Hippocampal, whole midbrain, red nucleus, and ventral tegmental area volumes are increased by selective breeding for high voluntary wheel-running behavior

Margaret P. Schmill [a,&], Zoe Thompson [a,b,&], Daisy Lee [c], Laurence Haddadin [c], Shaarang Mitra [c], Raymond Ezzat [c], Samantha Shelton [c], Phillip Levin [c], Sogol Behnam [c], Kelly J. Huffman [a, d], and Theodore Garland, Jr. [a,c,*]

[a] Neuroscience Graduate Program, University of California, Riverside, 92521, USA
[b] Present Address: Department of Biology, Utah Valley University, Orem, UT, 84058, USA
[c] Department of Evolution, Ecology, and Organismal Biology, University of California, Riverside, 92521, USA
[d] Department of Psychology, University of California, Riverside, 92521, USA
[&] Joint first authors.

Running title: Artificial selection on wheel-running behavior alters sizes of brain regions

[*] Corresponding author:
Theodore Garland, Jr.
Department of Evolution, Ecology, and Organismal Biology
University of California, Riverside
Riverside, CA 92521, USA
951-827-3524
tgarland@ucr.edu

Number of Tables: 3
Number of Figures: 6
Word count: 17,649 including Abstract and body text.

Keywords: Artificial selection, Brain size, Motivation, Plasticity, Voluntary exercise

Abstract

Uncovering relationships between neuroanatomy, behavior, and evolution is important for understanding the factors that control brain function. Voluntary exercise is one key behavior that both affects, and may be affected by, neuroanatomical variation. Moreover, recent studies suggest an important role for physical activity in brain evolution. We used a unique and ongoing artificial selection model in which mice are bred for high voluntary wheel-running behavior, yielding four replicate lines of High Runner (HR) mice that run ~3-fold more revolutions per day than four replicate non-selected Control (C) lines. Previous studies reported that, with body mass as a covariate, HR mice had heavier whole brains, non-cerebellar brains, and larger midbrains than C mice. We sampled mice from generation 66 and used high-resolution microscopy to test the hypothesis that HR mice have greater volumes and/or cell densities in nine key regions from either the midbrain or limbic system. In addition, half of the mice were given 10 weeks of wheel access from weaning, and we predicted that chronic exercise would increase the volumes of the examined brain regions via phenotypic plasticity. We replicated findings that both selective breeding and wheel access increased total brain mass, with no significant interaction between the two factors. In HR compared to C mice, adjusting for body mass, both the red nucleus (RN) of the midbrain and the hippocampus (HPC) were significantly larger, and the whole midbrain (WM) tended to be larger, with no effect of wheel access nor any interactions. Linetype and wheel access had an interactive effect on the volume of the periaqueductal gray (PAG), such that wheel access increased PAG volume in C mice but decreased volume in HR mice. Neither linetype nor wheel access affected volumes of the substantia nigra (SN), ventral

tegmental area (VTA), nucleus accumbens (NAc), ventral pallidum (VP) or basolateral amygdala (BLA). We found no main effect of either linetype or wheel access on neuronal densities (numbers of cells per unit area) for any of the regions examined. Taken together, our results suggest that the increased exercise phenotype of HR mice is related to increased red nucleus and hippocampal volumes, but that chronic exercise alone does not produce such phenotypes.

Introduction

The vertebrate brain is a complex organ, comprised of structures and areas with discrete functional specializations. Various studies comparing species have found that brain size or neuroanatomical structural volume correlates with behavioral ecology [Krebs et al. 1989; Hutcheon et al. 2002; Gonzalez-Voyer and Kolm 2010; Swanson et al. 2012; Liao et al. 2015; DeCasien and Higham 2019; Muller and Montgomery 2019]. For example, Raichlen and Gordon [2011] found a positive correlation between relative mammalian brain size and maximum aerobic metabolic rate (VO_2max), which they viewed as "a proxy for exercise frequency and capacity" [see also Albuquerque et al. 2015]. Species of insectivorous bats that rely on echolocation when pursuing prey have larger auditory nuclei than phytophagous bats [Hutcheon et al. 2002] and, among species of Carnivora, the relative volume of the cerebrum anterior to the cruciate sulcus is negatively related to both forelimb use in food processing and home range size [Swanson et al. 2012]. Brain-behavior relationships also exist in non-mammals; for example, species of food-storing birds that use long-lasting spatial memories for food retrieval have a larger hippocampal complex relative to their body size as compared with species that do not store food [Krebs et al. 1989] and food-caching birds have larger hippocampi relative to telencephalon size (or body size) [Sherry et al. 1989]. In lizards, the medial and dorsal cortices appear to be homologs of the mammalian hippocampus, and within the side-blotched lizard *Uta stansburiana*, dorsal cortical volume relative to the remainder of the telencephalon covaries with territory size [LaDage et al. 2009]. As another example, in chondrichthyans, relative sizes of major brain regions vary in relation to both ecological lifestyle and maternal investment [Mull et al. 2020].

