 9 wheel running	50	**0.0004+**			0.5998-		
Week 10 wheel running	50	**0.0010+**			0.5076-		
Week 1 home-cage activity	97	**0.0097+**	**0.0010-**	**0.0399**	0.9658+		
Week 2 home-cage activity	96	**0.0130+**	**<0.0001-**	**0.0009**	0.9551-		
Week 3 home-cage activity	95	0.0786+	**0.0005-**	**0.0218**	0.3143-		
Week 4 home-cage activity	95	0.0670+	**0.0008-**	**0.0580**	0.4333-		
Week 5 home-cage activity	95	0.0675+	**0.0002-**	**0.0213**	0.8137+		
Week 6 home-cage activity	97	**0.0248+**	**<0.0001-**	**0.0128**	0.8799-		
Week 7 home-cage activity	97	**0.0373+**	**0.0002-**	**0.0313**	0.8401-		
Week 8 home-cage activity	98	**0.0295+**	**<0.0001-**	**0.0092**	0.7243+		
Week 9 home-cage activity	98	0.0500+	**<0.0001-**	**0.0090**	0.4947+		
Week 10 home-cage activity	96	0.0963+	**0.0001-**	**0.0075**	0.5825+		
Whole midbrain volume	64	0.0713+	0.6070+	0.8002	0.5785-	0.3561+	0.0855-
Whole midbrain cells/unit area	80	0.7122+	0.3165+	0.4454	0.2613+		0.3305-
PAG volume	77	0.8347+	0.9172+	**0.0513**	0.6678-	0.8443+	**0.0004-**
PAG cells/unit area	81	0.6278-	0.1127+	0.4282	0.4281+		**0.0067+**

Substantia nigra volume	78	0.9321+	0.8080-	0.3563	0.6506-	0.7765+	0.6721-
Substantia nigra cells/unit area	83	0.8270+	0.3997+	0.2868	0.7323+		0.1736+
Ventral tegmental area volume	72	0.3743+	0.3012-	0.9002	0.4533-	0.0866-	**0.0052-**
Ventral tegmental area volume (no mass)	72	0.0820+	0.4180-	0.8184	0.2919-		**0.0061-**
Ventral tegmental area cells/unit area	83	0.8525+	0.4421+	0.7056	0.9590-		0.5053+

REFERENCES

Acosta, W., T. H. Meek, H. Schutz, E. M. Dlugosz, and T. Garland. 2017. Preference for Western diet coadapts in High Runner mice and affects voluntary exercise and spontaneous physical activity in a genotype-dependent manner. Behav. Processes 135:56–65.

Allen Institute for Brain Science. 2004. Allen Mouse Brain Atlas.

Behbehani, M. M. 1995. Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 46:575–605.

Byun, J., M. R. Verardo, B. Sumengen, G. P. Lewis, B. S. Manjunath, and S. K. Fisher. 2006. Automated tool for the detection of cell nuclei in digital microscopic images: application to retinal images. Mol Vis 12:949–960.

Cahill, L. S., P. E. Steadman, C. E. Jones, C. L. Laliberté, J. Dazai, J. P. Lerch, B. Stefanovic, and J. G. Sled. 2015. MRI-detectable changes in mouse brain structure induced by voluntary exercise. NeuroImage 113:175–183.

Colcombe, S. J., K. I. Erickson, P. E. Scalf, J. S. Kim, R. Prakash, E. McAuley, S. Elavsky, D. X. Marquez, L. Hu, and A. F. Kramer. 2006. Aerobic exercise training increases brain volume in aging humans. J. Gerontol. A. Biol. Sci. Med. Sci. 61:1166–1170.

Copes, L. E., H. Schutz, E. M. Dlugosz, W. Acosta, M. A. Chappell, and T. Garland, Jr. 2015. Effects of voluntary exercise on spontaneous physical activity and food consumption in mice: results from an artificial selection experiment. Physiol. Behav. 149:86–94.

Devoogd, T. J., J. R. Krebs, S. D. Healy, and A. Purvis. 1993. Relations between Song Repertoire Size and the Volume of Brain Nuclei Related to Song: Comparative Evolutionary Analyses amongst Oscine Birds. Proc. Biol. Sci. 254:75–82.

Dishman, R. K., H.-R. Berthoud, F. W. Booth, C. W. Cotman, V. R. Edgerton, M. R. Fleshner, S. C. Gandevia, F. Gomez-Pinilla, B. N. Greenwood, C. H. Hillman, and others. 2006. Neurobiology of exercise. Obesity 14:345–356.

Dubreucq, S., A. Durand, I. Matias, G. Bénard, E. Richard, E. Soria-Gomez, C. Glangetas, L. Groc, A. Wadleigh, F. Massa, and others. 2013. Ventral tegmental area cannabinoid type-1 receptors control voluntary exercise performance. Biol. Psychiatry 73:895–903.

Franklin, K. B. J., and G. Paxinos. 1997. A stereotaxic atlas of the mouse brain. San Diego: Academic.

Garland, Jr., T., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proc. R. Soc. B Biol. Sci. 278:574–581.

Garland, Jr., T., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267–1275.

Garland, Jr., T., M. Zhao, and W. Saltzman. 2016. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integr. Comp. Biol. 56:207–224.

Hannon, R. M., S. A. Kelly, K. M. Middleton, E. M. Kolb, D. Pomp, and T. Garland, Jr. 2008. Phenotypic effects of the “mini-muscle” allele in a large HR x C57BL/6J mouse backcross. J. Hered. 99:349–354.

Hickmott, P. W., and P. A. Steen. 2005. Large-scale changes in dendritic structure during reorganization of adult somatosensory cortex. Nat. Neurosci. 8:140–142.

Hiramatsu, L., J. C. Kay, Z. Thompson, J. M. Singleton, G. C. Claghorn, R. L. Albuquerque, B. Ho, B. Ho, G. Sanchez, and T. Garland. 2017. Maternal exposure to Western diet affects adult body composition and voluntary wheel running in a genotype-specific manner in mice. Physiol. Behav. 179:235–245.

Ilango, A., A. J. Kesner, K. L. Keller, G. D. Stuber, A. Bonci, and S. Ikemoto. 2014. Similar roles of substantia nigra and ventral tegmental dopamine neurons in reward and aversion. J. Neurosci. 34:817–822.

Johnson, R. A., J. S. Rhodes, S. L. Jeffrey, T. Garland Jr, and G. S. Mitchell. 2003. Hippocampal brain-derived neurotrophic factor but not neurotrophin-3 increases more in mice selected for increased voluntary wheel running. Neuroscience 121:1–7.

Jónás, I., K. A. Schubert, A. C. Reijne, J. Scholte, T. Garland, M. P. Gerkema, A. J. W. Scheurink, C. Nyakas, and G. van Dijk. 2010. Behavioral Traits are Affected by Selective Breeding for Increased Wheel-Running Behavior in Mice. Behav. Genet. 40:542–550.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacol. Biochem. Behav. 101:528–537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland, Jr. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812–820.

Kelly, S. A., F. R. Gomes, E. M. Kolb, J. L. Malisch, and T. Garland. 2017. Effects of activity, genetic selection and their interaction on muscle metabolic capacities and organ masses in mice. J. Exp. Biol. 220:1038–1047.

Kolb, E. M., E. L. Rezende, L. Holness, A. Radtke, S. K. Lee, A. Obenaus, and T. Garland. 2013. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. J. Exp. Biol. 216:515–523.

Krebs, J. R., D. F. Sherry, S. D. Healy, V. H. Perry, and A. L. Vaccarino. 1989. Hippocampal specialization of food-storing birds. Proc. Natl. Acad. Sci. 86:1388–1392.

Lefebvre, L., P. Whittle, E. Lascaris, and A. Finkelstein. 1997. Feeding innovations and forebrain size in birds. Anim. Behav. 53:549–560.

Lerch, J. P., A. P. Yiu, A. Martinez-Canabal, T. Pekar, V. D. Bohbot, P. W. Frankland, R. M. Henkelman, S. A. Josselyn, and J. G. Sled. 2011. Maze training in mice induces MRI-detectable brain shape changes specific to the type of learning. NeuroImage 54:2086–2095.

Li, G., J. S. Rhodes, I. Girard, S. C. Gammie, and T. Garland, Jr. 2004. Opioid-mediated pain sensitivity in mice bred for high voluntary wheel running. Physiol. Behav. 83:515–524.

Maguire, E. A., D. G. Gadian, I. S. Johnsrude, C. D. Good, J. Ashburner, R. S. Frackowiak, and C. D. Frith. 2000. Navigation-related structural change in the hippocampi of taxi drivers. Proc. Natl. Acad. Sci. 97:4398–4403.

Mercuri, N. B., P. Calabresi, and G. Bernardi. 1992. The electrophysiological actions of dopamine and dopaminergic drugs on neurons of the substantia nigra pars compacta and ventral tegmental area. Life Sci. 51:711–718.

Reader, S. M., and K. N. Laland. 2002. Social intelligence, innovation, and enhanced brain size in primates. Proc. Natl. Acad. Sci. 99:4436–4441.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. Mitchell, and T. Garland. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and

fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 158:120–131.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integr. Comp. Biol. 45:438–455.

Rhodes, J. S., and T. Garland. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 167:242–250.

Rhodes, J. S., and T. J. Kawecki. 2009. Behavior and neurobiology. Exp. Evol. Concepts Methods Appl. Sel. Exp. Univ. Calif. Press Berkeley 263–300.

Rhodes, J. S., H. van Praag, S. Jeffrey, I. Girard, G. S. Mitchell, T. Garland Jr, and F. H. Gage. 2003. Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. Behav. Neurosci. 117:1006.

Roeper, J. 2013. Dissecting the diversity of midbrain dopamine neurons. Trends Neurosci. 36:336–342.

Scholz, J., R. Allemang-Grand, J. Dazai, and J. P. Lerch. 2015a. Environmental enrichment is associated with rapid volumetric brain changes in adult mice. NeuroImage 109:190–198.

Scholz, J., Y. Niibori, P. W Frankland, and J. P Lerch. 2015b. Rotarod training in mice is associated with changes in brain structure observable with multimodal MRI. NeuroImage 107:182–189.

Schultz, W. 1998. Predictive reward signal of dopamine neurons. J. Neurophysiol. 80:1–27.

Sumiyoshi, A., Y. Taki, H. Nonaka, H. Takeuchi, and R. Kawashima. 2014. Regional gray matter volume increases following 7days of voluntary wheel running exercise: A longitudinal VBM study in rats. NeuroImage 98:82–90.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998a. Artificial selection for increased wheel-running behavior in house mice. Behav. Genet. 28:227–237.

Swallow, J. G., T. Garland, Jr, P. A. Carter, W.-Z. Zhan, and G. C. Sieck. 1998b. Effects of voluntary activity and genetic selection on aerobic capacity in house mice (Mus domesticus). J. Appl. Physiol. 84:69–76.

Swallow, J. G., P. Koteja, P. A. Carter, and T. Garland, Jr. 1999. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. J. Exp. Biol. 202:2513–2520.

Swallow, J. G., J. S. Rhodes, and T. Garland, Jr. 2005. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. Integr. Comp. Biol. 45:426–437.

Swallow, J., P. Koteja, P. Carter, and T. Garland. 2001. Food consumption and body composition in mice selected for high wheel-running activity. J. Comp. Physiol. B 171:651–659.

Thompson, Z., D. Argueta, T. Garland, Jr., and N. DiPatrizio. 2017. Circulating levels of endocannabinoids respond acutely to voluntary exercise, are altered in mice selectively bred for high voluntary wheel running, and differ between the sexes. Physiol. Behav. 170:141–150.

Wallace, I. J., and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evol. Anthropol. Issues News Rev. 25:98–104.

Wahlsten, D. 1990. Insensitivity of the analysis of variance to heredity-environment interaction. Behavioral and Brain Sciences 13:109–120.

Wahlsten, D. 1991. Sample size to detect a planned contrast and a one degree-of-freedom interaction effect. Psychological Bulletin 110:587–595.

Yopak, K. E., and T. J. Lisney. 2012. Allometric scaling of the optic tectum in cartilaginous fishes. Brain. Behav. Evol. 80:108–126.

CHAPTER 2

Circulating levels of endocannabinoids respond acutely to voluntary exercise, are altered in mice selectively bred for high voluntary wheel running, and differ between the sexes.

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ABSTRACT

The endocannabinoid system serves many physiological roles, including in the regulation of energy balance, food reward, and voluntary locomotion. Signaling at the cannabinoid type 1 receptor has been specifically implicated in motivation for rodent voluntary exercise on wheels. We studied four replicate lines of high runner (HR) mice that have been selectively bred for 81 generations based on average number of wheel revolutions on days five and six of a six-day period of wheel access. Four additional replicate lines are bred without regard to wheel running, and serve as controls (C) for random genetic effects that may cause divergence among lines. On average, mice from HR lines voluntarily run on wheels three times more than C mice on a daily basis. We tested the general hypothesis that circulating levels of endocannabinoids (i.e., 2-arachidonoylglycerol [2-AG] and anandamide [AEA]) differ between HR and C mice in a sex-specific manner. Fifty male and 50 female mice were allowed access to wheels for six days, while another 50 males and 50 females were kept without access to wheels (half HR, half C for all groups). Blood was collected by cardiac puncture during the time of peak running on the sixth night of wheel access or no wheel access, and later analyzed for 2-AG and AEA content by ultra-performance liquid chromatography coupled to tandem mass spectrometry. We observed a significant three-way interaction among sex, linetype, and wheel access for 2-AG concentrations, with females generally having lower levels than males and wheel access lowering 2-AG levels in some but not all subgroups.

The number of wheel revolutions in the minutes or hours immediately prior to sampling did not quantitatively predict plasma 2-AG levels within groups. We also observed a trend for a linetype-by-wheel access interaction for AEA levels, with wheel access lowering plasma concentrations of AEA in HR mice, while raising them in C mice. In addition, females tended to have higher AEA concentrations than males.

For mice housed with wheels, the amount of running during the 30 minutes before sampling was a significant positive predictor of plasma AEA within groups, and HR mice had significantly lower levels of AEA than C mice. Our results suggest that voluntary exercise alters circulating levels of endocannabinoids, and further demonstrate that selective breeding for voluntary exercise is associated with evolutionary changes in the endocannabinoid system.

1. Introduction

The endocannabinoid system is involved in a variety of physiological processes, including regulation of motor behavior (Dietrich and McDaniel 2004). Motor behavior is tremendously diverse, encompassing voluntary exercise, consummatory behaviors, spontaneous physical activity (SPA) of various types, including "fidgeting" (Garland, Jr. et al. 2011b), and performance during measures of forced-exercise capacity (Claghorn et al. 2016). Studies of cannabinoid effects on motor behavior have utilized a variety of approaches, involving variation in testing apparatus, length of observation period, time of day, etc., and, not surprisingly, this has led to conflicting results. Depending on the apparatus and length of test, these studies may be gauging multiple aspects of motor behavior, including, in some cases, reactivity to a novel environment. Generally, systemic administration of cannabinoids leads to a decrease in activity. In male rats, for example, doses of Δ-9-tetrahydrocannabinol higher than 1 mg/kg caused a decrease in ambulation and rearing as measured in a five-minute novel open-field test (Järbe et al. 2002). However, another study, also usingΔ-9-tetrahydrocannabinol in male rats, reported a triphasic effect, where very low and very high doses (0.2 mg/kg and 2.5 mg/kg, respectively) reduced the number of photobeam breaks produced in either a horizontal or vertical direction, and moderate doses (1-2 mg/kg) increased the number of beam breaks as measured over one hour in an activity chamber (Sañudo-Peña et al. 2000).

Cannabinoids activate the cannabinoid type-1 (CB_1) and type-2 receptor (CB_2), the former being found in high density in brain areas that control movement (basal ganglia, substantia nigra, etc., Tsou et al. 1998), including those involved in both spontaneous physical activity and voluntary exercise (Garland, Jr. et al. 2011b). Recent research suggests that the CB_1 receptor is specifically involved in voluntary exercise. For example, male CB_1 receptor knockout mice exhibit a 30-40% reduction in voluntary wheel running, but no change in number of horizontal squares crossed during five minutes in a large, dimly-illuminated, novel "activity cage" (Dubreucq et al. 2010). In a subsequent study, in which the CB_1 receptor was deleted only from brain GABAergic neurons, mice showed a 25-30% decrease in wheel running and no difference in habituated locomotor activity measured over five days in a cage with infrared sensors to detect horizontal beam breaks (Dubreucq et al. 2013). Previous studies of CB_1 knockout mice, however, *have* shown differences in other tests of locomotor activity. CB_1 knockout mice spend more time immobile in a test of catalepsy, and showed fewer beam breaks during an open-field test (with undescribed parameters)(Zimmer et al. 1999). A separate experiment found that CB_1 knockout mice had a significant decrease of ambulatory movements, as measured in a dimly-lit box with photocells placed to measure both horizontal and vertical movements (in this experiment, animals were measured for 15 minutes on several different days after being habituated to the cage) (Martin et al. 2000).

Limited evidence suggests that the endocannabinoid system may specifically affect motivation for voluntary exercise. The two primary endocannabinoids in humans and other mammals are 2-arachidonylglycerol (2-AG) and anandamide (AEA). Both 2-AG and AEA can cross the blood-brain barrier, bind to the CB_1 receptor, and cause dopamine release in areas involved in reward signaling (such as the nucleus accumbens, reviewed in Gardner 2005). After rats were trained to press a door to unlock wheel access (as a reward), systemic administration of 2-AG reduced the number of times that obese rats would press the door to gain wheel access before giving up, and also reduced the revolutions run once the rats were in the wheel [both obese and lean individuals (Smith and Rasmussen 2010)]. In a similar study, rimonabant (a CB_1 antagonist/inverse agonist) caused a similar reduction in number of door-presses prior to giving up, but did not affect number of revolutions run once rats did gain wheel access (Rasmussen and Hillman 2011). In addition, signaling through the CB_1 receptor produces analgesia in both peripheral and central sites (reviewed in Dietrich and McDaniel 2004). Analgesia might interact with motivation per se by reducing pain that could occur during exercise (e.g., see Li et al. 2004; Fuss et al. 2015).

The endocannabinoid system is also activated by exercise. Several studies have examined how circulating levels of both 2-AG and AEA respond to exercise. In general, exercise increases levels of AEA in the blood, but does not seem to affect levels of 2-AG (Sparling et al. 2003; Feuerecker et al. 2012;

Heyman et al. 2012; Raichlen et al. 2012, 2013). Most previous studies of circulating endocannabinoid levels have involved forced exercise, which may cause "stress" relative to voluntary exercise, or confound the effects of stress with exercise (Girard and Garland, Jr. 2002; Brown et al. 2007). Arguably, no (ethical) study of humans involves “forced” exercise, but an examination of the studies cited above shows that subjects were not allowed to choose the quantity, length of time, and/or the speed at which they exercised (but see Feuerecker et al. 2012), which may cause stress in either the psychological or physical sense. Voluntary exercise has been examined in two animal studies. One small-scale study (n = 3 per group; sex not reported) measured mice after they were allowed wheel access for 3 h/day for 8 days, with samples taken after 30 minutes of running. Plasma levels of both endocannabinoids tended to be lower in the running mice as compared with mice housed with locked wheels, although the differences were non-significant (Chaouloff et al. 2012). One additional study of rats used voluntary exercise, and although they did not measure circulating endocannabinoids, they did find an increase in AEA in the hippocampus after 8 days of wheel access (Hill et al. 2010).

The endocannabinoid system may also be altered in response to selective breeding for voluntary exercise behavior. An ongoing artificial selection experiment (currently in generation 81) has produced four replicate high-runner (HR) lines while also maintaining four non-selected control (C) lines (Swallow et al. 1998; Wallace and Garland, Jr. 2016; Garland, Jr. et al. 2017). In the routine

breeding protocol, mice are allowed access to wheels for six days as young adults, and mice in the HR lines are selected to breed based on the average number of wheel revolutions on days five and six. Mice in the control lines are bred without regard to how much they run. Mice from the HR lines run about three times as much on a daily basis as compared with C mice, which they accomplish primarily by running faster (Girard et al. 2001; Garland, Jr. et al. 2011a; Careau et al. 2013). In addition, female mice run more than male mice, which they also accomplish by running faster (Koteja and Garland, Jr. 2001). When given a systemic injection of a CB_1 receptor antagonist (SR141716; rimonabant), HR female mice decreased their running more than C female mice over the next hour, which was accomplished by a decrease in total revolutions, average speed and maximum speed, but not amount of time spent running (Keeney et al. 2008). Male HR and C mice did not show a differential response. In contrast, when given a systemic injection of a CB_1 receptor agonist (WIN 55,212-2), both female and male HR mice showed a differential decrease in running (compared to C mice; (Keeney et al. 2012). For two hours after injection, female HR mice had a reduction in total revolutions, average speed, and maximum speed. In the second hour they also decreased their time spent running. Male HR mice showed decreased total revolutions, average speed and maximum speed during the entire two hours after injection. As a CB_1 receptor agonist and antagonist both decreased running in HR mice in different ways in a

sex-specific manner, it is difficult to infer from these studies alone precisely how the endocannabinoid system has changed in response to selective breeding.

One possible cause of differential responses to pharmacological manipulation could be differences in circulating levels of endocannabinoids (alternatively, tissue-specific receptor densities or receptor sensitivity might have evolved). In addition, circulating concentrations of endocannabinoids might show different quantitative responses to exercise, either between the sexes or potentially between HR and control lines of mice. The purpose of the present study was, therefore, to measure circulating endocannabinoid levels in mice given access to wheels, as compared with those housed in standard cages. We studied both male and female HR and C mice given access to wheels for six days (as used in the regular selection protocol), or housed without wheels for six days, and sampled during the time of peak running. We expected that circulating levels of endocannabinoids (2-AG and AEA) would differ between HR and C mice, possibly in a sex-specific manner. We also predicted that the amount of wheel running might be a quantitative predictor of circulating endocannabinoid levels. We also tested the relationship between the plasma concentrations of 2-AG and AEA at the level of individual variation within groups as well as among the average values for the eight subgroups (combinations of sex, linetype, and wheel access).

2. Materials & procedures

2.1. Ethical approval

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

2.2. Experimental animals

Mice were taken from a long-term artificial selection experiment on voluntary wheel running that was started in 1993 (Swallow et al. 1998). Originally, outbred Hsd:ICR mice (*Mus domesticus*) were obtained from Harlan Sprague Dawley (Indianapolis, Indiana, USA). These mice were randomly split into eight closed lines, with four designated to become “high runner” (HR) and four designated as “control” (C) lines. All mice are allowed access to wheels for six days and HR mice are chosen to breed based on their average number of wheel revolutions on days five and six. Control mice are bred without regard to wheel running. HR mice now run on average three times as much as C mice do, primarily by running faster.

For the present study, 50 male and 50 female mice from generation 74 (half HR and half C) were allowed access to wheels for six days, while another 50 males and 50 females (also half HR and half C) were kept without access to wheels. Wheel revolutions for mice with wheels were recorded for 23 hours per day, and home-cage activity was recorded for all mice. On the sixth day of the study, animals were anesthetized with isoflurane and blood samples were taken

by cardiac puncture. Animals were on a reversed photoperiod, with lights off from 7 am to 7 pm, so that sampling could occur during the time of peak wheel running, which starts approximately 2 hours after lights are turned off (Girard et al. 2001; Girard and Garland, Jr. 2002; Malisch et al. 2008, 2009). Sampling occurred from ~9 am to 1 pm (from 2 hours after lights off to 6 hours after lights off). The age range at the time of sampling was 71-91 days old.

Mini-muscle status was determined for each mouse by dissection and weighing of the triceps surae muscles at the end of the experiment. The "mini-muscle" phenotype is caused by a recessive allele that, when homozygous, reduces triceps surae and total hindlimb muscle mass by ~50% (Garland, Jr. et al. 2002). However, the mass-specific aerobic capacity of the muscles is approximately doubled (Houle-Leroy et al. 2003). Mini-muscle individuals tend to run faster, but for fewer minutes per day, as compared with unaffected individuals (Garland, Jr. et al. 2002; Houle-Leroy et al. 2003; Swallow et al. 2005; Syme et al. 2005; Kelly et al. 2006; Hannon et al. 2008). In addition, mini-muscle individuals have larger internal organs than normal-muscled mice (liver, kidneys, and heart ventricles: (Garland, Jr. et al. 2002; Swallow et al. 2005)). As the mini-muscle phenotype clearly has pleiotropic effects, mini-muscle status was used as a cofactor in all analyses.

2.3. Wheel running

Mice were housed individually during the experimental period and half were provided with cages with a hole for wheel access. The wheels used were the same as in the regular selection protocol (Swallow et al. 1998) – Wahman-type activity wheels with a circumference of 1.12 meters. Wheel revolutions are recorded automatically for 23 hours per day (one hour is used to download data and check mice), and a measure of wheel freeness was used as a covariate in all analyses of wheel running. We did not choose to provide locked wheels for the mice without wheel access, as we have shown that when HR mice are housed with locked wheels, they climb more than do mice from the non-selected Control lines (Koteja et al. 1999), so locked wheels, unfortunately, provide more than just "environmental enrichment."

2.4. Home-cage activity

Home-cage activity (HCA) was measured using passive infrared sensors that detect motion. These sensors give readings 3 times per second – either 0 (no movement) or 1 (movement). Readings are then averaged over a one-minute interval and reported using arbitrary units. We used a similar setup and calibration procedure as previously reported (Acosta et al. 2015; Copes et al. 2015). HCA was measured for 23 hours per day and all analyses were performed using a measure of sensor sensitivity as a covariate (Acosta et al. 2015; Copes et al. 2015).

2.5. Measurement of plasma 2-AG and AEA

Isoflurane was used to anesthetize animals prior to tissue harvest. Blood was collected by cardiac puncture and stored in EDTA-lined tubes on ice, and then plasma was obtained by centrifugation (1500 *g* for 10 minutes, maintained at 4°C). All samples were stored at -80° C until processing. One hundred microliters (µL) of plasma was used for extraction of lipids in one milliliter (mL) of methanol containing the following internal standards: [$^2H_5$]-2-AG and [$^2H_4$]-AEA (Cayman Chemical, Ann Arbor, MI, USA). 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 (LC/MS/MS) 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. Lipids were separated using an Acquity UPLC BEH C_{18} column (50 x 2.1 mm; i.d. 1.7 µm), eluted by a gradient of methanol (0.25% acetic acid, 5 millimolar [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 were maintained in the sample manager at 10˚ C. Argon was used as collision gas. 2-AG, [$^2H_5$] 2-AG,

AEA, and [$^2H_4$]-AEA were identified in the positive ionization mode, based on their retention times and MS^2 properties, using authentic standards (Cayman Chemical) as references. Multiple reaction monitoring was used to acquire full-scan tandem MS spectra of selected ions. Extracted ion chromatograms were used to quantify 2-AG (*m/z* = 379.3>287.3), AEA (*m/z* = 348.3>62.04), and [$^2H_5$] 2-AG (*m/z* = 384.3 > 93.4) and [$^2H_4$]-AEA (*m/z* = 352.3>66.11), which were used as internal standards.

2.6. Statistical analyses

Following numerous previous studies of these eight lines of mice (Swallow et al. 1998; Rhodes et al. 2000), plasma endocannabinoid concentrations were analyzed by nested analysis of covariance (ANCOVA), with line nested within linetype (HR vs C) as a random effect, and with covariates of age and time of day that plasma sampling occurred (SAS Procedure Mixed).

For the individuals with wheel access, we repeated the foregoing analyses with amount of wheel running (revolutions/unit time) as a covariate. Although we expected a possible quantitative relationship with the amount of exercise prior to sampling, we could not predict the precise nature of this relationship. Therefore, we computed the number of wheel revolutions in each minute before plasma sampling, from 1 to 10 minutes before, and then in 10-minute bins from 10 to 120 minutes before sampling. We fitted models using each of these alternative covariates and examined the restricted maximum log-likelihood (REML) values

from each analysis to determine which time period of summed running effort provided the best fit. For wheel running, the number of revolutions in the previous 30 minutes before plasma sampling provided the best fit and was used in all analyses with wheel running as a covariate. For consistency, we also used the amount of home-cage activity in the previous 30 minutes as a covariate in certain analyses.

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 $p \leq 0.05$. 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 are presented in figures and 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 Proc Mixed, unadjusted for multiple comparisons.

3. Results

3.1. Wheel running

Day five of the wheel access period was analyzed, as mice were removed and blood collected in the middle of night six (Fig. 2.1). As expected, HR mice ran more than C mice (p = 0.0004). Neither the effect of sex (p = 0.1431) nor the sex by linetype interaction (p = 0.1576) was significant. Wheel freeness, mini-muscle status, and age were not statistically significant predictors of wheel running (results not shown).

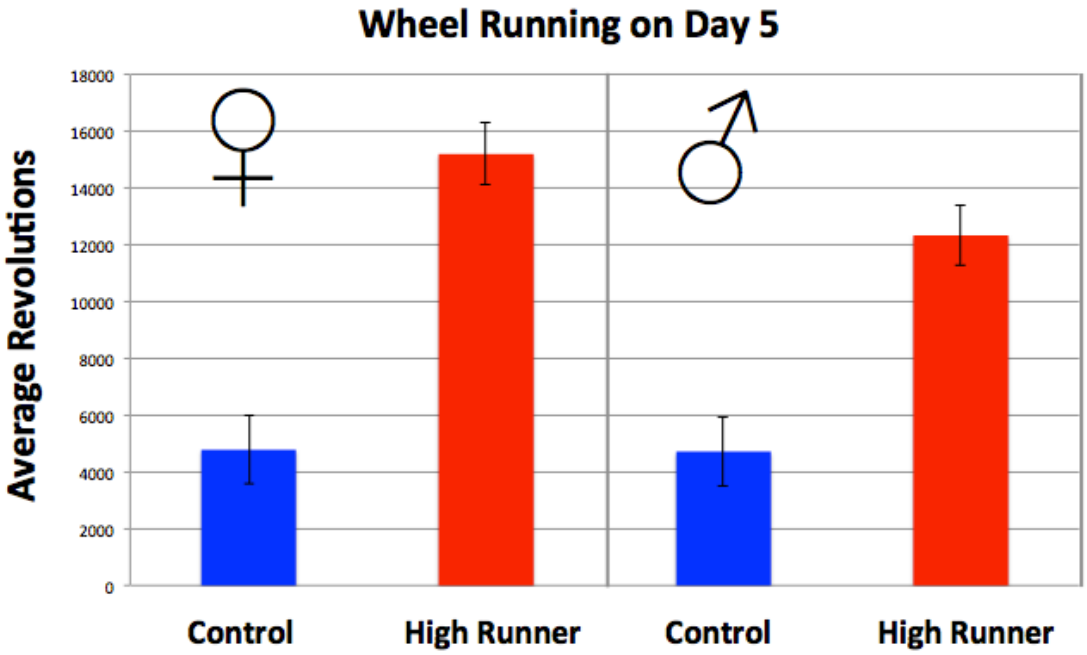

Figure 2.1. Wheel running on day five of experiment. Values are LS means +/- standard error from SAS Proc Mixed. N = 90.

3.2. Home-cage activity

Data from day five of the experiment were analyzed, as day six was interrupted when mice were removed from cages for collection of blood. Values from each minute were summed over 23 hours, then $\log_{10}$ transformed to improve normality of the residuals. In the three-way analysis of covariance (Fig. 2.2, Table 2.1), mice with wheel access always had reduced HCA ($p = 0.0001$), this reduction was greater in HR than in C mice (wheel access by linetype interaction, $p = 0.0558$), females always had higher HCA than males ($p = 0.0047$), and HR mice always had higher HCA than C mice ($p = 0.007$). In addition, mice with the mini-muscle trait had lower HCA than those that did not ($p = 0.018$). Age and a measure of sensor sensitivity were not significant predictors of home-cage activity. We also tested body mass as an additional covariate, but it had no statistical effect ($p > 0.50$) and caused little change in the significance levels of the other factors and covariates (results not shown).

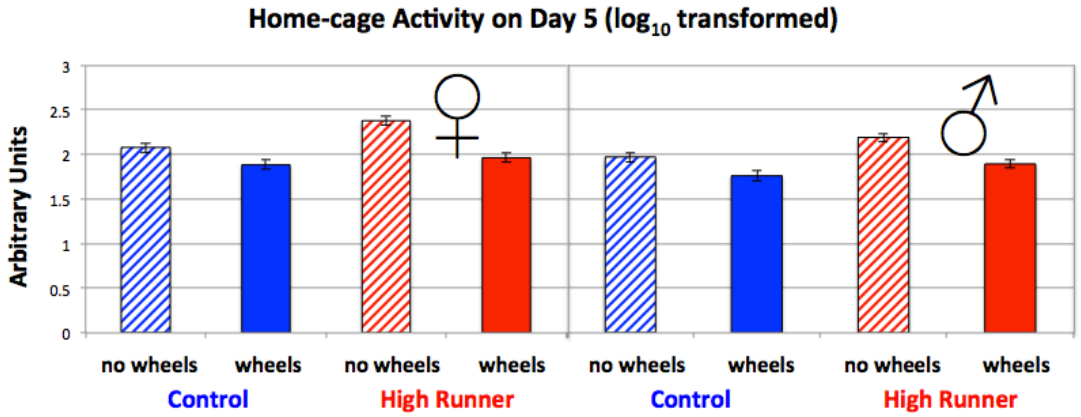

Figure 2.2. HCA on day 5 of experiment. See Table 2.1 for statistical results. Mice with wheel access always had reduced HCA (p = 0.0001), this reduction was greater in HR than in C mice (linetype by wheel access interaction, p = 0.0558), females always had higher HCA than males (p = 0.0047), and HR mice always had higher HCA than C mice (p = 0.007). Values are LS means (log_{10} transformed) +/- standard error from SAS Proc Mixed. N = 190.

Table 2.1. Three-way analysis of covariance of home-cage activity on day 5 (log_{10} transformed) (N = 190). See Figure 2 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	19.22	0.0047
Linetype	1,6	16.08	0.0070
Wheel Access	1,6	73.52	0.0001
Sex*Linetype	1,6	0.07	0.8043
Sex*Wheel Access	1,6	0.81	0.4029
Linetype*Wheel Access	1,6	5.60	0.0558
Sex*Linetype*Wheel Access	1,6	1.49	0.2681
Mini-muscle	1,155	5.72	0.0180
Age	1,155	0.53	0.4695
HCA sensor sensitivity	1,155	0.62	0.4306

3.3. Plasma 2-AG concentrations

Females had lower levels of 2-AG than males in all four experimental groups, and the main effect of sex was significant (p = 0.0265). Figure 2.3 illustrates the significant three-way interaction among sex, linetype, and wheel access (p = 0.0408). Levels of 2-AG are lower in mice with wheel access, except for C males. However, when examining the pairwise comparisons, 2-AG levels were significantly lower for mice with wheel access only for the C female group (p = 0.0301). The main effect of linetype was not significant (p = 0.4604).

Separate analyses of mice with (N = 92) and without (N = 94) wheels (not including amount of physical activity as covariates) indicated that females had significantly lower levels of 2-AG only when they had wheel access (p = 0.0169 for mice with wheels, p = 0.1235 for mice without wheels).

Mini-muscle status was not a significant factor for any analyses, but both time of day that plasma sampling occurred and age at time of plasma sampling were significant in the overall analysis and the analysis of mice without wheel access. Only time of day was significant for the analysis of mice with wheels. Plasma 2-AG concentrations tended to be higher later in the sampling period, which ranged from ~9 am to 1 pm. Age was positively correlated with 2-AG levels (age range was 71-91 days old).