Although neuroanatomical features can evolve in concert with behavior and ecology, they can also be affected by experiences and exposures throughout the lifetime. Interestingly, regional volumetric changes occur in the brains of rodents provided varying durations of voluntary exercise. For example, rats with just 7 days of wheel running showed significant increases in volumes of motor, somatosensory, association, and visual cortices [Sumiyoshi et al. 2014]. In another study, mice with 4 weeks of exercise had a significantly increased volume of the hippocampus [Cahill et al. 2015]. After a period of 6-8 weeks of housing with wheels, Biedermann et al. [2012] found increases in hippocampal volumes in the brains of these mice. These examples of phenotypic plasticity are consistent with an emerging view that the human brain may have coadapted with relatively high levels of aerobic activity [Raichlen and Alexander 2020]. Rodent wheel running is viewed as a model for some forms of human voluntary exercise [Eikelboom 1999; Garland, Jr. et al. 2011b]. Importantly, Meijer and Robbers [2014] showed that wild rodents will run on wheels placed in nature, indicating that wheel running is a motivated and elective behavior, not solely a lab artefact [see also Sherwin 1998; Novak et al. 2012; Greenwood and Fleshner 2019].

Our unique High Runner mouse model, which includes four replicate selectively bred High Runner (HR) lines and four non-selected Control (C) lines [Swallow et al. 1998], is well-poised to help answer questions about neuroanatomical responses to selection that acts on behavior, as well as phenotypic plasticity induced by physical activity. Since reaching selection limits around generation 17-27 (depending on line and sex), mice from the HR lines have run ~2.5-3 times as many revolutions per day as those from C lines [Keeney et al. 2008; Careau et al. 2013; Acosta et al. 2017; Hiramatsu et al.

2017; Kelly et al. 2017], primarily due to increased average (and maximum) running speeds [Garland et al. 2011a]. HR mice also run more intermittently on wheels [Girard et al. 2001] and are more active in home cages when wheels are not available [Malisch et al. 2009a; Copes et al. 2015]. Not surprisingly, both endurance capacity and maximal oxygen consumption (VO_2max) measured during forced treadmill exercise are enhanced in HR mice [Rezende et al. 2006; Meek et al. 2009; Kolb et al. 2010]. Various behavioral phenotypes differ between HR and C mice. Compared with C mice, HR mice build smaller nests when housed either with or without wheels [Carter et al. 2000]; HR mice make fewer turns in an open-field test [Bronikowski et al. 2001]; HR males have lower latencies to attack crickets in a test of predatory aggression [Gammie et al. 2003]; HR mice have reduced responsiveness to wheel-running rewards of shorter duration [Belke and Garland 2007]; HR males spend more time immobile in the forced-swim test when wheel deprived, suggesting a predisposition for depression-like behavior or increased fear responsivity [Malisch et al. 2009a]; finally, HR males spent more time in the closed arms of an elevated plus maze, suggesting increased anxiety, increased fear responsivity or decreased risk-taking [Singleton and Garland 2019]. The genetic and epigenetic basis for differences between the HR and C lines are an area of active investigation [Saul et al. 2017; Hillis et al. 2020; Nguyen et al. 2020; Latchney et al. 2022; Hillis and Garland 2023].

Additional neurobiological phenotypes have occurred from selective breeding. For example, HR mice have an altered dopaminergic system [Rhodes et al. 2001, 2005; Rhodes and Garland 2003; Mathes et al. 2010; Waters et al. 2013], unusual 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 HR mice show increased brain-derived neurotrophic factor (BDNF) acutely after running [Johnson et al., 2003]. We are interested in identifying neuroanatomical correlates of the HR behavioral phenotypes, as few studies have examined the neuroanatomy of HR mice. Kolb et al. [2013c] found that non-cerebellar brain mass of both sexes was significantly increased (as much as 7.0% for dry mass in males) when compared to C mice (with body mass as a covariate). These authors additionally used ex-vivo MRI to reveal that HR mice had a significantly larger midbrain volume than C mice (~13% with body mass as a covariate). Hiramatsu et al. [2017] reported that whole brain mass tended to be larger in HR mice and Cadney et al. [2021] found the brain to be statistically significantly larger in HR mice (both analyses with body mass as a covariate).

In the present study, we investigate midbrain and limbic regions associated with motor control, motivation, and/or reward. Although changes in brain region size do not necessarily show that a larger region is responsible for increased exercise behavior, it does provide evidence of a correlated response – i.e., these things have evolved together. In addition, with our experimental setup, we are able to tell if chronic exercise alone is responsible for an increase in brain region size. Our nine ROIs include the whole midbrain (WM) and, within the midbrain, the substantia nigra (SN), ventral tegmental area (VTA), red nucleus (RN), and periaqueductal gray (PAG). Within the limbic system, we assess the hippocampus (HPC), nucleus accumbens (NAc), ventral pallidum (VP), and basolateral amygdala (BLA). Our rationale for examining these regions follows (see also the Discussion).

Several previous studies of the HR 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]. For example, HR mice have decreased activity levels when administered the dopamine transporter blocker methylphenidate (Ritalin) and decreased sensitivity to D1-type receptor antagonist SCH 23390 [Rhodes and Garland 2003; Rhodes et al. 2005]. As an important part of both dopamine pathways, the 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 VTA 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 four studies have shown that the endocannabinoid system is altered in the HR mice [Keeney et al. 2008, 2012; Thompson et al. 2017; Schmill et al. 2022].