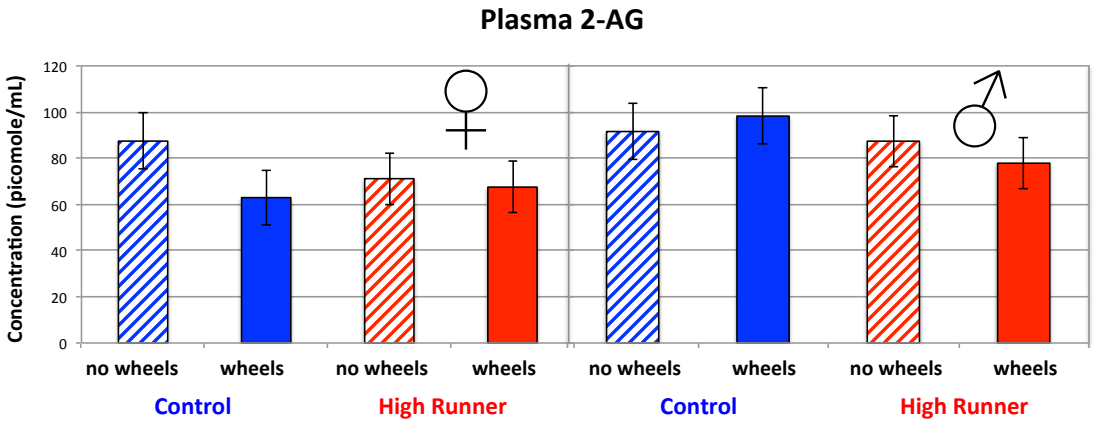

Figure 2.3. Levels of 2-AG in mouse plasma collected during peak activity on the 6th night of wheel running. See Table 2.2 for statistical results. The three-way interaction among sex, linetype, and wheel access was statistically significant (p = 0.0408), with females also having lower levels than males (p = 0.0265). Values are LS means +/- standard error from SAS Proc Mixed. N = 189.

Table 2.2. Three-way analysis of covariance of plasma concentration of 2-AG (N = 189). See Figure 2.3 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	8.56	0.0265
Linetype	1,6	0.62	0.4604
Wheel Access	1,6	2.42	0.1711
Sex*Wheel Access	1,6	3.04	0.1316
Sex*Linetype	1,6	0.33	0.5883
Linetype*Wheel Access	1,6	0.05	0.8244
Sex*Linetype*Wheel Access	1,6	6.75	0.0408
Mini-muscle	1,147	0.71	0.4021
Age	1,147	11.34	0.0010
Time of Day	1,147	8.23	0.0047

3.4. Plasma 2-AG concentrations with wheel running as a covariate

The first analysis was restricted to mice housed with wheel access. The amount of wheel running (transformed to the 0.4 power to reduce positive skew) in the previous 30 minutes (before the cardiac puncture and plasma sample) was added as a covariate to the statistical models indicated above. Amount of prior running was not a significant predictor of 2-AG levels, nor was the amount of home-cage activity during this period (also transformed to the 0.4 power), but females still had significantly lower 2-AG concentrations than males (p = 0.0274), with no difference between HR and Control lines (Table 2.3). Age was still a significant positive predictor, but time and mini-muscle status were not.

Table 2.3. Plasma concentration of 2-AG with wheel running as a covariate (N = 92).

Effect	d.f.	F	P
Sex	1,6	8.39	0.0274
Linetype	1,6	0.24	0.6446
Sex*Linetype	1,6	2.41	0.1713
Mini-muscle	1,64	3.77	0.0567
Age	1,64	19.12	<.0001
Time of Day	1,64	3.30	0.0742
Running in previous 30 min	1,64	0.57	0.4518
HCA in previous 30 min	1,64	1.40	0.2405

To test whether acute physical activity had a different effect than the 5 days of wheel access, we reran the analysis with the amount of wheel running and home-cage activity in the previous 30 minutes as covariates, but this time also including the mice housed without wheels and assigning values of zero for their wheel running (as in Copes et al. 2015). In this analysis (N = 186), neither measure of physical activity was a significant predictor of 2-AG concentrations (both $p > 0.5$), and the effects of sex ($p = 0.0252$) and the 3-way interaction ($p = 0.0544$) remained similar to those reported in Table 2.2.

3.5. Plasma AEA concentrations

Females tended to have higher levels of AEA than males (p = 0.0599, Figure 2.4, Table 2.4). A linetype by wheel access interaction (p = 0.0628) indicated that wheel access tended to increase levels of AEA in C mice (and decrease levels in HR males).

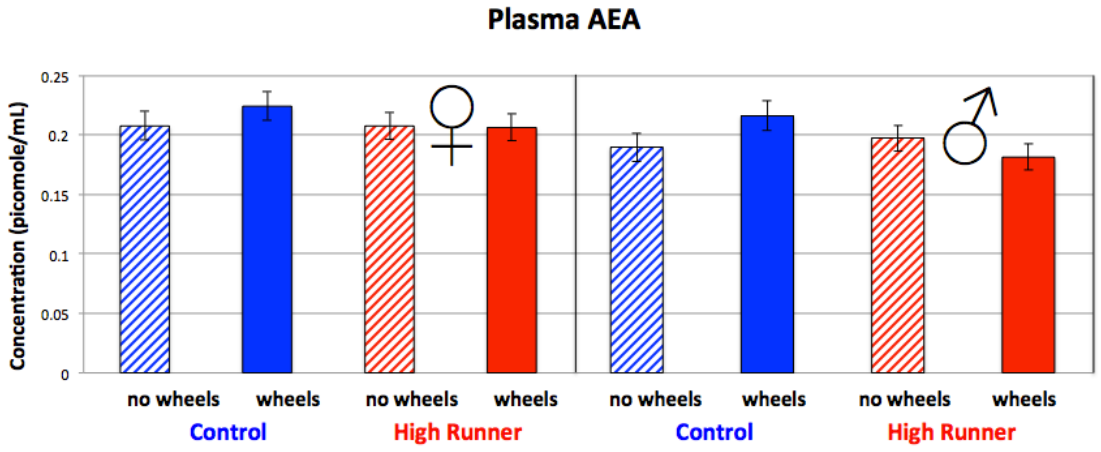

Figure 2.4. Levels of AEA in mouse plasma collected during peak activity on the 6th night of wheel running. See Table 2.4 for statistical results, which indicated a linetype by wheel access interaction (p = 0.0628). Values are LS means +/- standard error from SAS Proc Mixed. N = 185.

Table 2.4. Three-way analysis of covariance of plasma concentration of AEA (N = 185). See Figure 2.4 for graphical representation of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	5.36	0.0599
Linetype	1,6	2.29	0.1809
Wheel Access	1,6	0.92	0.3741
Sex*Wheel Access	1,6	0.04	0.8550
Sex*Linetype	1,6	0.12	0.7451
Linetype*Wheel Access	1,6	5.20	0.0628
Sex*Linetype*Wheel Access	1,6	0.86	0.3886
Mini-muscle	1,143	1.35	0.2476
Age	1,143	0.00	0.9484
Time of Day	1,143	82.97	<.0001

Separate analyses of mice with and without wheels revealed a trend for females to have higher levels only when they were housed without wheels (p = 0.0683 without wheels, p = 0.1637 with wheels). In addition, there was a trend for HR mice to have lower levels when housed with wheels (p = 0.0883 with wheels, p = 0.8950 without wheels).

Mini-muscle status and age at time of plasma sampling were not significant factors for any analyses. Time of day that plasma sampling occurred was significant for all three analyses (overall, mice with wheels, mice without wheels), indicating reduced values later in the sampling period.

3.6. Plasma AEA concentrations with wheel running as a covariate

The amount of wheel running (raised to the 0.4 power) was a highly significant positive predictor (p = 0.0043) of the amount of plasma AEA (Table 2.5, Fig. 2.5), home-cage activity was a negative predictor (also raised to the 0.4 power, p = 0.0383), and HR mice had significantly lower plasma AEA concentrations than C mice (p = 0.0235), with no difference between the sexes. Both age (positive) at the time of plasma sampling and time of day that sampling occurred (negative) were also significant predictors of plasma AEA.

Table 2.5. Plasma concentration of AEA with wheel running as a covariate (N = 89; see also Fig. 2.5).

Effect	d.f.	F	P
Sex	1,6	2.97	0.1354
Linetype	1,6	9.11	0.0235
Sex*Linetype	1,6	0.33	0.5859
Mini	1,61	0.02	0.9029
Age	1,61	4.89	0.0307
Time of Day	1,61	33.95	<.0001
Running in previous 30 min	1,61	8.79	0.0043
HCA in previous 30 min	1,61	4.49	0.0383

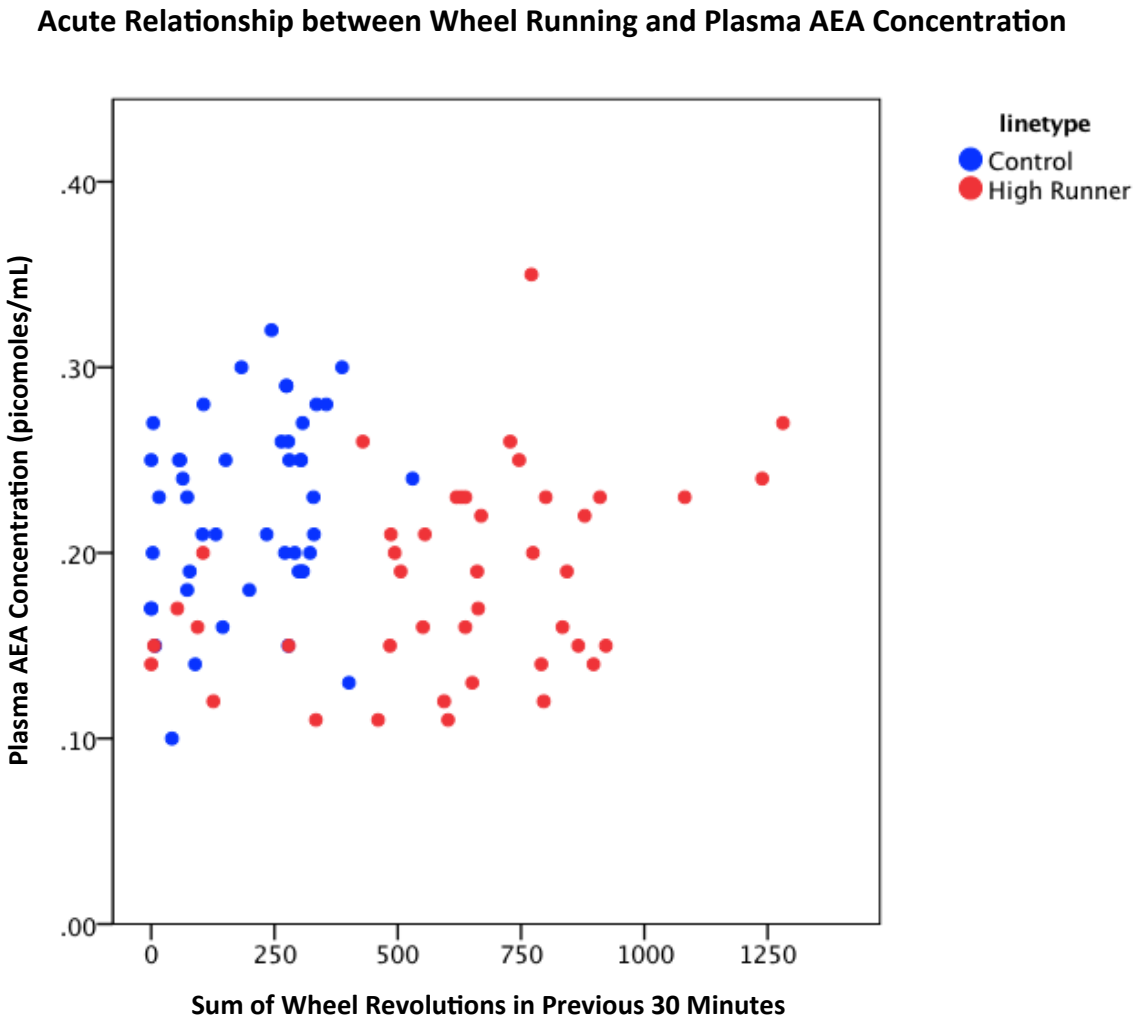

Figure 2.5. Plasma AEA concentration from mice with wheel access as a function of the number of wheel revolutions in the 30 minutes prior to plasma sampling (N = 89). The number of wheel revolutions (transformed to the 0.4 power for statistical analyses [Table 2.5], but shown here as raw values) was a significant positive predictor of AEA values (p = 0.0043), home-cage was a negative predictor (p = 0.0383), and HR mice had lower levels than C mice after adjusting for these relationships (Table 2.5). The interactions between linetype and amount of wheel running or home-cage activity were not statistically significant (results not shown and these terms not included in final statistical model).

For the analysis including mice housed without wheel access (assigning them values of zero revolutions, as also done for plasma 2-AG concentrations - see *Section 3.4* above), the amount of wheel running was still a significant positive predictor of AEA concentrations (p = 0.0044), home-cage activity became non-significant (p = 0.1954, negative effect), the effects of sex (p = 0.0548) and wheel access (p = 0.0503) were marginally non-significant, and the wheel access by linetype interaction was significant (p = 0.0110). In this analysis, wheel access lowered AEA levels in all mice, but much more so in HR lines than in Control lines (Table 2.6, Figure 2.6).

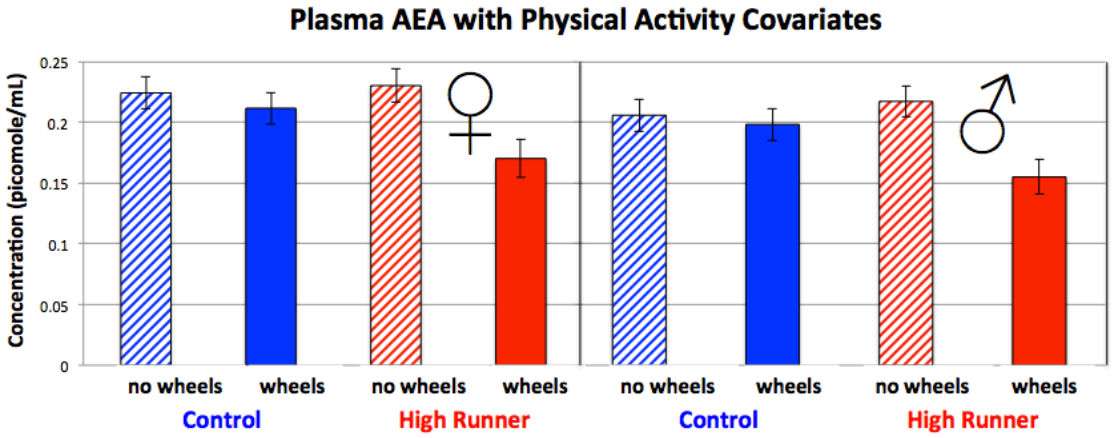

Figure 2.6. Levels of AEA in mouse plasma collected during peak activity on the 6th night of wheel running, with amount of wheel running and home-cage activity used as covariates (see text), and including mice without wheels in the analysis by assigning them zero for wheel revolutions run. Values are LS means +/- standard error from SAS Proc Mixed, based on analyses presented in Table 2.6. N = 183.

Table 2.6. Analysis of covariance of plasma concentration of AEA with physical activity covariates (N = 183). See Figure 2.6 for graph of adjusted group means.

Effect	d.f.	F	P
Sex	1,6	5.66	0.0548
Linetype	1,6	3.93	0.0946
Wheel Access	1,6	5.97	0.0503
Sex*Wheel Access	1,6	0.01	0.9080
Sex*Linetype	1,6	0.02	0.8906
Linetype*Wheel Access	1,6	13.17	0.0110
Sex*Linetype*Wheel Access	1,6	0.09	0.7796
Mini-muscle	1,139	1.08	0.3011
Age	1,139	1.01	0.3175
Time of Day	1,139	72.65	<.0001
Running in previous 30 min	1,139	8.38	0.0044
HCA in previous 30 min	1,139	1.69	0.1954

3.7. Relationship between plasma 2-AG and AEA concentrations

We examined the relationship between circulating levels of these two endocannabinoids in several ways, as also shown in the online Supplemental material. Overall, these analyses indicate that 2-AG and AEA concentrations tend to be positively related.

First, considering the raw values for all 191 mice, the Pearson correlation was 0.175, 2-tailed $p = 0.0155$. Second, we analyzed the correlation within each of the eight subgroups (N = 23-24 per group), and found values ranging from -

0.07 to + 0.54, with only one of the eight correlations differing significantly from zero by a 2-tailed test (Control females housed with wheel access r = 0.535, N = 24, p = 0.007). The mean value of the eight correlations was 0.207 with a standard error of 0.0815 (Supplemental material), suggesting an overall positive correlation, on average. Third, we computed simple mean values for each of the eight subgroups (linetype by sex by wheel access) and found no statistically significant correlation (r = -0.199, p = 0.636).

Fourth, we used the model presented in Table 2.2 for 2-AG, and added concentrations of AEA as an additional covariate. In this model, AEA is a highly significant positive predictor of 2-AG levels (p < 0.0001). We then performed the reciprocal analysis, adding 2-AG levels as an additional independent variable for the model presented in Table 2.4, and found the same thing: 2-AG is a highly significant positive predictor of AEA levels (p < 0.0001).

Fifth, we repeated this procedure for the model shown in Table 2.3 for 2-AG, and found that AEA levels were again a highly significant positive predictor of 2-AG (p < 0.0001). We did the same for the model in Table 2.5 for AEA, and again found that 2-AG levels were a positive predictor (p = 0.0003).

Sixth, we compared residuals from the models shown in Tables 2 and 4, and found the following correlation: r = 0.347, N = 184, p = 0.000001. Seventh, we compared residuals from Tables 3 and 5, and found r = 0.430, N = 89, p = 0.000026.