The RN is a midbrain region that plays a prominent role in locomotion. Within the RN, the rubrospinal tract originates and projects axons to the brain stem, cerebellum, and spinal cord to function in the control of muscle tone and limb movement. The rubrospinal system receives somatosensory inputs with information processed by the cerebellum and the basal ganglia, and from cortical motor areas [Schieber and Baker 2013]. To our knowledge, no previous study has examined the size of the RN in relation to any specific behavior, either among or within species.

The PAG plays a major role in both ascending and descending pain transmission [Behbehani 1995]. A previous study [Li et al. 2004] found no statistical difference in the response of HR and C mice to opioid antagonists (naloxone, naltrexone); specifically, they showed an equal decrease in tail-flick latency and a proportionally equal decrease in running. However, 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 pain sensitivity 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].

We also chose to analyze regions associated with the limbic system, because of their functions in reward-processing, motivation, and learning & memory. Size differences related to behavior or genetic factors have been reported for various limbic structures. For example, Makris et al. [2008] showed a decrease in volume in reward-related areas in alcoholic men, Gilman et al. [2014] found greater gray matter density in the nucleus accumbens of young adult marijuana users, and Seifert et al. [2015] observed a decrease in nucleus accumbens volume in heroin-dependent users undergoing treatment [see also Discussion as well as Rapuano et al. 2017]. Importantly, some evidence suggests the possibility of exercise addiction in the HR lines of mice [Malisch et al. 2009b; Kolb et al. 2013a]. These regions are also among those that respond to chronic exercise [Biedermann et al. 2012; Cahill et al. 2015; Yamamoto et al. 2017]. The additional regions studied here (HPC, BLA, NAc, and VP) have been implicated in not only affective behaviors, but also goal-directed and reward-seeking motivated behavior [Ambroggi et al. 2008; Stuber et al. 2011; Lee et al. 2016; Yang and Wang 2017; LeGates et al. 2018]. Moreover, previous studies of HR mice that examined the HPC found evolved differences in gene expression [Bronikowski et al. 2004], increased hippocampal brain-derived neurotrophic factor (BDNF) following a week of wheel running compared to C mice [Johnson et al. 2003], and wheel-running activation of dentate gyrus cells by Fos-IR that reached a plateau for HR but not C mice [Rhodes et al. 2003a].

The purposes of the present study were to uncover evolved differences in regional brain volume in relation to physical activity levels and to examine whether the regions of interest respond to chronic aerobic exercise experienced during a critical developmental window. Overall, this study contributes to our understanding of both brain evolution in response to selection and neuroplasticity.

Materials & Methods

Ethical approval

All experimental procedures were approved by the UC Riverside Institutional Animal Care and Use Committee (20080018).

Experimental Animals

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

One hundred females from generation 66, half HR and half C, were weaned at 3 weeks of age and placed directly into individual cages. For the next 10 weeks, half of the mice from each linetype were provided 24/7 access to running wheels [1.12 m in diameter, as in the routine selection experiment: Swallow et al. 1998] and the other half had no access to wheels. Passive infrared sensors mounted within each cage measured home-cage activity (HCA) [Copes et al. 2015]. During the final week of wheel access (or lack thereof) and into the 13th week, brains were removed following transcardial perfusion and subsequently preserved. Age at the time of perfusion ranged from 84 to 113 days, and averaged 99.0 days (SD =7.48 days, N = 100).

Tissue Processing and Imaging

Following removal, the brains were stored in 30% sucrose in 4% paraformaldehyde (PFA) for a minimum of 48 hours. Tissue was sectioned coronally at 40 micrometers thickness on a Leica CM1850 cryostat, with alternating slices placed on a slide designated for this experiment or a slide for future testing. Following sectioning, tissue was stained for Nissl substance using Cresyl Violet and digital images were taken for each region using a Zeiss Discovery V.12 stereo microscope and an attached Zeiss AxioCam. Magnification for each brain region was chosen for the best differentiation of region borders and for clarity. Sample sizes are <100 due to various artefacts of slicing and staining, e.g., tears or damage that made it impossible to demarcate regions accurately.

Regional Area Measurements

ImageJ software (NIH) was used to import each photograph and the polygon-selection tool provided the means to free-hand outline each region of interest. The Franklin and Paxinos mouse brain atlas [Franklin and Paxinos 1997, 2008] and online Allen Mouse Brain Atlas [Allen Institute for Brain Science 2021] were used as guides. A digital micrometer for each magnification was used to set the scale of pixels/μm, which was used to calculate the area of the outlined brain region in each image. For the whole midbrain (WM), periaqueductal gray (PAG) and nucleus accumbens (NAc), some images centrally contained the cerebral aqueduct (WM and PAG) or anterior commissure (NAc), which was also traced, then later subtracted from the area, volume, and cell count measurements. Images of the tracing details for individual regions are presented in the Online Supplementary Material.

Regional Cell Density Measurements

The Image-based Tool for Counting Nuclei (ITCN) was used in ImageJ to measure the number of cells within each photographed and outlined brain ROI [Byun et al. 2006]. Settings within the ITCN for each region included the average pixel width of cells, the minimum distance between cells, and dark peaks to be detected. The total number of cells was then summed and divided by the total cross-sectional area, generating the measure of average cell count per unit area. For the WM, PAG and NAc, the total cell number and area of their inner component (cerebral aqueduct or anterior commissure) were subtracted before calculating the cell count per unit area.