4. Discussion

Much remains to be understood regarding the role of the endocannabinoid system in exercise behavior and physiology. In the present study, we used selectively bred lines of mice to test four specific hypotheses regarding circulating levels of endocannabinoids, i.e., that they would *(i)* differ between the sexes, *(ii)* be affected by selective breeding for high levels of voluntary exercise on wheels, *(iii)* change following six days of wheel access, and *(iv)* be affected by the acute amount of wheel running or home-cage activity immediately prior to sampling. We measured plasma concentrations of two endocannabinoids, 2-AG and AEA, during the time of normal peak wheel running. Our results indicate that circulating levels of 2-AG and AEA differ *(i)* between sexes, *(ii)* between selectively bred HR and non-selected C lines of mice, *(iii)* are affected by six days of wheel access (training effect), and *(iv)* are affected acutely by physical activity. Furthermore, some of these effects differ between 2-AG and AEA. Finally, we tested the relationship between the plasma concentrations of 2-AG and AEA at the level of individual variation within groups as well as among the average values for the eight subgroups, and found evidence that the two endocannabinoids tend to covary positively.

4.1. Physical activity

As expected from numerous previous studies (e.g., Rhodes et al. 2000, 2003; Girard et al. 2001; Malisch et al. 2009; Copes et al. 2015), HR mice of both

sexes ran much more than Control mice on day five of wheel access (Fig. 2.1). Also, as reported previously for HR and Control mice (Acosta et al. 2015; Copes et al. 2015), housing with wheel access reduced the amount of home-cage activity measured simultaneously (Fig. 2.2 and Table 2.1). Irrespective of housing condition, HR mice had higher HCA than did Control mice, again consistent with previous reports that studied mice only when housed without wheel access (Rhodes et al. 2001b; Malisch et al. 2008, 2009). In addition, irrespective of housing condition, females of both linetypes had higher HCA than males, a difference not reported previously when mice were housed and tested without wheels, using a different apparatus (Malisch et al. 2009). Finally, mice with the mini-muscle phenotype had lower HCA than those that did not (Table 2.1), an effect not observed previously (Copes et al. 2015).

4.2. Training effects on plasma endocannabinoid levels

To our knowledge, the effects of several days of training (physical conditioning) on plasma endocannabinoid levels have not previously been reported for both male and female mice. Our combined analyses of all groups indicated a three-way interaction among sex, linetype, and wheel access for 2-AG levels (Table 2.2). However, it is important to note that our sampling design included 2-4 hours of acute exercise prior to blood sampling, in addition to the five prior days of wheel access, which could confound training effects with acute exercise effects. Hence, we also conducted analyses of 2-AG levels that

included the acute amounts of wheel running and home-cage activity as covariates, and found that they showed the same three-way interaction (see Results Section 3.4). Thus, our results show that, with respect to circulating 2-AG concentrations, even when taking differences in acute exercise into account, the endocannabinoid system responds to exercise differently based on sex and genetic background, and these differences may be a result of the exercise training undergone by the animal.

For plasma AEA levels, we found an interaction between linetype and wheel access, with wheel access lowering AEA levels for HR mice but raising them for C mice (Figure 2.4, Table 2.4). Analyses that included the acute amounts of wheel running and home-cage activity as covariates indicated a stronger two-way interaction and a trend for an overall decrease in AEA levels for mice housed with wheel access (Table 2.6, Fig. 2.6). Interestingly, however, the effect of acute wheel running was highly significant and positive within groups (Fig. 2.6). Thus, our results show that, for AEA, the effects of voluntary exercise on the endocannabinoid system differ acutely versus chronically.

Physical conditioning in response to aerobic exercise has been studied extensively in both rodents and humans, although typically over the course of weeks rather than days (Harpur 1980; Saltin and Gollnick 1983; Scribbans et al. 2016; Stanford and Goodyear 2016). Only two previous studies of rodents have examined endocannabinoids in relation to voluntary exercise that lasts for days, and these support our general finding that the endocannabinoid system can

"train" in response to physical activity. Eight days of wheel access for male rats was associated with increased AEA levels in the hippocampus, but not the prefrontal cortex, and no effects on 2-AG levels in either brain region (Hill et al. 2010). In addition, CB_1 receptor density was increased in the hippocampus. However, the authors did not examine plasma levels of endocannabinoids. An additional small study measured endocannabinoid levels in mice after they were allowed to run for three hours per day for eight days, and compared them to mice who were in locked wheels for the same amount of time (Chaouloff et al. 2012). They did not find any significant differences in plasma endocannabinoid levels, although AEA was decreased in the hippocampus of running mice. However, since these animals also experienced acute exercise right before measurements were done, there is no way to tell if these differences are due to the acute or chronic effects of exercise.

Aside from training effects per se, several studies have now shown that AEA levels are raised after acute exercise (Sparling et al. 2003; Feuerecker et al. 2012; Heyman et al. 2012; Raichlen et al. 2012, 2013). The same studies generally suggest that 2-AG levels are not raised after acute exercise (Heyman et al. 2012), although three studies have reported a non-significant trend for raised 2-AG levels in humans and dogs (Sparling et al. 2003; Feuerecker et al. 2012; Raichlen et al. 2012). Finally, dogs and ferrets did not show significant increases in plasma 2-AG levels following treadmill exercise (Raichlen et al. 2012). Thus, it appears that species of mammals may differ in the extent to

which exercise acutely alters circulating 2-AG levels. Alternatively, some of the apparent species differences could be explained by methodological differences related to the exercise intensity imposed on, or chosen by, the running subjects. For example, Raichlen and colleagues (2013) measured four different levels of exercise intensity in humans and found that AEA levels only increased for the two middle intensities. In addition, the amount of training experienced by the individual may affect how the endocannabinoid system reacts to acute exercise. Although plasma AEA was positively correlated with the amount of wheel running for both C and HR mice, there was a significant effect of linetype, with HR mice having lower levels of AEA even though they run more. This finding may also indicate that selective breeding for voluntary exercise has caused associated evolutionary changes in how the endocannabinoid system responds to exercise.

4.3. Sex differences in plasma endocannabinoid levels

We found that female mice have lower levels of 2-AG than males, especially when exercising, and tend to have higher levels of AEA (Figs. 3-5). Sex differences are not unexpected, given previous studies on these mice that show sex differences in the response of wheel running to CB_1 agonists and antagonists (Keeney et al. 2008, 2012). Aside from those studies on the HR and C lines of mice, most previous studies of plasma endocannabinoids have used only males (Sparling et al. 2003; Feuerecker et al. 2012; Heyman et al. 2012). Two studies of humans included both males and females, but did not test for sex

differences (Raichlen et al. 2012, 2013). Studies in rats show that females, especially adolescents, are more vulnerable than males to disruption of CB_1 signaling by repeated exposure to THC (Burston et al. 2010), and studies of humans indicate that females are more sensitive than males to the effects of cannabis (Craft et al. 2013).

4.4. Time-of-day effects

Plasma 2-AG concentrations tended to be higher later in the sampling period, which ranged over approximately four hours during the early part of the dark phase, the time when mice are normally most active. This pattern is similar to one seen in a study of human subjects, where their levels of 2-AG also increased during the morning and peaked in midafternoon (Hanlon et al. 2014). For plasma AEA, time of day was significant for all three analyses (overall, mice with wheels, mice without wheels), indicating reduced values later in the sampling period. This pattern is similar to a study of AEA in the cerebrospinal fluid of rats, which found that it decreased during the dark phase (Murillo-Rodriguez et al. 2006). Thus, although our study was not intended to sample over a time period long enough to address circadian patterns per se, the time-related variation that we observed is consistent with previous reports for other species.

4.5. Conclusions & Future Directions

Overall, our results demonstrate that voluntary physical exercise affects circulating endocannabinoid levels differently, depending on sex, recent activity, and genetic background. More specifically, we found that acute voluntary exercise was associated with plasma AEA concentrations in a way similar to the effects of forced exercise reported in previous studies, in that the amount of wheel running done by a rodent before plasma sampling was a positive predictor of the level of AEA found in the blood (Raichlen et al. 2012, 2013). We also found differences in circulating 2-AG and AEA levels between the sexes and between lines of mice bred for high levels of voluntary exercise, when compared to their non-selected control lines. Furthermore, the effects of five days of wheel access on endocannabinoid levels varied between the sexes and/or between HR and C mice.

Although not considered in the present study, receptor density or sensitivity may also have evolved in our mice. Broad-scale (macroevolutionary) patterns of endocannabinoid receptor evolution have been discussed elsewhere (Elphick and Egertová 2009), but we do not know of studies that have considered microevolutionary variation in CB receptors (e.g., among closely related species). Although microevolutionary studies are lacking, laboratory mice and rats can show changes in CB receptor gene expression and sensitivity over the course of several days. For example, female mice given wheel access for 10 days had increased CB_1 receptor gene expression in the hippocampus as compared with

sedentary controls (Wolf et al. 2010). In another study, male rats that had wheel access for eight days had an increase in CB_1 receptor binding site density in the hippocampus (Hill et al. 2010).

In future studies, we will further examine acute and chronic effects of voluntary exercise on the endocannabinoid system, including possible changes in receptor densities in various target organs (brain, muscle, gut). Interestingly, although HR mice run much more than C mice, they had lower circulating levels of AEA when the acute effect of wheel running was taken into account (Fig. 2.6). This finding suggests that the HR endocannabinoid system is differentially regulated, although it is unknown at what level changes have occurred (receptor density, number of converting or recycling enzymes, etc.). Therefore, future studies will also explore the mechanisms and genetics underlying the evolved endocannabinoid system of these unique, high-activity lines of mice, and how this system may interact with neurotransmitter and endocrine systems that are also known to have evolved in the HR mice (Rhodes et al. 2001a; Girard and Garland, Jr. 2002; Malisch et al. 2008).

Conflict of Interest Statement

The authors declare there is no conflict of interest.

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REFERENCES

Acosta, W., T. H. Meek, H. Schutz, E. M. Dlugosz, K. T. Vu, and T. Garland, Jr. 2015. Effects of early-onset voluntary exercise on adult physical activity and associated phenotypes in mice. Physiology & Behavior 149:279–286.

Brown, D. A., M. S. Johnson, C. J. Armstrong, J. M. Lynch, N. M. Caruso, L. B. Ehlers, M. Fleshner, R. L. Spencer, and R. L. Moore. 2007. Short-term treadmill running in the rat: what kind of stressor is it? Journal of Applied Physiology 103:1979–1985.

Burston, J. J., J. L. Wiley, A. A. Craig, D. E. Selley, and L. J. Sim-Selley. 2010. Regional enhancement of cannabinoid CB1 receptor desensitization in female adolescent rats following repeated Δ9-tetrahydrocannabinol exposure. British Journal of Pharmacology 161:103–112.

Careau, V., M. E. Wolak, P. A. Carter, and T. Garland, Jr. 2013. Limits to behavioral evolution: the quantitative genetics of a complex trait under directional selection. Evolution 67:3102–3119.

Chaouloff, F., S. Dubreucq, I. Matias, and G. Marsicano. 2012. Physical activity feel-good effect. Pp. 71–87 *in* Routledge Handbook of Physical Activity and Mental Health. Routledge, London.

Claghorn, G. C., I. A. T. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiology & Behavior 161:145–154.

Copes, L. E., H. Schutz, E. M. Dlugosz, W. Acosta, M. A. Chappell, and T. Garland, Jr. 2015. Effects of voluntary exercise on spontaneous physical activity and food consumption in mice: results from an artificial selection experiment. Physiology & Behavior 149:86–94.

Craft, R. M., J. A. Marusich, and J. L. Wiley. 2013. Sex differences in cannabinoid pharmacology: a reflection of differences in the endocannabinoid system? Life Sciences 92:476–481.

Dietrich, A., and W. F. McDaniel. 2004. Endocannabinoids and exercise. British Journal of Sports Medicine 38:536–541.

DiPatrizio, N. V., G. Astarita, G. Schwartz, X. Li, and D. Piomelli. 2011. Endocannabinoid signal in the gut controls dietary fat intake. Proceedings of the National Academy of Sciences 108:12904–12908.

Dubreucq, S., A. Durand, I. Matias, G. Bénard, E. Richard, E. Soria-Gomez, C. Glangetas, L. Groc, A. Wadleigh, F. Massa, and others. 2013. Ventral tegmental area cannabinoid type-1 receptors control voluntary exercise performance. Biological Psychiatry 73:895–903.

Dubreucq, S., M. Koehl, D. N. Abrous, G. Marsicano, and F. Chaouloff. 2010. CB1 receptor deficiency decreases wheel-running activity: consequences on emotional behaviours and hippocampal neurogenesis. Experimental Neurology 224:106–113.

Elphick, M. R., and M. Egertová. 2009. Cannabinoid receptor genetics and evolution. Pp. 123–149 *in* P. H. Reggio, ed. The Cannabinoid Receptors. Humana Press, Totowa, NJ.

Feuerecker, M., D. Hauer, R. Toth, F. Demetz, J. Hölzl, M. Thiel, I. Kaufmann, G. Schelling, and A. Chouker. 2012. Effects of exercise stress on the endocannabinoid system in humans under field conditions. European Journal of Applied Physiology 112:2777–2781.

Fuss, J., J. Steinle, L. Bindila, M. K. Auer, H. Kirchherr, B. Lutz, and P. Gass. 2015. A runner's high depends on cannabinoid receptors in mice. Proceedings of the National Academy of Sciences 112:13105–13108.

Gardner, E. L. 2005. Endocannabinoid signaling system and brain reward: emphasis on dopamine. Pharmacology Biochemistry and Behavior 81:263–284.

Garland, Jr., T., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proceedings of the Royal Society B: Biological Sciences 278:574–581.

Garland, Jr., T., M. T. Morgan, J. G. Swallow, J. S. Rhodes, I. Girard, J. G. Belter, and P. A. Carter. 2002. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution 56:1267–1275.

Garland, Jr., T., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. Van Dijk, and others. 2011b. The biological control of voluntary exercise, spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. Journal of Experimental Biology 214:206–229.

Garland, Jr., T., M. Zhao, and W. Saltzman. 2017. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integrative and Comparative Biology 57:in press.

Girard, I., and T. Garland, Jr. 2002. Plasma corticosterone response to acute and chronic voluntary exercise in female house mice. Journal of Applied Physiology 92:1553–1561.

Girard, I., M. W. McAleer, J. S. Rhodes, and T. Garland, Jr. 2001. Selection for high voluntary wheel-running increases speed and intermittency in house mice (*Mus domesticus*). Journal of Experimental Biology 204:4311–4320.

Hanlon, E. C., E. Tasali, R. Leproult, K. L. Stuhr, E. Doncheck, H. De Wit, C. J. Hillard, and E. Van Cauter. 2014. Circadian rhythm of circulating levels of the endocannabinoid 2-arachidonoylglycerol. The Journal of Clinical Endocrinology & Metabolism 100:220–226.

Hannon, R. M., S. A. Kelly, K. M. Middleton, E. M. Kolb, D. Pomp, and T. Garland, Jr. 2008. Phenotypic effects of the “mini-muscle” allele in a large HR x C57BL/6J mouse backcross. Journal of Heredity 99:349–354.

Harpur, R. P. 1980. The rat as a model for physical fitness studies. Comparative Biochemistry and Physiology Part A: Physiology 66:553–574.

Heyman, E., F.-X. Gamelin, M. Goekint, F. Piscitelli, B. Roelands, E. Leclair, V. Di Marzo, and R. Meeusen. 2012. Intense exercise increases circulating endocannabinoid and BDNF levels in humans—possible implications for reward and depression. Psychoneuroendocrinology 37:844–851.

Hill, M. N., A. K. Titterness, A. C. Morrish, E. J. Carrier, T. T.-Y. Lee, J. Gil-Mohapel, B. B. Gorzalka, C. J. Hillard, and B. R. Christie. 2010. Endogenous cannabinoid signaling is required for voluntary exercise-induced enhancement of progenitor cell proliferation in the hippocampus. Hippocampus 20:513–523.

Houle-Leroy, P., H. Guderley, J. G. Swallow, and T. Garland, Jr. 2003. Artificial selection for high activity favors mighty mini-muscles in house mice. American Journal of Physiology-Regulatory, Integrative and Comparative Physiology 284:R433–R443.

Järbe, T. U., M. E. Andrzejewski, and N. V. DiPatrizio. 2002. Interactions between the CB1 receptor agonist Δ 9-THC and the CB1 receptor antagonist SR-141716 in rats: open-field revisited. Pharmacology Biochemistry and Behavior 73:911–919.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in

mice selectively bred for high voluntary wheel-running behavior. Pharmacology Biochemistry and Behavior 101:528–537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland, Jr. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behavioural Pharmacology 19:812–820.

Kelly, S. A., P. P. Czech, J. T. Wight, K. M. Blank, and T. Garland, Jr. 2006. Experimental evolution and phenotypic plasticity of hindlimb bones in high-activity house mice. Journal of Morphology 267:360–374.

Koteja, P., T. Garland Jr, J. K. Sax, J. G. Swallow, and P. A. Carter. 1999. Behaviour of house mice artificially selected for high levels of voluntary wheel running. Animal Behaviour 58:1307–1318.

Koteja, P., and T. Garland, Jr. 2001. Response to R. Eikelboom. Animal Behaviour 61:F25–F26.

Li, G., J. S. Rhodes, I. Girard, S. C. Gammie, and T. Garland, Jr. 2004. Opioid-mediated pain sensitivity in mice bred for high voluntary wheel running. Physiology & Behavior 83:515–524.

Malisch, J. L., C. W. Breuner, F. R. Gomes, M. A. Chappell, and T. Garland, Jr. 2008. Circadian pattern of total and free corticosterone concentrations, corticosteroid-binding globulin, and physical activity in mice selectively

bred for high voluntary wheel-running behavior. General and Comparative Endocrinology 156:210–217.

Malisch, J. L., C. W. Breuner, E. M. Kolb, H. Wada, R. M. Hannon, M. A. Chappell, K. M. Middleton, and T. Garland, Jr. 2009. Behavioral despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations. Behavior Genetics 39:192–201.

Martin, M., C. Ledent, M. Parmentier, R. Maldonado, and O. Valverde. 2000. Cocaine, but not morphine, induces conditioned place preference and sensitization to locomotor responses in CB1 knockout mice. European Journal of Neuroscience 12:4038–4046.