Volume Calculations

Brain region volumes were calculated using the area (μm^2) output (from the ITCN) multiplied by 80μm (the distance between each section). Values for missing or damaged tissue sections were replaced by taking the average of the previous and next section, or as follows for multiple sections: e.g., if sections 8, 9 & 10 were missing, the volume of section 7 was subtracted from section 11 to get number x, and then x was divided by 4 to get number y, which was added to the volume of section 7 to get the volume of section 8, then added to the volume of section 8 to get the area of section 9, etc. The volumes from each section in each ROI were then summed to get an overall estimate of the region volume for each mouse.

Except for WM and PAG, the volumes were multiplied by two because images were taken unilaterally to produce higher magnified photographs for accurate cell counts. Mice that were judged to be missing sections at the anterior or posterior end(s) of a region, as well as mice with lost or particularly damaged tissue (sometimes region specific) from the slicing and staining processes, were excluded from the analyses. Thus, some final sample sizes were considerably smaller than the starting number of 100.

Statistical Analyses

Following numerous previous studies using mice from the selection experiment, mixed models were implemented in SAS Procedure Mixed, with wheel access and linetype as main effects, and replicate line nested within linetype (HR vs. C) as a random effect. The interaction between wheel access and linetype was tested relative to the wheel*line(linetype) random effect term. Additionally, a subset of HR mice have a "mini-muscle" phenotype caused by a recessive allele which reduces hindlimb muscle mass by about half, and pleiotropically affects a variety of other traits [Garland et al. 2002; Kelly et al. 2013]. Mini-muscle mice were discerned at dissection by examination of the triceps surae muscles, then included as an additional main effect in all analyses. Body mass, age, the amount of time brains spent in paraformaldehyde prior to sectioning, and how long the sections spent in a freezer ($\log_{10}$ transformed) before Nissl staining were included as covariates for all volume analyses. Aside from body mass, all of these are viewed as nuisance variables, so they are not presented in the text or discussed. All analyses were then repeated with brain mass, rather than body mass, as a covariate. Neither body nor brain mass was used as a covariate for the cell density analyses. When needed, dependent variables were transformed to improve normality of residuals. Mice that had residual values greater than approximately three standard deviations from the mean were excluded from the final analyses. P-values ≤ 0.05 were treated as statistically significant. For interactions, P-values ≤ 0.1 were treated as significant, as the power to detect interactions is considerably lower than that for detecting main effects [Wahlsten 1990, 1991].

Results

Wheel Running

As expected, HR always ran more than C mice (Fig. 1), with the difference reaching statistical significance in weeks 2-10 (Table 1). The average ratio of wheel running over all 10 weeks for HR/C was 3.27 (LS Means from SAS). The effect of mini-muscle status was significant only in week 2 (Table 1), and this may represent a Type I error.

Home-cage Activity

The linetype-by-wheel access interaction was significant for home-cage activity during all weeks (Table 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 (Fig. 2). Mini-muscle status did not significantly affect HCA.

Body Mass and Whole-brain Mass

Mice from the HR lines tended to weigh less than C mice (25.1 vs. 27.8 grams, P = 0.0912, Fig. 3A), but wheel access did not significantly affect body mass (P = 0.1533), and the linetype-by-wheel access interaction was not significant (P = 0.2825). Body mass was positively related to age (P = 0.0447), but was not related to mini-muscle status (P = 0.6895).

With body mass as a covariate (P = 0.0056), HR mice tended to have heavier brains than C mice (LS means: 0.494 grams vs. 0.471 grams, P = 0.0676, Table 2, Fig.

3B), and mice with wheel access for 10 weeks had heavier brains than those without wheels (0.492 grams vs. 0.473 grams, P = 0.0127), with no interaction and no effect of mini-muscle status. Without body mass as a covariate, only the positive effect of wheel access remained (P = 0.0267).

Brain Region Volumes and Densities in Relation to Mini-muscle Status

The effect of the mini-muscle phenotype was never statistically significant (see Tables 2 and 3), regardless of whether body mass or brain mass was used as a covariate, and so is not mentioned further in the Results or in the Discussion.

Whole Midbrain

With body mass as a covariate, the total volume of the midbrain tended to be larger in the HR mice (P = 0.0713, Table 2, Fig. 4), an effect that was not apparent with brain mass as a covariate or with no covariate. The number of cells per unit area in the whole midbrain was not significantly affected by linetype, wheel access or their interaction (Table 3).

Substantia Nigra

Neither linetype nor wheel access affected the volume of the substantia nigra, with no interaction, regardless of the covariate used. Neither linetype nor wheel access affected the number of cells per unit area in the SN, with no significant interaction (Table 3).

Ventral Tegmental Area

Without body or brain mass as a covariate, HR mice tended to have a larger ventral tegmental area (P = 0.0820, Fig. 5A), with no effect of wheel access (P = 0.4180) and no interaction (P = 0.8184). Neither linetype (Table 3) nor wheel access had a significant effect on the number of cells per unit area in the VTA, with no significant interaction.

Red Nucleus

With body mass as a covariate, the total volume of the red nucleus was significantly larger in HR compared to C mice (P = 0.0151, Fig. 5B, Table 2), with no statistical effect of wheel access or the interaction between linetype and wheel access. Similar results were obtained without body mass as a covariate, although the linetype effect became statistically non-significant (P = 0.0988) when brain mass was used as a covariate. The number of cells per unit area in the RN was not significantly affected by linetype, wheel access or their interaction (Table 3).

Periaqueductal Gray

Linetype and wheel access had an interactive effect on the volume of the periaqueductal grey (interaction P = 0.0253, Fig. 5C): wheel access increased the volume of the PAG in C mice, while decreasing it in HR mice. This significant interaction was robust to using brain mass as the covariate or having no covariate. Neither linetype (Table 3) nor wheel access affected cells per unit area in the PAG, with no interaction.

Hippocampus

With body mass as a covariate, the total volume of the hippocampus was significantly larger in HR mice (P = 0.0134, Fig. 5D, Table 2), with no effect of wheel access nor an interaction. Results were similar with brain mass as a covariate (linetype P = 0.0876) or no covariate (linetype P = 0.0273). The number of cells per unit area in the HPC was not significantly affected by linetype, wheel access or the interaction (Table 3).

Basolateral Amygdala

The total volume of the basolateral amygdala was not significantly affected by linetype (P = 0.1416), wheel access or their interaction (Table 2), regardless of the covariate used. Brain mass was a significant positive predictor of basolateral amygdala volume (P = 0.0227). No effects on the number of cells per unit area were observed (Table 3).

Nucleus Accumbens

Nucleus accumbens total volume was not statistically affected by linetype, wheel access or their interaction (Table 2). The number of cells per unit area (excluding the anterior commissure) was also not significantly affected by linetype, wheel access or body mass, but the linetype-by-wheel access interaction (P = 0.0935) (Table 3) indicated that wheel access slightly decreased counts in C mice (-1%) but increased them in HR mice (+3%).

Ventral Pallidum

The total volume of the ventral pallidum was not statistically affected by linetype (P = 0.1546), wheel access or their interaction (Table 2), regardless of the covariate used, but brain mass was a positive predictor. No effect on the number of cells per unit area was observed (Table 3).

Discussion

Our primary goal was to test for differences in the size of key brain regions between four replicate lines of mice that have been bred for voluntary exercise since 1993 (66 generations in the present study) and their four non-selected control lines. In addition, we tested for effects of 10 weeks of wheel access, beginning at weaning, on the same brain regions. We also compared neuronal densities for each region. Our main findings were that mice with wheel access for 10 weeks had significantly heavier brains than those without wheels (regardless of whether body mass was used as a covariate), and that HR mice also tended to have relatively heavier brains than C mice (P = 0.0676 with body mass as a covariate). In addition, the volumes of some regions were either significantly larger (red nucleus, hippocampus: whether or not body mass was a covariate) or tended to be relatively larger (whole midbrain: P = 0.0713 with body mass was a covariate) in the HR mice. We also found one region with a significant interactive effect – wheel access increased the volume of the PAG in C mice but decreased it in HR mice (both with and without body mass or brain mass as a covariate).

Wheel Running and Home-cage Activity

Wheel-running (Fig. 1) and home-cage activity results were largely as previously reported [e.g., Malisch et al. 2009a; Garland et al. 2011b; Careau et al. 2013; Copes et al. 2015], with HR mice running ~3-fold more than C over the course of the experiment. When housed with wheels, mice from the HR and C lines have similar activity levels in their home cages, but without wheels HCA is increased in both linetypes, and the increase is much greater for HR mice (Fig. 2). Thus, HR mice appear to "transfer" their elevated wheel activity to cage activity when deprived of wheels [Rhodes et al. 2005; Copes et al. 2015; Acosta et al. 2017].

Body Mass and Whole-brain Mass

As expected from previous studies, HR mice tended to weigh less than C mice at the conclusion of the experiment (Fig. 3A, Table 2). However, 10 weeks of wheel access did not significantly affect body mass, with no interaction between linetype and wheel access. Female mice from these lines do tend to have smaller changes in body mass than males when given chronic 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; but see Hiramatsu and Garland 2018 regarding 6 days of wheel access]. 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 to consider because linetype differences are somewhat confounded with body size differences in the HR and C mice [Kolb et al. 2013c].

With body mass as a covariate, total brain mass (wet) tended to be larger in female HR mice (+5%, P = 0.0676), consistent with results of a previous study [Kolb et al.

2013c], which found that HR females from generation 34 had larger whole brains than C mice (wet mass +11%, P = 0.0768; dry mass +11%, P = 0.0650). However, the magnitude of the difference we measured was reduced to non-significance without body mass as a covariate (Table 2). Wheel access for 10 weeks increased total brain mass in both HR and C mice (+4%), a result seen previously in these lines, even with only 3 weeks of juvenile exercise [Cadney et al. 2021]. This result was robust to not using body mass as a covariate. 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]. Difference in brain-derived neurotrophic factor have been implicated previously in comparisons of the HR and C mice [Johnson et al. 2003; Latchney et al. Unpublished results].

Relationships of Whole Brain Mass to Regional Brain Volumes

The "mosaic" model of brain evolution posits that the size of individual structures is related to particular behaviors [Barton and Harvey 2000; DeCasien and Higham 2019] and contrasts with a model of coordinated structural evolution, in which the size of the whole brain evolves with little change in the relative size of individual regions [Finlay and Darlington 1995]. We observed both patterns in our comparisons [see also Kolb et al. 2013c]. Brain mass was a statistically significant positive predictor of RN, PAG, HPC, BLA, and VP volume, but not of whole midbrain, SN, VTA, or NAc volume (Table 2). Thus, the former structures generally covary with whole brain size, whereas the latter show somewhat independent variation in these lines of mice.