Murillo-Rodriguez, E., F. Désarnaud, and O. Prospéro-García. 2006. Diurnal variation of arachidonoylethanolamine, palmitoylethanolamide and oleoylethanolamide in the brain of the rat. Life Sciences 79:30–37.

Raichlen, D. A., A. D. Foster, G. L. Gerdeman, A. Seillier, and A. Giuffrida. 2012. Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the “runner’s high.” Journal of Experimental Biology 215:1331–1336.

Raichlen, D. A., A. D. Foster, A. Seillier, A. Giuffrida, and G. L. Gerdeman. 2013. Exercise-induced endocannabinoid signaling is modulated by intensity. European Journal of Applied Physiology 113:869–875.

Rasmussen, E. B., and C. Hillman. 2011. Naloxone and rimonabant reduce the reinforcing properties of exercise in rats. Experimental and Clinical Psychopharmacology 19:389.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. Mitchell, and T. Garland. 2001a. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 158:120–131.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology 45:438–455.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behavioral Neuroscience 117:1243.

Rhodes, J. S., G. R. Hosack, I. Girard, A. E. Kelley, G. S. Mitchell, and T. Garland, Jr. 2001b. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology 158:120–131.

Rhodes, J. S., P. Koteja, J. G. Swallow, P. A. Carter, and T. Garland, Jr. 2000. Body temperatures of house mice artificially selected for high voluntary wheel-running behavior: repeatability and effect of genetic selection. Journal of Thermal Biology 25:391–400.

Saltin, B., and P. D. Gollnick. 1983. Skeletal muscle adaptability: significance for metabolism and performance. Comprehensive Physiology 555–631.

Sañudo-Peña, M. C., J. Romero, G. E. Seale, J. J. Fernandez-Ruiz, and J. M. Walker. 2000. Activational role of cannabinoids on movement. European Journal of Pharmacology 391:269–274.

Scribbans, T. D., S. Vecsey, P. B. Hankinson, W. S. Foster, and B. J. Gurd. 2016. The effect of training intensity on VO2max in young healthy adults: a meta-regression and meta-analysis. International Journal of Exercise Science 9:230.

Smith, S. L., and E. B. Rasmussen. 2010. Effects of 2-AG on the reinforcing properties of wheel activity in obese and lean Zucker rats. Behavioural Pharmacology 21:292–300.

Sparling, P. B., A. Giuffrida, D. Piomelli, L. Rosskopf, and A. Dietrich. 2003. Exercise activates the endocannabinoid system. Neuroreport 14:2209–2211.

Stanford, K. I., and L. J. Goodyear. 2016. Exercise regulation of adipose tissue. Adipocyte 5:153–162.

Swallow, J. G., P. A. Carter, and T. Garland, Jr. 1998. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics 28:227–237.

Swallow, J. G., J. S. Rhodes, and T. Garland, Jr. 2005. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. Integrative and Comparative Biology 45:426–437.

Syme, D. A., K. Evashuk, B. Grintuch, E. L. Rezende, and T. Garland, Jr. 2005. Contractile abilities of normal and “mini” triceps surae muscles from mice (*Mus domesticus*) selectively bred for high voluntary wheel running. Journal of Applied Physiology 99:1308–1316.

Tsou, K., S. Brown, M. C. Sanudo-Pena, K. Mackie, and J. M. Walker. 1998. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. Neuroscience 83:393–411.

Wahlsten, D. 1990. Insensitivity of the analysis of variance to heredity-environment interaction. Behavioral and Brain Sciences 13:109–120.

Wahlsten, D. 1991. Sample size to detect a planned contrast and a one degree-of-freedom interaction effect. Psychological Bulletin 110:587–595.

Wallace, I. J., and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: insights from experimental evolution. Evolutionary Anthropology 25:98–104.

Wolf, S. A., A. Bick-Sander, K. Fabel, P. Leal-Galicia, S. Tauber, G. Ramirez-Rodriguez, A. Müller, A. Melnik, T. P. Waltinger, O. Ullrich, and others. 2010. Cannabinoid receptor CB1 mediates baseline and activity-induced survival of new neurons in adult hippocampal neurogenesis. Cell Communication and Signaling 8.

Zimmer, A., A. M. Zimmer, A. G. Hohmann, M. Herkenham, and T. I. Bonner. 1999. Increased mortality, hypoactivity, and hypoalgesia in cannabinoid CB1 receptor knockout mice. Proceedings of the National Academy of Sciences 96:5780–5785.

CHAPTER 3

High-runner mice have reduced incentive salience for a sweet-taste reward when housed with wheel access

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ABSTRACT

To explore reward substitution in the context of voluntary exercise, female mice from four replicate high-runner (HR) lines (bred for wheel running) and four non-selected control (C) lines were given simultaneous access to wheels and palatable solutions as competing rewards (two doses of sucrose [3.5, 10.5% w/v]; two doses of saccharin [0.1, 0.2% w/v]; two doses of common artificial sweetener blends containing saccharin [Sweet 'N Low®: 0.1, 0.2% w/v], aspartame [Equal®: 0.04, 0.08% w/v], sucralose [Splenda®: 0.08, 0.16% w/v]). In a separate set of mice, the experiment was repeated without wheel access. Access to the artificial sweeteners did not have any significant effect on wheel running. However, based on proportional responses, both doses of sucrose significantly elevated wheel running in C but not HR mice. In contrast, the high dose of sucrose suppressed home-cage activity. Fluid consumption generally increased in a dose-dependent manner with sucrose and the blends. As compared with C, HR had a significantly smaller increase in consumption of artificial sweetener blends when they had access to wheels, but not when housed without wheels. Overall, these results suggest that HR mice have a reduced incentive salience for some artificial sweetener blends, likely attributable to the stronger competing reward of wheel running in these lines.

1. Introduction

Animals tend to seek out activities that are rewarding and avoid those that are detrimental or not pleasurable. The brain's reward pathways are responsible for determining which behaviors should be avoided and which should be repeated, possibly by imbuing them with incentive salience. As discussed by Berridge and Robinson (1998, p. 313), incentive salience transforms "the neural representation of a stimulus into an object of attraction that animals will work to acquire." Activities are generally said to be rewarding when they increase activity in reward nuclei (such as the ventral tegmental area or nucleus accumbens) or if they increase dopamine transmission in those areas. The reward system in mammals is especially well-studied, and activities which activate it include social-play behavior (Vanderschuren et al. 1997), sexual behavior (López and Ettenberg 2002), and pair-bonding in rodents (Aragona et al. 2003), as well as listening to music (Menon and Levitin 2005) and gambling in humans (Dodd et al. 2005). However, many questions remain, especially in comparing the value of the incentive salience of one reward to another. Are different rewards attributed different strengths? How do species or individuals within species differ in their response to various rewarding activities or substances?

Studies on rodents and humans have shown that some rewarding activities or substances can substitute for others, but this substitution is not always complete or reciprocal. For example, in a study with rats, saccharin acted

as a competing reward with cocaine, but not with morphine (Schulze et al. 2002). Sucrose was also able to partially substitute for wheel-running reinforcement in rats, but the converse was not true (Belke et al. 2006). In addition, five minutes of access to a glucose-saccharin mixture significantly reduced cocaine-seeking behavior in rats (Liu and Grigson 2005). In humans, this reward substitution is often used as therapy – those that are suffering from withdrawal can use exercise to substitute for the reward they would usually get from alcohol or nicotine and decrease the "craving" they feel (Ussher et al. 2001, 2004; Daniel et al. 2004; Taylor et al. 2007).

For over 80 generations, we have been selecting for voluntary wheel running behavior in four replicate lines of mice (Swallow et al. 1998, 2009; Wallace and Garland, Jr. 2016). Over time, there has been a three-fold increase in wheel running in the high runner (HR) lines as compared with four non-selected control (C) lines. The HR mice run more primarily by running faster, especially in females, and not by increasing their time spent running (Swallow et al. 1998; Koteja et al. 1999). HR mice also have higher endurance during forced exercise (Meek et al. 2009), higher aerobic capacity (Rezende 2006; Kolb et al. 2010) lower body mass (Swallow et al. 1999, 2001; Meek et al. 2009), and a variety of other traits beneficial to sustained locomotor performance (Swallow et al. 2001; Rezende 2006; Kolb et al. 2010).

In addition to physiological or morphological changes, neurobiological changes have also occurred in the HR mice, including in the dopaminergic (Rhodes and Garland, Jr. 2003) and endocannabinoid (Keeney et al. 2008; Thompson et al. 2017) systems, which are both involved in reward pathways. When HR mice are prevented from running after 6 days of exercise, they show differential brain activation, when compared to C mice, in areas of the brain tied to reward and motivation (Rhodes et al. 2003).

Exercise, including wheel running in mice, is a rewarding behavior (Premack et al. 1964; Timberlake and Wozny 1979; Belke and Heyman 1994; Belke 1996; Sherwin and Nicol 1996; Sherwin 1998; Belke and Garland, Jr. 2007). Studies have attempted to quantify its incentive salience via operant conditioning protocols (Belke and Heyman 1994; Belke 1996; Belke and Garland, Jr. 2007); including operant conditioning with reinforcers (Premack et al. 1964; Timberlake and Wozny 1979) and also by increasing the effort needed to reach a running wheel (Sherwin and Nicol 1996). If wheel running is attributed a higher incentive salience in HR mice, then the response to a competing reward may be reduced.

As explained above, palatable solutions are rewarding because they cause an increased release of dopamine in reward nuclei (Hernandez and Hoebel 1988; Spangler et al. 2004). Therefore, they should compete with wheel running as a reward. We gave both HR and C mice access to two competing

rewards, in an attempt to evaluate whether the incentive salience of exercise has evolved in the HR mice.

2. Materials and procedures

2.1 Experimental animals

Mice for this study were initially derived from a base population of 224 outbred Hsd:ICR mice (*Mus domesticus*) purchased from Harlan Sprague Dawley (Indianapolis, IN). After random mating for two generations, mice were randomly allotted into 8 lines, four designated as high runner (HR) and four as non-selected control (C) lines. The HR lines have been bred for high wheel revolutions on days 5 and 6 of a 6-day period of wheel access, while the C lines have been bred without regard to the amount of running during the test (Swallow et al. 1998). Each generation, mice are wheel-tested at approximately 6-8 weeks of age, and within-family selection is used in choosing breeders. During wheel testing, mice are individually housed with access to Wahman-type running wheels (1.12 m circumference; Lafayette Instruments, Lafayette, Indiana, USA). A minimum of 10 mating pairs from each line produces litters every generation, and pups are weaned at 21 days of age.

2.2 Experimental design

For generations 42 and 53, the experiment started with access to wheels for two weeks, along with free access to tap water, so that mice could acclimate to their new cages. For generation 75, mice were allowed to acclimate to single-housing for two weeks before the solutions were administered. For generation 75, home-cage activity was recorded using infrared sensors for 23h/day for the portion of the study when solutions were administered (Acosta et al. 2015; Thompson et al. 2017). Water bottles were weighed when changing solutions. Additionally, bottles were placed in empty cages to serve as evaporative controls, with the amount lost to evaporation averaged and subtracted from the average fluid consumption. All subjects had free access to food during the entire experiment (Harlan Teklad Rodent Diet [W] 8604, Madison, WI, USA).

Mice from generation 42 (n = 72, 9 per line) were given saccharin, sucrose, and Splenda®, whereas those from generation 53 (n = 75, approximately 9 per line) had access to Sweet 'N Low® and Equal®. All mice in generation 75, which were tested without wheel access, received all compounds. Different generations were used for logistical reasons. The low and high doses of each compound were mixed with tap water and placed in water bottles so that the mice had free access. Each mouse received 2 days of the low dose, 2 days of the high dose, and a 2-day tap water "sham" dose. Two to four days of "washout" were given between each solution (Ramirez and Fuller 1976). For

example, a mouse during the sucrose trial might have seen the following sequence: two days of the high dose of sucrose, two days of tap water, and two days of the low dose of sucrose.

2.3 Palatable solutions

Solution doses were based on relative concentrations of active ingredients found in the artificial sweetener blends, as well as known palatability of these blends (and sucrose) in mice (Pelz et al. 1973; Fuller 1974; Ramirez and Fuller 1976; Blizard et al. 1999; Bachmanov et al. 2001; Spangler et al. 2004; Lewis et al. 2005; Belke et al. 2006). These concentrations were: sodium saccharin (0.1%, 0.2%), Sweet 'N Low® (0.1%, 0.2%), Equal® (0.04%, 0.08%), Splenda® (0.08%, 0.16%), and sucrose (3.5%, 10.5%).

Commercial artificial sweetener blends (as opposed to pure aspartame, sucralose, etc.) were included in this study based on results from Dr. Craig Davis indicating that some mice have a strong preference for Sweet 'N Low® (personal communication). Artificial sweetener blends also maximize sweetness while avoiding the bitter tastes that tend to occur at high concentrations with pure artificial sweeteners (see Dess et al. 2008 for a discussion of rats preferring Splenda to its main sweetener sucralose). In addition, using commercially available blends maximizes the relevance of our results to humans.

2.4 Statistical analyses

Wheel running, home-cage activity, and fluid consumption were analyzed for each dose (3 doses per compound). Additionally, wheel running, home-cage activity and fluid consumption were also analyzed using repeated-measures ANCOVAs, with linetype and dose as main effects. Covariates were included where appropriate, including wheel freeness and age for wheel-running analyses and body mass and age for fluid-consumption analyses. When necessary, data were log transformed to improve normality of residuals.

3. Results

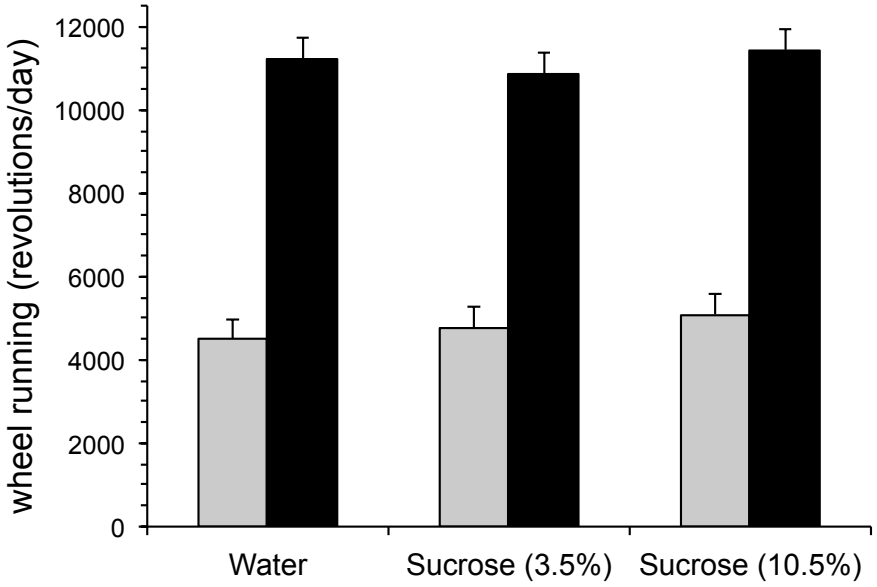
3.1 Biometric data

Mice ranged from 61-76 days old (generation 42), 71-75 days old (generation 53), or 58-72 days old (generation 75) at the start of each experiment. As previously reported, HR mice weighed less than C mice (Swallow et al. 1999, 2001; Meek et al. 2009).

3.2 Wheel running

As seen in Figure 3.1A, HR mice always ran more than C mice. Solution concentration for the artificial sweeteners did not affect wheel running (Table 3.1). However, sucrose increased total wheel revolutions (Table 3.1, dose $P = 0.0386$). Separate analyses of the revolutions run at individual doses (Fig. 3.1B)

indicated that the proportional response of C mice was significantly higher than for HR mice at the 3.5% sucrose dose (linetype P = 0.0474), but not at the 10.5% sucrose dose (linetype P = 0.2343). Moreover, the increased running by C mice given sucrose was statistically significant for both doses (3.5%: +7.3%, P = 0.0484; 10.5%: +12.0%, P = 0.0187), whereas it was not for HR mice (3.5%: -3.0%, P = 0.3361; 10.5%: +4.9%, P = 0.2345).


wheel running (revolutions/day)
12000
10000
8000
6000
4000
2000
0
Water
Sucrose (3.5%)
Sucrose (10.5%)

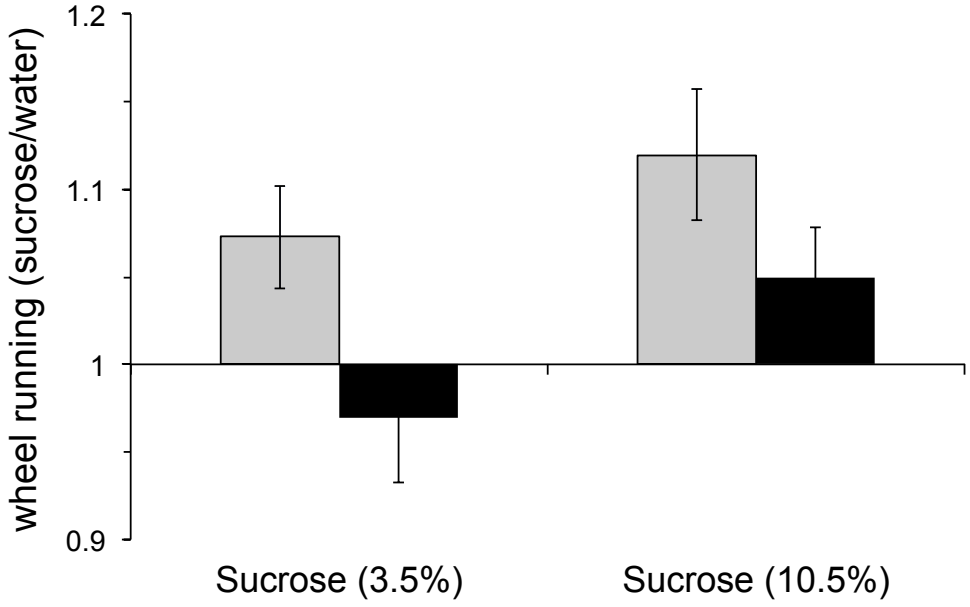

wheel running (sucrose/water)
1.2
1.1
1
0.9
Sucrose (3.5%)
Sucrose (10.5%)

Figure 3.1. Wheel running during sucrose trials, absolute and proportional. **A)** Wheel revolutions during sucrose trials (least squares means +/- S.E. derived from a repeated-measures, two-way ANCOVA with linetype and sucrose dose as the main effects). Gray bars are C mice and black bars are HR mice. Effects of linetype (P<0.0001) and sucrose dose (P=0.0386) were statistically significant, but their interaction was not (P=0.1598; see Table 1). Sucrose was the only substance that had a statistically significant effect on wheel revolutions (Table 3.1). Averaging across HR and C mice, least squares means (+ S.E.) for wheel revolutions were 7,863 (+ 353), 7,819 (+ 353), and 8,268 (+ 353) for water, low dose, and high dose sucrose. Thus, high-dose sucrose increased wheel revolutions by 5.4% relative to the average values for water and low-dose sucrose. **B)** Proportional response in voluntary wheel running (revolutions/day) during sucrose trials. Values are least squares means (+/- S.E.) from separate one-way ANCOVAs of running with sucrose/with water, with linetype as the main effect and age and wheel freeness as covariates. See Results section 3.2 for statistical details.