Midbrain: Whole Midbrain

We also supported another major result of Kolb et al. [2013c], who found that HR mice had larger midbrains than C mice (only with body mass as a covariate), though our result here (P = 0.0713) did not reach statistical significance. Although our sample size 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 (i.e., a direct genetic effect), rather than an effect of their higher physical activity acting as an intermediate phenotype (Fig. 6). However, HR mice have increased home-cage activity when they do not have access to wheels (Fig. 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 through phenotypic plasticity.

Midbrain: Ventral Tegmental Area

In absolute terms, 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; Schmill et al. 2022].

Midbrain: Red Nucleus

Given that the RN plays an important role in locomotion and that a previous study found HR midbrains to be enlarged [Kolb et al. 2013c], we predicted larger red nuclei in HR mice. Plastic changes in response to exercise often improve organ function (adaptive plasticity) [e.g., see Garland and Kelly 2006; Swallow et al. 2010; Anderson et al. 2014; Kelly et al. 2017]; therefore, we also expected that chronic locomotion on wheels might enlarge the RN, perhaps especially in HR mice because they run much more than C mice. Although the RN was significantly larger in HR mice (Table 2), its volume was not affected by wheel running.

In support of its role in locomotor behavior, Kolpakwar et al. [2021] found that patients with early-onset Parkinson's disease (PD) had significantly larger RN volumes compared to patients in a late-onset disease group [see also Colpan and Slavin 2010]. Whether this difference is related to the genetic basis of PD is unknown; however, literature suggests the existence of genetic effects that are unique to either early- or late-onset PD [Hicks et al. 2002; Billingsley et al. 2018; Blauwendraat et al. 2020]. Environmental risk factors for PD have also been identified [Kieburtz and Wunderle 2013; Delamarre and Meissner 2017], but only one study has shown that physically active individuals have a decreased risk of PD [Thacker et al. 2008]. Given that PD is characterized by the loss of midbrain dopamine, the presence of dopamine and dopamine receptor mRNA in the RN is noteworthy [Jellinger et al. 1981; Hurd et al. 2001]. [For a review of evidence pertaining to the role of dopaminergic pathways in regulating voluntary activity, see Ruiz-Tejada et al. 2022.]

The evolution and differentiation of the RN was primarily driven by quadrupedal locomotion, and its primary function involves the execution of voluntary movements by forelimbs and hindlimbs [Basile et al. 2021]. Hence, a larger red nucleus in HR mice may indicate enhanced control of limb movement. Furthermore, the RN contains sensory neurons that respond to painful stimulation, and connectivity suggests that the RN may contribute to the body's analgesic response via the descending antinociceptive system [Prado et al. 1984; Basile et al. 2021]. Interestingly, the PAG (see next section) is part of the same system, and PAG volume was influenced by a linetype-by-wheel access interaction.

In addition, a recent study [He et al. 2022] identified a pathway from the RN to the VTA that is involved specifically in exercise reward. Glutamatergic neurons in the magnocellular region of the RN had increased c-fos expression after a wheel-running program that stretched over 10 days, synapse onto dopaminergic VTA neurons, and are rewarding when activated. Given this information and the VTA results noted above, it is perhaps not surprising that we also found an increase in RN volume. He et al. [2022] also showed that there is a reciprocal pathway from the dopaminergic VTA neurons to the RN.

We hypothesize that volume differences in the RN & VTA contribute to the previously reported alterations in dopamine signaling in the HR mice [Rhodes and Garland 2003; Bronikowski et al. 2004; Rhodes et al. 2005; Mathes et al. 2010; Garland et al. 2011b]. These changes in HR dopamine function may be a basis for increased motivation to exercise.

Various lines of evidence suggest that HR mice are more highly motivated to run on wheels compared to C mice [Rhodes et al. 2005; Belke and Garland 2007]. Exercise is a rewarding behavior [Sher 1998; Ekkekakis et al. 2005; Dishman et al. 2006; Brené et al. 2007; Garland et al. 2011; Novak et al. 2012] and one possible reason for HR increased motivation could be a modified sensitivity to exercise reward, an idea supported by research showing HR mice have altered D1-type receptor signaling [Rhodes and Garland 2003]. Interestingly, a study using an operant conditioning paradigm, where mice were trained to lever-press in order to gain access to running wheels, found that a short (90 second) amount of running time was sufficient in C mice, but HR mice only learned the paradigm when the amount of running time allowed was 30 minutes [Belke and Garland 2007]. This suggests that the motivational system may have been altered in a way that reduces the value of short running durations. These results match one theory of attention deficit hyperactivity disorder – that those with ADHD have an elevated reward threshold – and ADHD likely also involves changes in dopamine signaling [Sharma and Couture 2014; Klein et al. 2019].

Midbrain: Periaqueductal Gray

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 (Fig. 5C), 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 has other functions besides pain transmission, such as the processing of fear and anxiety, and the production of vocalizations [Behbehani 1995]. Adult male HR mice from generation 68 spent more time in the closed arms of an elevated plus maze, suggesting increased anxiety [Singleton and Garland 2019] [see also test Jónás et al. 2010], although this result was not obtained for males at generation 72 [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.

Limbic Regions: Hippocampus

We might expect both evolutionary history and exercise during ontogeny to impact hippocampal volume. In general, hippocampal volume in mammals increases as a function of brain volume [e.g., see Finlay and Darlington 1995; Patzke et al. 2015]. As mentioned in the Introduction, Biedermann et al. [2012] and Cahill et al. [2015] found increased hippocampal volumes in mice provided 4-8 weeks of wheel access. Additionally, two studies by Scholz et al. [2015a, 2015b] found an increase in whole HPC volume following environmental enrichment that included a running wheel for either 24 h (1.4% increase) or three weeks (3.8% increase) and a larger HPC after 80 5-min trials of rotarod training compared to mice that went without. In addition, both a review and a meta-analysis have noted that aerobic exercise often increases whole or partial hippocampal volumes in both mice and humans [Li et al. 2017; Rendeiro and Rhodes 2018].

In the current study, we found that HR mice had larger hippocampal volumes than C mice, but 10 weeks of wheel access did not affect the volume of the whole HPC (Table 2: all $P > 0.7$). This is different from the effects observed for the dentate gyrus by Rhodes et al. [2003b], who found that wheel access for 40 days (beginning at 4 weeks of age) significantly increased the volume of the dentate gyrus (a part of the hippocampal formation) in both HR and C mice, in association with increased neurogenesis and BDNF concentration. Interestingly, in a mouse model of Alzheimer's disease, four months of wheel access can protect against myelin sheath degeneration within mouse CA1, which is associated with increased CA1 volume [Chao et al. 2018]. Therefore, understanding the subregional differences for HR mice could provide important insights for some neurobiological diseases.

Limbic Regions: Basolateral Amygdala, Nucleus Accumbens, and Ventral Pallidum

The BLA plays an integral role in anxiety, and excitatory projections from BLA to ventral HPC are sufficient to mediate anxiety [Yang and Wang 2017]. HR mice of both sexes have resting corticosterone levels approximately twice those of C mice [Malisch et al. 2007] and males have increased anxiety-like behavior in an elevated plus maze [Singleton and Garland 2019]. The BLA, as well as the NAc and VP, are crucial to reward behaviors [Schultz 1998; Ambroggi et al. 2008; Smith et al. 2009; Stuber et al. 2011; Berridge and Kringelbach 2015; Lee et al. 2016; Yang and Wang 2017] and, therefore, make prime targets for study because of the alterations in dopamine signaling in the HR lines noted above.

To our knowledge, no studies have correlated volume differences in the BLA or VP with variations in either general or exercise-related reward. However, one study showed that children who are genetically at risk for obesity showed elevated reward-related responses to food cues in the NAc, and this area was also larger in these children

[Rapuano et al. 2017]. LIn addition, in a study of elderly males (without cognitive impairment), self-reported exercise habits were associated with greater volume of the bilateral NAc [Yamamoto et al. 2017]. In this context, it is worth noting that the HR mice show some evidence for exercise addiction [Kolb et al. 2013b].

We did not observe statistically significant volumetric differences in BLA, NAc or VP in HR as compared with C mice, which suggests that changes in the HR brain reward and motivational systems either do not involve these regions, or are restricted to lower levels, e.g., neurotransmitter release and reception. However, the HR ventral tegmental area tended to be larger in absolute size as compared with C mice (P = 0.0820), and dopaminergic connections from this region may be influencing BLA, NAc, and VP in a downstream fashion. Furthermore, mice with wheel access did not differ from sedentary mice for volumes of these regions (Table 2). However, underlying effects of exercise on brain plasticity may be contributing to HR motivation in ways that do not affect volume but rather circuits and/or neuromodulation.

Cell Densities

Aside from a linetype-by-wheel access interaction in the nucleus accumbens (Table 3), we did not observe any significant differences in the number of cells per unit area in any of the regions investigated. Because our measurements were total number of cells per unit area, we may not have been able to see more subtle changes – such as those of increased cell proliferation and hippocampal neurogenesis, which have previously been shown with wheel running [van Praag et al. 1999], including in these lines of mice [Rhodes et al. 2003b]. Correlates of brain plasticity can also include changes in dendritic structure [Hickmott and Steen 2005]. Lin et al. [2012] found that both treadmill exercise and wheel running increased the size of the dendritic field and increased dendritic spine density in the hippocampus. These alterations may be a more common consequence of aerobic exercise, rather than an increase in total cell number or cell density.

Concluding Remarks and Future Directions

In this study, our goal was to determine whether selection for increased wheel-running behavior has produced changes in either brain structure size or cell density, and whether either of these would change with chronic exercise. Our primary results were that the red nucleus and hippocampus were significantly larger in HR mice. We also found that chronic wheel running increased brain mass in both HR and C mice (regardless of the covariate used), and that HR mice tended to have increased total brain mass compared to C mice (with body mass as a covariate).