3.3 Consumption of sweeteners with wheel access

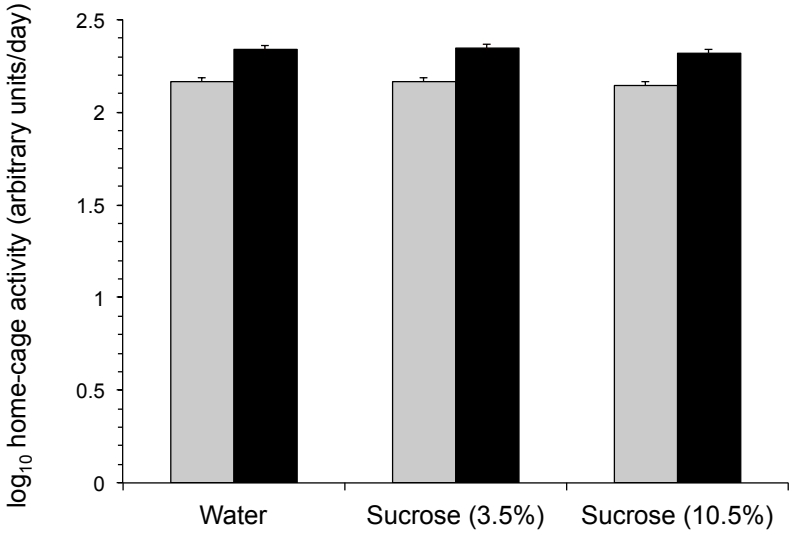
Consumption of saccharin was significantly increased in both HR and C mice when they had access to wheels (Table 3.1, Fig. 3.3A). Significant linetype-by-dose interactions, with C mice showing larger increases in fluid consumption, were seen for Sweet 'N Low, Equal, and Splenda (Table 3.1, Fig.3). Sucrose also increased fluid consumption (Table 3.1, Fig. 3.3I), but the linetype*dose interaction caused a somewhat different pattern than for the artificial sweeteners.

3.4 Home-cage activity

HR mice always had higher home-cage activity (total activity per day) than C mice (Table 3.1, all linetype $P < 0.006$), regardless of which solution they were consuming. The higher activity of HR mice was attributable to both the duration (for linetype, saccharin $P = 0.0066$, Sweet 'N Low $P = 0.0050$, Equal $P = 0.0205$, Splenda $P = 0.0086$, sucrose $P = 0.0045$) and the average intensity of activity when mice were active (for linetype, with all values log-transformed to improve normality of residuals, saccharin $P = 0.0100$, Sweet 'N Low $P = 0.0024$, Equal $P = 0.0052$, Splenda $P = 0.0051$, sucrose $P = 0.0052$).

Dose of the artificial sweeteners did not have a statistically significant effect on home-cage activity, and we observed no linetype*dose interactions (Table 3.1). However, sucrose dose did affect activity ($P=0.0203$), primarily due to a suppression of activity by the higher dose of sucrose (Fig. 3.2B). Sucrose

dose significantly affected the average intensity of activity (P = 0.0020, lowest at the higher dose), but not the duration of activity (P = 0.1544), with no significant linetype*dose interaction (P = 0.49 for intensity, P = 0.75 for duration). Separate analyses of the proportional changes in home-cage activity at individual doses (Fig. 3.2B) indicated that the proportional response did not differ between HR and C mice for either sucrose dose (linetype P = 0.8156 and P = 0.8562, respectively), and neither HR or C mice differed significantly from unity for either dose.


log10 home-cage activity (arbitrary units/day)
2.5
2
1.5
1
0.5
0
Water
Sucrose (3.5%)
Sucrose (10.5%)

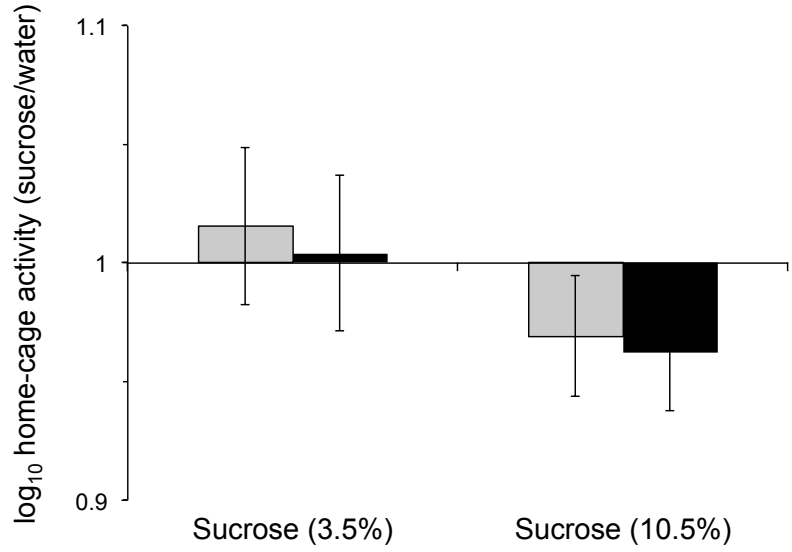
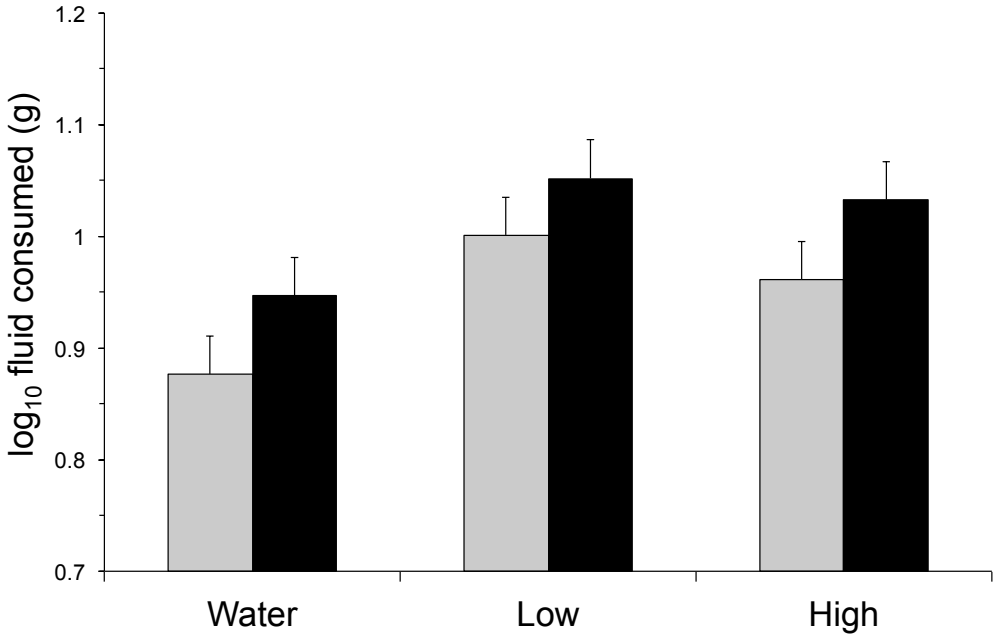

log10 home-cage activity (sucrose/water)
1.1
1
0.9
Sucrose (3.5%)
Sucrose (10.5%)

Figure 3.2. Home-cage activity during sucrose trials, absolute and proportional.
A) Home-cage activity during sucrose trials (least squares means +/- S.E. derived from a repeated-measures, two-way ANCOVA with linetype and sucrose dose as the main effects). Gray bars are C mice and black bars are HR mice. Effects of linetype (P=0.0012) and sucrose dose (P=0.0203) were statistically significant, but their interaction was not (P=0.9207; see Table 3.1). **B)** Proportional response in home-cage activity during sucrose trials when mice did not have access to wheels. Values are least squares means (+/- S.E.) from separate one-way ANCOVAs of activity with sucrose/with water, with linetype as the main effect and age and sensor sensitivity as covariates. See Results section 3.4 for statistical details.

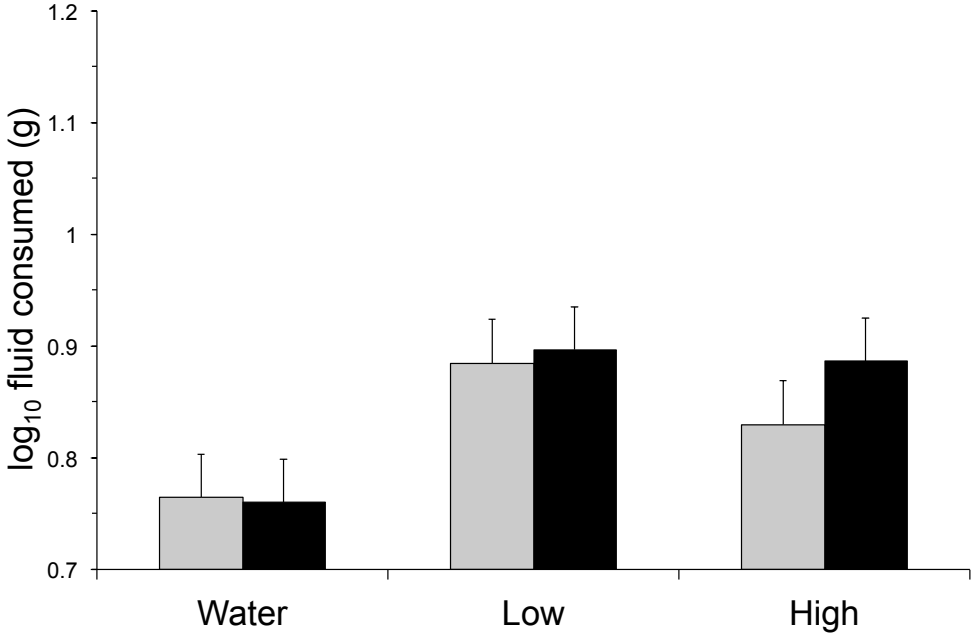
3.5 Consumption of sweeteners without wheel access

Without wheels, dose significantly affected fluid consumption for all solutions (all $P<0.0001$: Table 3.1), and as shown in Fig. 3.3, mice always drank more of the sweet-tasting solutions than they did water. However, without access to wheels (Fig. 3.3, bottom of each page), none of the linetype*dose interactions were significant (all $P > 0.28$: Table 3.1), which contrasts sharply with the results when mice had wheel access (Fig. 3.3, top of each page).

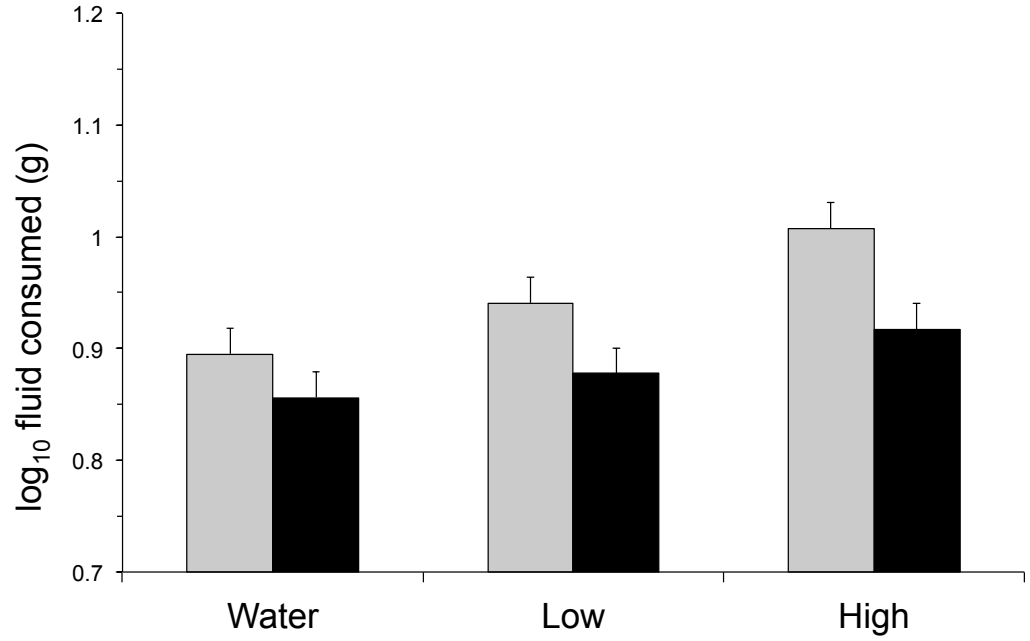
A) Saccharin Consumption with wheel access
Linetype X Dose P = 0.77



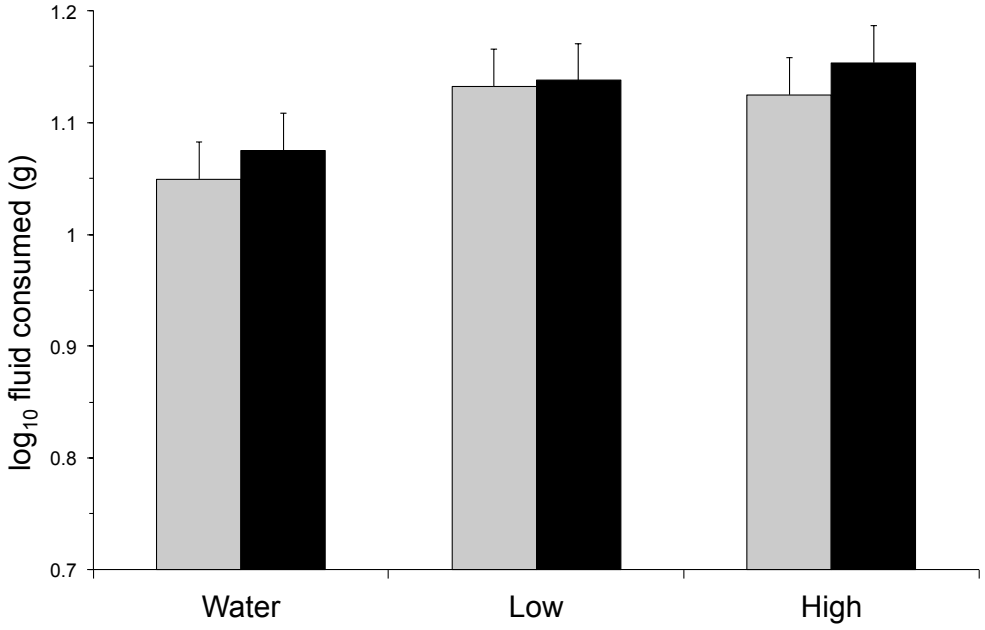
B) Saccharin Consumption without wheels
Linetype X Dose P = 0.37



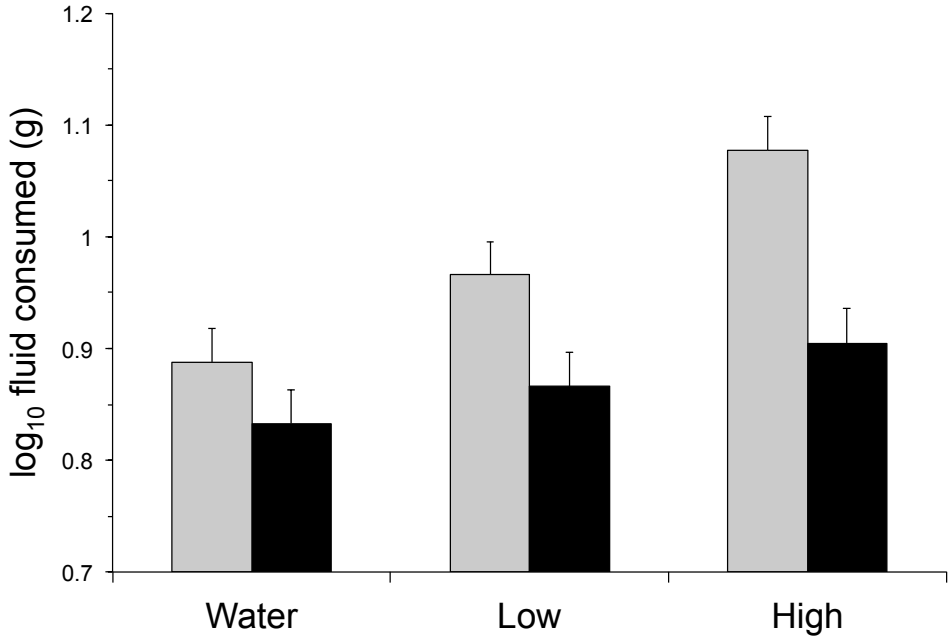
C) Sweet 'N Low Consumption with wheel access
Linetype X Dose P = 0.017*



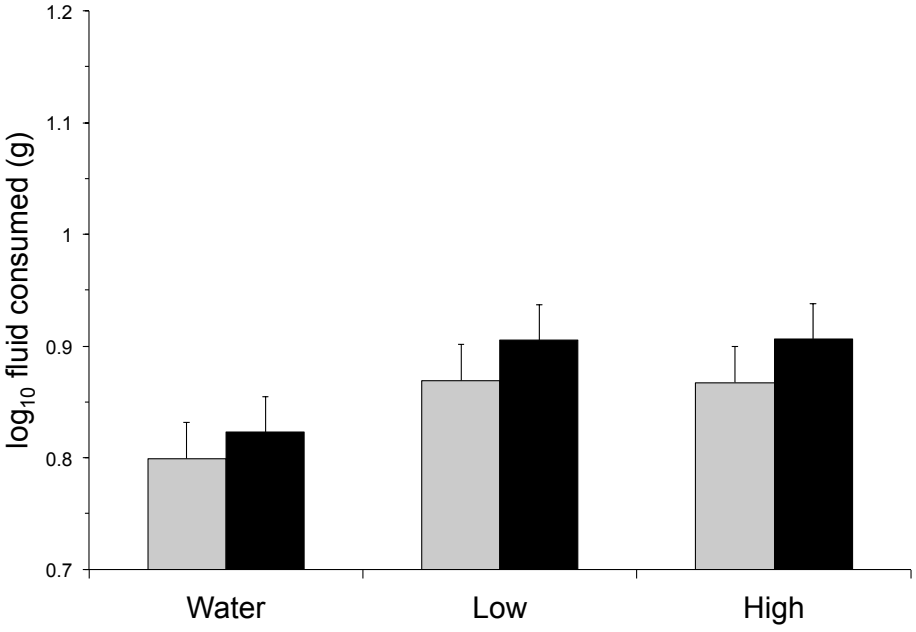
D) Sweet 'N Low Consumption without wheels
Linetype X Dose P = 0.72



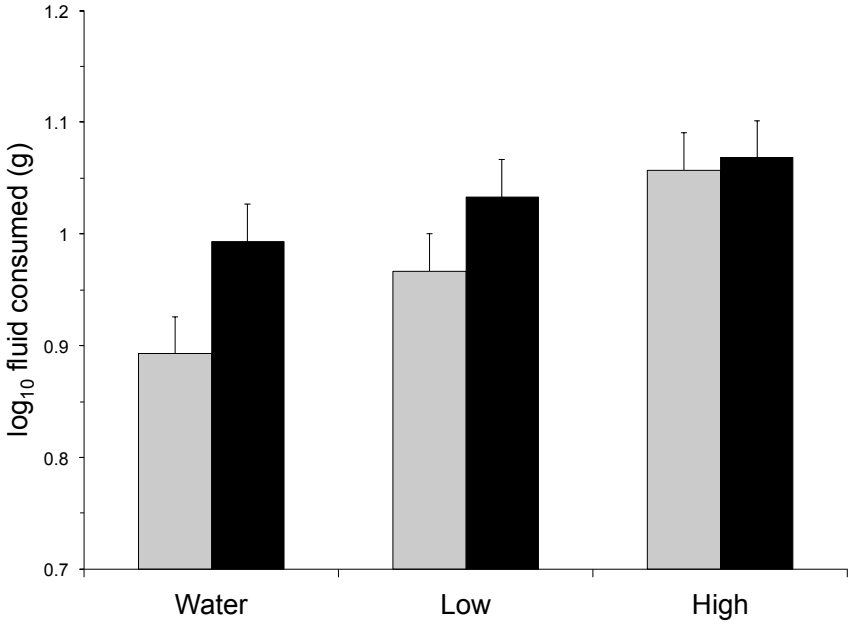
E) Equal Consumption with wheel access
Linetype X Dose P = 0.012*



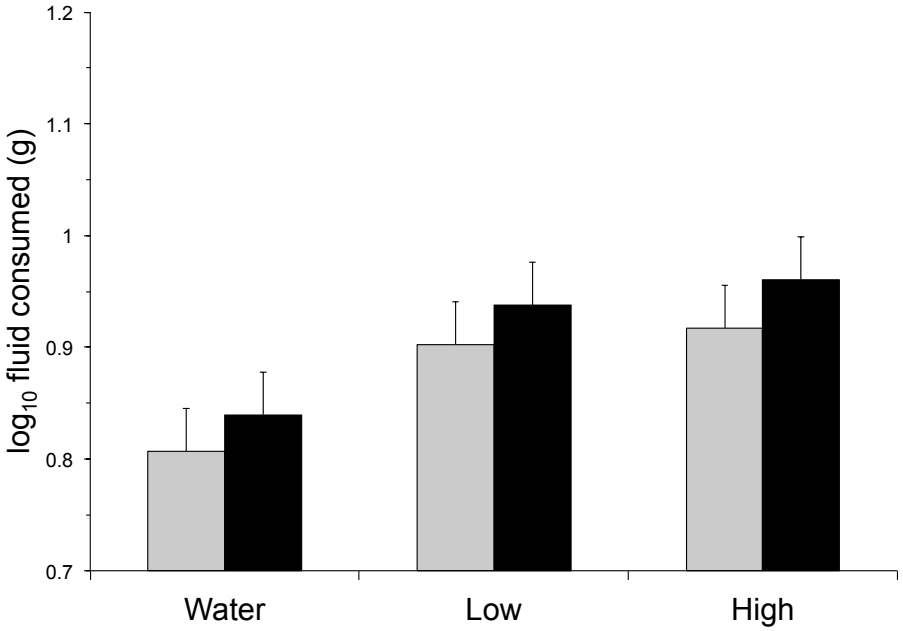
F) Equal Consumption without wheels
Linetype X Dose P = 0.74



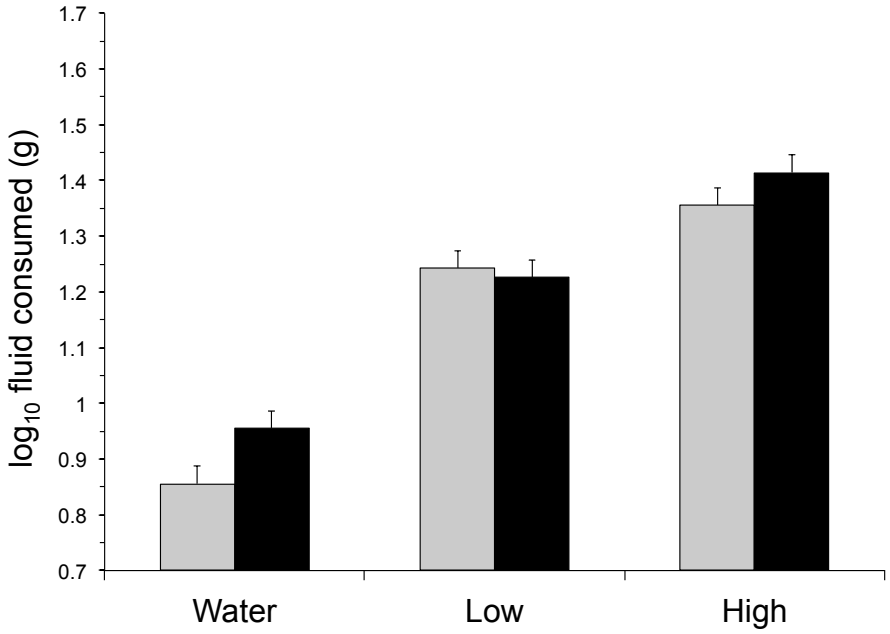
G) Splenda Consumption with wheel access
Linetype X Dose P = 0.008*



H) Splenda Consumption without wheels
Linetype X Dose P = 0.87



I) Sucrose Consumption with wheel access
Linetype X Dose P = 0.023*



J) Sucrose Consumption Without wheels
Linetype X Dose P = 0.28

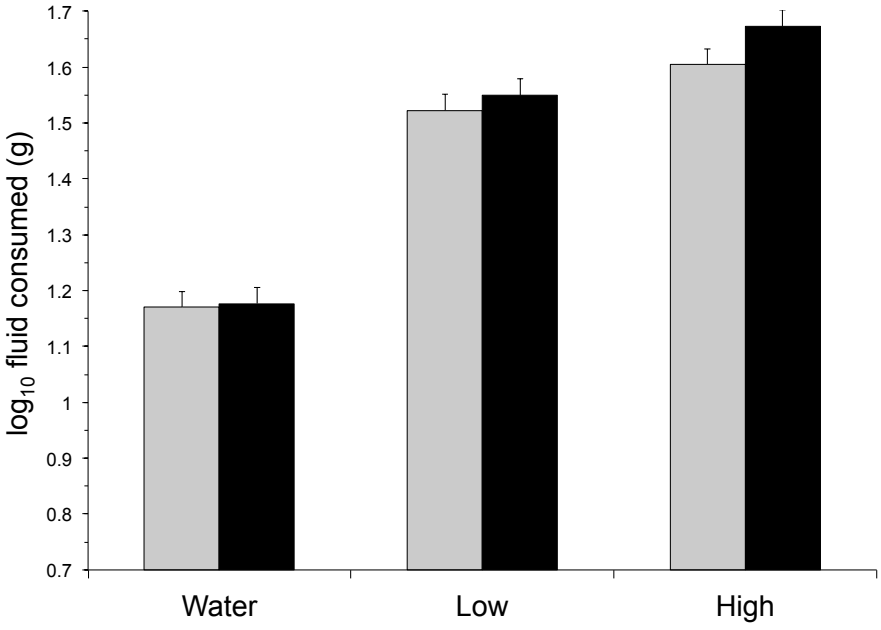


Figure 3.3. Consumption of sweet-tasting solutions, with and without access to wheels. Consumption of sweet-tasting solutions during access to wheels (**A, C, E, G, I**) or without access to wheels (**B, D, F, H, J**). Values are least-squares means (+/- standard errors) of log-transformed data from SAS Proc Mixed (Table 3.1). Gray bars are C mice and black bars are HR mice. For four of the sweet solutions, when mice had wheel access the linetype*dose interaction was statistically significant (Table 3.1), such that the increases in consumption were mainly in C mice at the higher doses.

Table 3.1. Significance levels for voluntary wheel-running behavior, home-cage activity (in the absence of wheels), and fluid consumption (log-transformed) based on repeated-measures, two-way nested ANCOVAs.

Treatment	Wheel Running (revolutions/day)[a]			Fluid Consumption With Wheel Access[b]		
	$P_{Linetype}$	P_{Dose}	$P_{Linetype \times Dose}$	$P_{Linetype}$	P_{Dose}	$P_{Linetype \times Dose}$
Saccharin	**<0.0001**	0.8552	0.3755	0.2155	**<0.0001**	0.7692
Sweet 'N Low	**0.0031**	0.6728	0.3627	0.0903	**<0.0001**	**0.0172**
Equal	**0.0040**	0.6869	0.3208	**0.0324**[c]	**<0.0001**	**0.0116**
Splenda	**<0.0001**	0.9909	0.9525	0.2518	**<0.0001**	**0.0076**
Sucrose	**<0.0001**	**0.0386**	0.1598	0.2792	**<0.0001**	**0.0226**
Treatment	Home-cage Activity (total/day)[d]			Fluid Consumption Without Wheel Access[b]		
	$P_{Linetype}$	P_{Dose}	$P_{Linetype \times Dose}$	$P_{Linetype}$	P_{Dose}	$P_{Linetype \times Dose}$
Saccharin	**0.0010**	0.1691	0.4708	0.5075	**<0.0001**	0.3688
Sweet 'N Low	**0.0012**	0.8833	0.6206	0.7123	**<0.0001**	0.7218
Equal	**0.0054**	0.6899	0.5970	0.5959	**<0.0001**	0.7405
Splenda	**0.0036**	0.7193	0.8981	0.5491	**<0.0001**	0.8699
Sucrose	**0.0012**	**0.0203**	0.9207	0.2971	**<0.0001**	0.2812

P values were considered significant at $P < 0.05$ (in bold).
[a]For wheel running, age and wheel freeness were used as covariates, but neither was ever statistically significant (results not shown).
[b]In the fluid consumption analyses, age and body mass were used as covariates (results not shown).
[c]HR mice drank significantly less of both doses of Equal, but not of water.
[d]For home-cage activity, age and sensor sensitivity were used as covariates, but neither was ever statistically significant (results not shown).

4. Discussion

The main purpose of this study was to explore reward substitution in the context of voluntary exercise, by use of a novel mouse model. Natural and non-nutritive sweeteners were offered as competing rewards for wheel running, and we studied females from four replicate High Runner lines, selectively bred for voluntary wheel running, as well as four non-selected Control lines. None of the artificial sweeteners (Table 1) had a statistically significant effect on wheel running or home-cage activity, the latter measured for mice that did not have wheel access, in either HR or C mice. However, sucrose increased wheel running in C mice without affecting the running of HR mice. In contrast, sucrose suppressed home-cage activity for both HR and C mice housed without wheels. Looking specifically at sweetener consumption, we found that HR mice (as compared with C mice) had a significantly smaller increase in consumption for artificial sweetener blends when they had access to wheels, but not when housed without wheels. These results suggest that HR mice have a reduced incentive salience for some artificial sweetener blends, possibly because wheel running is a stronger competing reward.

For C mice, sucrose increased the amount of wheel running by increasing the time spent running, rather than running speed, based on analyses of proportional responses (Fig. 3.1). Both C and HR mice run for many hours each night (Garland, Jr. et al. 2011a), and sucrose in drinking water may serve as a

fuel for that exercise (see also Claghorn et al. 2017). Alternatively, the increased wheel running observed in C mice with sucrose, but not with artificial sweeteners, may arise from the differential processing of a natural sugar in the brain's reward pathways. For example, an oral rinse of a glucose solution was able to increase maximal oxygen consumption in human cyclists, but saccharin solutions did not have the same effect (Chambers et al. 2009). The glucose solution also caused activation of regions in the brain associated with reward-processing, such as the striatum and cingulate cortex. The striatum has been specifically tied to wheel running in mice (Rhodes et al. 2003; Waters et al. 2013; Saul et al. 2017). Whatever the mechanism underlying the effect of sucrose on physical activity in C mice, these effects were not observed in HR mice, thus providing further evidence that they have evolved in terms of motivation and/or ability for voluntary exercise (Garland, Jr. et al. 2016; Wallace and Garland, Jr. 2016).

When housed without wheels, sucrose significantly affected home-cage activity for both HR and C mice, primarily due to a suppression of activity by the high dose (Fig. 3.2). The high dose decreased the average intensity of activity, without affecting the number of minutes per day the mice were active. Recent studies have shown that fructose can suppress home-cage activity in rodents (Rendeiro et al. 2015) and decrease energy expenditure in humans (Cox et al. 2012). The mechanism underlying such effects is not yet known, but may involve how and where fructose is metabolized (Samuel 2011).

With the exception of saccharin, all mice drank more of the sweet solutions in a dose-dependent manner, both with and without wheel access (Fig. 3.3). However, as compared with C lines, mice from HR lines had a significantly smaller increase in consumption for artificial sweetener blends when they had access to wheels, but not when housed without wheels. These results indicate that the HR lines have evolved with respect to the incentive salience of a reward (some sweet-tasting solutions) when an important competing reward (wheel running) is present.

Because the HR mice have been selected for voluntary exercise, it makes sense that motivation for the performance of exercise behavior may have changed (Rhodes et al. 2005; Garland, Jr. et al. 2011b, 2016). Because our results indicate that the HR mice have reduced incentive salience for sweet-taste rewards, we argue that they have an enhanced incentive salience for voluntary exercise. Belke and Garland (2007) suggest that the reward system may be up-regulated for one particular aspect or type of behavior, and therefore have a trade-off with other aspects of the same behavior or other behaviors.

Although a certain level of ability is necessary for high levels of voluntary exercise, the results from this study highlight that motivation can impact its control as well, even in rodents (see also Garland, Jr. et al. 2011b). The brain has an important role in both the ability and motivation for voluntary exercise

(Kayser 2003; Baden 2005; Noakes 2007, 2008; Rose and Parfitt 2007; Claghorn et al. 2016).

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REFERENCES

Acosta, W., T. H. Meek, H. Schutz, E. M. Dlugosz, K. T. Vu, and T. Garland, Jr. 2015. Effects of early-onset voluntary exercise on adult physical activity and associated phenotypes in mice. Physiol. Behav. 149:279–286.

Aragona, B. J., Y. Liu, J. T. Curtis, F. K. Stephan, and Z. Wang. 2003. A critical role for nucleus accumbens dopamine in partner-preference formation in male prairie voles. J. Neurosci. 23:3483–3490.

Bachmanov, A. A., M. G. Tordoff, and G. K. Beauchamp. 2001. Sweetener preference of C57BL/6ByJ and 129P3/J mice. Chem. Senses 26:905–913.

Baden, D. A. 2005. Effect of anticipation during unknown or unexpected exercise duration on rating of perceived exertion, affect, and physiological function * Commentary. Br. J. Sports Med. 39:742–746.

Belke, T. W. 1996. Investigating the reinforcing properties of running: Or, running as its own reward. Pp. 45–56 *in* Activity anorexia: Theory, research and treatment.

Belke, T. W., and T. Garland, Jr. 2007. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. J. Exp. Anal. Behav. 88:199–213.

Belke, T. W., and G. M. Heyman. 1994. Increasing and signaling background reinforcement: Effect on the foreground response—reinforcer relation. J. Exp. Anal. Behav. 61:65–81.

Belke, T. W., W. D. Pierce, and I. D. Duncan. 2006. Reinforcement value and substitutability of sucrose and wheel running: Implications for activity anorexia. J. Exp. Anal. Behav. 86:131–158.

Berridge, K. C., and T. E. Robinson. 1998. What is the role of dopamine in reward: hedonic impact, reward learning, or incentive salience? Brain Res. Rev. 28:309–369.

Blizard, D. A., B. Kotlus, and M. E. Frank. 1999. Quantitative trait loci associated with short-term intake of sucrose, saccharin and quinine solutions in laboratory mice. Chem. Senses 24:373–385.

Chambers, E. S., M. W. Bridge, and D. A. Jones. 2009. Carbohydrate sensing in the human mouth: effects on exercise performance and brain activity: Oral carbohydrate and exercise performance. J. Physiol. 587:1779–1794.

Claghorn, G. C., I. A. T. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol. Behav. 161:145–154.

Claghorn, G. C., Z. Thompson, K. Wi, L. Van, and T. Garland, Jr. 2017. Caffeine stimulates voluntary wheel running in mice without increasing aerobic capacity. Physiol. Behav. 170:133–140.

Cox, C. L., K. L. Stanhope, J. M. Schwarz, J. L. Graham, B. Hatcher, S. C. Griffen, A. A. Bremer, L. Berglund, J. P. McGahan, P. J. Havel, and others. 2012. Consumption of fructose-sweetened beverages for 10

weeks reduces net fat oxidation and energy expenditure in overweight/obese men and women. Eur. J. Clin. Nutr. 66:201–208.

Daniel, J., M. Cropley, M. Ussher, and R. West. 2004. Acute effects of a short bout of moderate versus light intensity exercise versus inactivity on tobacco withdrawal symptoms in sedentary smokers. Psychopharmacology (Berl.) 174.

Dess, N. K., C. D. Chapman, and D. Monroe. 2008. Consumption of SC45647 and Sucralose by Rats Selectively Bred for High and Low Saccharin Intake. Chem. Senses 34:211–220.

Dodd, M. L., K. J. Klos, J. H. Bower, Y. E. Geda, K. A. Josephs, and J. E. Ahlskog. 2005. Pathological gambling caused by drugs used to treat Parkinson disease. Arch. Neurol. 62:1377–1381.

Fuller, J. L. 1974. Single-locus control of saccharin preference in mice. J. Hered. 65:33–36.

Garland, Jr., T., S. A. Kelly, J. L. Malisch, E. M. Kolb, R. M. Hannon, B. K. Keeney, S. L. Van Cleave, and K. M. Middleton. 2011a. How to run far: multiple solutions and sex-specific responses to selective breeding for high voluntary activity levels. Proc. R. Soc. B Biol. Sci. 278:574–581.

Garland, Jr., T., H. Schutz, M. A. Chappell, B. K. Keeney, T. H. Meek, L. E. Copes, W. Acosta, C. Drenowatz, R. C. Maciel, G. van Dijk, C. M. Kotz, and J. C. Eisenmann. 2011b. The biological control of voluntary exercise,

spontaneous physical activity and daily energy expenditure in relation to obesity: human and rodent perspectives. J. Exp. Biol. 214:206–229.

Garland, Jr., T., M. Zhao, and W. Saltzman. 2016. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integr. Comp. Biol. 56:207–224.

Hernandez, L., and B. G. Hoebel. 1988. Food reward and cocaine increase extracellular dopamine in the nucleus accumbens as measured by microdialysis. Life Sci. 42:1705–1712.

Kayser, B. 2003. Exercise starts and ends in the brain. Eur. J. Appl. Physiol. 90:411–419.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland, Jr. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812–820.

Kolb, E. M., S. A. Kelly, K. M. Middleton, L. S. Sermsakdi, M. A. Chappell, and T. Garland. 2010. Erythropoietin elevates but not voluntary wheel running in mice. J. Exp. Biol. 213:510–519.

Koteja, P., J. G. Swallow, P. A. Carter, and T. Garland, Jr. 1999. Energy cost of wheel running in house mice: Implications for coadaptation of locomotion and energy budgets. Physiol. Biochem. Zool. 72:238–249.

Lewis, S. R., S. Ahmed, C. Dym, E. Khaimova, B. Kest, and R. J. Bodnar. 2005. Inbred mouse strain survey of sucrose intake. Physiol. Behav. 85:546–556.

López, H. H., and A. Ettenberg. 2002. Sexually conditioned incentives:: Attenuation of motivational impact during dopamine receptor antagonism. Pharmacol. Biochem. Behav. 72:65–72.

Meek, T. H., B. P. Lonquich, R. M. Hannon, and T. Garland. 2009. Endurance capacity of mice selectively bred for high voluntary wheel running. J. Exp. Biol. 212:2908–2917.

Menon, V., and D. J. Levitin. 2005. The rewards of music listening: Response and physiological connectivity of the mesolimbic system. NeuroImage 28:175–184.

Noakes, T. D. 2008. Testing for maximum oxygen consumption has produced a brainless model of human exercise performance. Br. J. Sports Med. 42:551–555.

Noakes, T. D. 2007. The limits of human endurance: What is the greatest endurance performance of all time? Which factors regulate performance at extreme altitude? Pp. 255–276 *in* Hypoxia and the Circulation. Springer, Boston, MA.

Pelz, W. E., G. Whitney, and J. C. Smith. 1973. Genetic influences on saccharin preference of mice. Physiol. Behav. 10:263–265.

Premack, D., R. W. Schaeffer, and A. Hundt. 1964. Reinforcement of drinking by running: effect of fixed ratio and reinforcement time. J. Exp. Anal. Behav. 7:91–96.

Ramirez, I., and J. L. Fuller. 1976. Genetic influence on water and sweetened water consumption in mice. Physiol. Behav. 16:163–168.

Rendeiro, C., A. M. Masnik, J. G. Mun, K. Du, D. Clark, R. N. Dilger, A. C. Dilger, and J. S. Rhodes. 2015. Fructose decreases physical activity and increases body fat without affecting hippocampal neurogenesis and learning relative to an isocaloric glucose diet. Sci. Rep. 5.

Rezende, E. L. 2006. Maximal oxygen consumption in relation to subordinate traits in lines of house mice selectively bred for high voluntary wheel running. J. Appl. Physiol. 101:477–485.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integr. Comp. Biol. 45:438–455.

Rhodes, J. S., and T. Garland, Jr. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 167:242–250.

Rhodes, J. S., T. Garland, Jr., and S. C. Gammie. 2003. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behav. Neurosci. 117:1243.

Rose, E. A., and G. Parfitt. 2007. A quantitative analysis and qualitative explanation of the individual differences in affective responses to prescribed and self-selected exercise intensities. J. Sport Exerc. Psychol. 29:281–309.

Samuel, V. T. 2011. Fructose induced lipogenesis: from sugar to fat to insulin resistance. Trends Endocrinol. Metab. 22:60–65.

Saul, M. C., P. Majdak, S. Perez, M. Reilly, T. Garland, Jr., and J. S. Rhodes. 2017. High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice. Genes Brain Behav. 16:328–341.

Schulze, K., M. Dadmarz, and W. H. Vogel. 2002. Voluntary self-administration of both morphine and cocaine by rats. Pharmacology 64:113–118.

Sherwin, C. M. 1998. Voluntary wheel running: a review and novel interpretation. Anim. Behav. 56:11–27.

Sherwin, C. M., and C. J. Nicol. 1996. Reorganization of behaviour in laboratory mice, Mus musculus, with varying cost of access to resources. Anim. Behav. 51:1087–1093.

Spangler, R., K. M. Wittkowski, N. L. Goddard, N. M. Avena, B. G. Hoebel, and S. F. Leibowitz. 2004. Opiate-like effects of sugar on gene expression in reward areas of the rat brain. Mol. Brain Res. 124:134–142.

Swallow, J. G., P. A. Carter, and T. Garland Jr. 1998. Artificial selection for increased wheel-running behavior in house mice. Behav. Genet. 28:227–237.

Swallow, J. G., J. P. Hayes, P. Koteja, and T. Garland, Jr. 2009. Selection experiments and experimental evolution of performance and physiology. Exp. Evol. Concepts Methods Appl. Sel. Exp. 301–351.

Swallow, J. G., P. Koteja, P. A. Carter, and T. Garland, Jr. 1999. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. J. Exp. Biol. 202:2513–2520.

Swallow, J., P. Koteja, P. Carter, and T. Garland. 2001. Food consumption and body composition in mice selected for high wheel-running activity. J. Comp. Physiol. B 171:651–659.

Taylor, A. H., M. H. Ussher, and G. Faulkner. 2007. The acute effects of exercise on cigarette cravings, withdrawal symptoms, affect and smoking behaviour: a systematic review. Addiction 102:534–543.

Thompson, Z., D. Argueta, T. Garland, Jr., and N. DiPatrizio. 2017. Circulating levels of endocannabinoids respond acutely to voluntary exercise, are altered in mice selectively bred for high voluntary wheel running, and differ between the sexes. Physiol. Behav. 170:141–150.

Timberlake, W., and M. Wozny. 1979. Reversibility of reinforcement between eating and running by schedule changes: A comparison of hypotheses and models. Anim. Learn. Behav. 7:461–469.

Ussher, M., P. Nunziata, M. Cropley, and R. West. 2001. Effect of a short bout of exercise on tobacco withdrawal symptoms and desire to smoke. Psychopharmacology (Berl.) 158:66–72.

Ussher, M., A. K. Sampuran, R. Doshi, R. West, and D. C. Drummond. 2004. Acute effect of a brief bout of exercise on alcohol urges. Addiction 99:1542–1547.

Vanderschuren, L. J. M. J., R. J. M. Niesink, and J. M. Van Pee. 1997. The neurobiology of social play behavior in rats. Neurosci. Biobehav. Rev. 21:309–326.

Wallace, I. J., and T. Garland, Jr. 2016. Mobility as an emergent property of biological organization: Insights from experimental evolution. Evol. Anthropol. Issues News Rev. 25:98–104.

Waters, R. P., R. B. Pringle, G. L. Forster, K. J. Renner, J. L. Malisch, T. Garland, Jr., and J. G. Swallow. 2013. Selection for increased voluntary wheel-running affects behavior and brain monoamines in mice. Brain Res. 1508:9–22.

CONCLUSION

Exercise is a complex, rewarding behavior with a multitude of positive effects on the body and brain. Aspects of exercise are modulated by several different neurotransmitters. For example, dopamine is a main neurotransmitter in both the reward and locomotor pathways of the brain. Microdialysis studies with rats have shown that exercise increases the concentration of dopamine in the striatum (Meeusen et al. 2001). Depletion of dopamine from the substantia nigra (similar to what occurs in Parkinson's disease), causes loss of motor control. Serotonin, or the ratio of serotonin to dopamine, is implicated in cessation of exercise behavior due to central fatigue (Claghorn et al. 2016). Endogneous opioids, particularly endorphins, increase with exercise and have long been held responsible for the phenomenon of the "runner's high," although more recent studies have shown that the endocannabinoid system is also at least partially responsible (Fuss and Gass 2010; Raichlen et al. 2012). In addition, targeted deletion of endocannabinoid receptor neurons in the ventral tegmental area decreases voluntary wheel-running in mice (Dubreucq et al. 2010).

Mice from the selectively-bred high runner (HR) lines discussed in this dissertation voluntarily exercise approximately three times as much as do those from non-selected control (C) lines. Previous research has shown that the dopaminergic system in HR mice differs from that of C mice. For example, three different dopamine reuptake blockers, administered peripherally, decreased wheel running in HR mice while having no effect or increasing wheel running in C

mice (Rhodes et al. 2001; Rhodes and Garland 2003). As blocking dopamine reuptake increases dopamine in the synapse, and this decreased running in the HR mice, these results suggest that HR mice normally have reduced dopamine function, similar to humans with ADHD (Rhodes et al. 2005). HR mice also differ in their response to dopamine receptor agonists and antagonists, in a way that specifically implicates reduced function of the D1-type dopamine receptor (Rhodes and Garland 2003). As compared with one C line, mice from one HR line also showed lower concentrations of dopamine and one of its metabolites in the dorsal raphe nucleus and substantia nigra (Waters et al. 2013).

Waters et al. (2013) also showed that mice from one HR line had lower concentrations of serotonin and one of its metabolites in the dorsal striatum and the substantia nigra. HR mice respond differently to a serotonin antagonist during endurance exercise, and to a serotonin agonist during voluntary exercise (Claghorn et al. 2016). However, HR mice do not display an altered wheel-running response to a serotonin reuptake inhibitor (Rhodes et al. 2001).

Only one study has examined the opioid system in the HR mice, and found that naloxone (an opioid receptor antagonist), increased the pain sensitivity of HR and C mice in a similar manner, and decreased wheel running in a similar proportion in both HR and C mice (Li et al. 2004). These results suggest that the opioid system has not differentially evolved in the HR mice.

Finally, both female and male HR mice differentially decrease their running after administration of a cannabinoid type-1 (CB_1) receptor agonist

(Keeney et al. 2012). When given a CB_1 receptor antagonist, only female HR mice showed a differential decrease in running (Keeney et al. 2008).

How do the results of this dissertation help us understand the evolution of voluntary exercise that has occurred in the HR mice? In Chapter 1, I was able to support a previous finding that the HR mice have a larger midbrain (Kolb et al. 2013). The midbrain contains several regions involved in both locomotion and reward. Using female HR and C mice, both with and without wheel access for 10 weeks, I found volumetric differences in two specific regions – the periaqueductal grey (PAG) and the ventral tegmental area (VTA). The volume of the PAG was positively affected by wheel access in C mice and negatively affected by wheel access in HR mice. The PAG contains neurons which release opioids, gamma-aminobutyric acid (GABA), and glutamate (Behbehani 1995). Interestingly, the VTA (which tended to be larger in HR mice) also releases GABA and glutamate. Based on previous results showing no difference in opioid-mediated pain transmission, further study of GABA and glutamate function in the HR mice may prove fruitful.

In Chapter 2, I provided further evidence that the endocannabinoid system is altered in HR mice. Plasma concentrations of 2-arachidonylglycerol (2-AG) were lower in females, and there was a significant interaction between the factors of sex, linetype, and wheel access for five days. Plasma levels of anandamide (AEA) were higher in females, and there was a significant linetype-by-wheel access interaction. In addition, although running in the previous 30 minutes

before sampling was a significant positive predictor of anandamide (AEA) concentration, HR mice had significantly lower levels than C mice, even though they ran more than C. More detailed analyses of cannabinoid receptor density and function will likely help us understand more about how the endocannabinoid system modulates voluntary exercise. Also, as seen here, the inclusion of both sexes in experiments, although it doubles the sample size, helps provide a more complete picture of the evolution of the HR phenotype.

In Chapter 3, I showed that HR mice have reduced incentive salience for a sweet-taste reward, but only when they have access to wheels. HR and C mice were given various sweet-tasting solutions, with and without access to wheels as a competing reward. All mice drank more of the sweet-tasting solutions, as evidenced by a strong dose effect for all analyses, but there was no difference in consumption between HR and C mice when they did not have wheel access. However, when HR mice had access to the competing reward of exercise, they showed a significantly smaller increase in consumption for the artificial sweeteners when compared to the C mice. This suggests that the processing of the reward from exercise in HR mice may differ from that of other rewards.

Overall, results from this dissertation have furthered our knowledge of the complexity of the increased voluntary exercise phenotype seen in the HR mice. New questions are also provided for study, including what might be the mechanism of increased volume in the midbrain, how the endocannabinoid

system has changed at the receptor level, and how the reward from exercise might compare to other types of rewards.

REFERENCES

Behbehani, M. M. 1995. Functional characteristics of the midbrain periaqueductal gray. Prog. Neurobiol. 46:575–605.

Claghorn, G. C., I. A. T. Fonseca, Z. Thompson, C. Barber, and T. Garland, Jr. 2016. Serotonin-mediated central fatigue underlies increased endurance capacity in mice from lines selectively bred for high voluntary wheel running. Physiol. Behav. 161:145–154.

Dubreucq, S., M. Koehl, D. N. Abrous, G. Marsicano, and F. Chaouloff. 2010. CB1 receptor deficiency decreases wheel-running activity: consequences on emotional behaviours and hippocampal neurogenesis. Exp. Neurol. 224:106–113.

Fuss, J., and P. Gass. 2010. Endocannabinoids and voluntary activity in mice: runner's high and long-term consequences in emotional behaviors. Exp. Neurol. 224:103–105.

Keeney, B. K., T. H. Meek, K. M. Middleton, L. F. Holness, and T. Garland Jr. 2012. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacol. Biochem. Behav. 101:528–537.

Keeney, B. K., D. A. Raichlen, T. H. Meek, R. S. Wijeratne, K. M. Middleton, G. L. Gerdeman, and T. Garland, Jr. 2008. Differential response to a selective cannabinoid receptor antagonist (SR141716: rimonabant) in

female mice from lines selectively bred for high voluntary wheel-running behaviour. Behav. Pharmacol. 19:812–820.

Kolb, E. M., E. L. Rezende, L. Holness, A. Radtke, S. K. Lee, A. Obenaus, and T. Garland. 2013. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. J. Exp. Biol. 216:515–523.

Li, G., J. S. Rhodes, I. Girard, S. C. Gammie, and T. Garland Jr. 2004. Opioid-mediated pain sensitivity in mice bred for high voluntary wheel running. Physiol. Behav. 83:515–524.

Meeusen, R., M. F. Piacentini, and K. De Meirleir. 2001. Brain microdialysis in exercise research. Sports Med. 31:965–983.

Raichlen, D. A., A. D. Foster, G. L. Gerdeman, A. Seillier, and A. Giuffrida. 2012. Wired to run: exercise-induced endocannabinoid signaling in humans and cursorial mammals with implications for the “runner’s high.” J. Exp. Biol. 215:1331–1336.

Rhodes, J., G. Hosack, I. Girard, A. Kelley, G. Mitchell, and T. Garland. 2001. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 158:120–131.

Rhodes, J. S., S. C. Gammie, and T. Garland, Jr. 2005. Neurobiology of mice selected for high voluntary wheel-running activity. Integr. Comp. Biol. 45:438–455.

Rhodes, J. S., and T. Garland. 2003. Differential sensitivity to acute administration of Ritalin, apormorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology (Berl.) 167:242–250.

Waters, R. P., R. B. Pringle, G. L. Forster, K. J. Renner, J. L. Malisch, T. Garland, Jr., and J. G. Swallow. 2013. Selection for increased voluntary wheel-running affects behavior and brain monoamines in mice. Brain Res. 1508:9–22.