Our experimental system provides a unique way to relate increased exercise behavior to the evolution of particular brain regions. Artificial selection provides a means to breed for a behavior of interest and use the resultant evolved organisms to test hypotheses about correlated evolution of other traits, including the brain and its function [Roderick et al. 1976; Rhodes and Kawecki 2009; Garland et al. 2016]. Uncovering correlated responses to selection on behavior in real time (i.e., across generations) in a “top-down” fashion, from an altered behavior to potentially altered organs to tissues to proteins to DNA [Saul et al. 2017; Hillis et al. 2020; Nguyen et al. 2020; Latchney et al. 2022], may ultimately provide insights about specific genetic and epigenetic mechanisms that underlie individual and species differences in brain morphology and function, including the possibility of "multiple solutions" in response to selection [Garland et al.

2011a; Hillis and Garland 2023]. For example, results of the present study support the idea that the human brain may have coadapted with the evolution of aerobically supported physical activity [Raichlen and Alexander 2020] and that it indeed needs aerobic exercise to function optimally [Raichlen and Alexander 2020]. They also support the positive correlation between relative mammalian brain size and VO_2max [Raichlen and Gordon 2011]. The present results also provide new insights to brain evolution, in particular the finding that the red nucleus (RN) of the midbrain is associated with inherent propensity and/or ability for sustained, endurance-type physical activity. Although these results do not provide direct evidence of a cause-effect relationship, they do help generate hypotheses that could be tested with direct manipulation of a particular brain region.

Overall, no changes in regional neuronal density were uncovered. Therefore, neither wheel running nor selective breeding appear to be driving neurogenesis in the nine investigated brain regions [with the exception of dentate gyrus: see Rhodes et al. 2003b]. Rather, the volumetric changes observed may be caused by hypertrophic (or hypotrophic in the case of HR PAG) effects on the neurons and/or glial cells of these regions. Future studies should test for differences in neuronal growth, glial activity, dendritic spines, and microstructural changes in the HR brain, particularly in the red nucleus and hippocampus [see also Roth et al. 2010].

One limitation of the present study is that it did not include males. Given that a number of sex differences in both the direct (i.e., wheel running itself) and correlated responses to selection have been documented [e.g., Carter et al. 2000; Bronikowski et al. 2001, 2002; Garland et al. 2011a; Keeney et al. 2012; Acosta et al. 2017; Latchney et al.

2022], it will be important to include both sexes in any future studies of brain regional volumes.

Finally, we note that the present study included only one cohort of mice, whereas wheel-running behavior shows strong seasonal variation [Careau et al. 2013]. Given that at least one species of bird shows seasonal changes in the size of the hippocampus that have been argued to be causally related to seasonal changes in food caching [Sherry and Hoshooley 2010], it would be of interest to test for seasonal changes in the size of the hippocampus in the HR and C lines of mice.

Acknowledgments

We thank members of the Garland and Huffman labs for assistance.

Statement of Ethics

Procedures were approved by the UCR Institutional Animal Care and Use Committee (20080018).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

Supported by NSF grants to T.G., most recently IOS-2038528.

Author Contributions

The research was designed by MPS, ZT, KJH, and TG. MPS and ZT conducted the research, with assistance from DL, LH, SM, RE, SS, PL, and SB. MPS, ZT, and TG performed statistical analyses. MPS, ZT, and TG wrote the initial draft, then KJH edited.

All authors approved the final version.

Data Availability Statement

The data are available from the corresponding author on request.

References

Acosta W, Meek TH, Schutz H, Dlugosz EM, Garland, Jr. T. Preference for Western diet coadapts in High Runner mice and affects voluntary exercise and spontaneous physical activity in a genotype-dependent manner. Behavioural Processes. 2017 Feb 1;135:56–65.

Albuquerque RL, Sanchez G, Garland, Jr. T. Relationship between maximal oxygen consumption (VO_2max) and home range area in mammals. Physiological and Biochemical Zoology. 2015 Nov;88[6]:660–7.

Allen Institute for Brain Science. Allen mouse brain atlas [Internet]. Interactive Atlas Viewer. 2021. Available from: http://mouse.brain-map.org/experiment/thumbnails/100048576?image_type=atlas

Ambroggi F, Ishikawa A, Fields HL, Nicola SM. Basolateral amygdala neurons facilitate reward-seeking behavior by exciting nucleus accumbens neurons. Neuron. 2008 Aug 28;59[4]:648–61.

Anderson PS, Renaud S, Rayfield EJ. Adaptive plasticity in the mouse mandible. BMC Evolutionary Biology. 2014 Apr 18;14[1]:85.

Barton RA, Harvey PH. Mosaic evolution of brain structure in mammals. Nature. 2000;405[6790]:1055–8.

Basile GA, Quartu M, Bertino S, Serra MP, Boi M, Bramanti A, et al. Red nucleus structure and function: From anatomy to clinical neurosciences. Brain Struct Funct. 2021 Jan 1;226[1]:69–91.

Behbehani MM. Functional characteristics of the midbrain periaqueductal gray. Progress in Neurobiology. 1995 Aug 1;46[6]:575–605.

Belke TW, Garland, Jr. T. A brief opportunity to run does not function as a reinforcer for mice selected for high daily wheel-running rates. Journal of the Experimental Analysis of Behavior. 2007 Sep 1;88[2]:199–213.

Berridge KC, Kringelbach ML. Pleasure systems in the brain. Neuron. 2015 May 6;86[3]:646–64.

Biedermann S, Fuss J, Zheng L, Sartorius A, Falfán-Melgoza C, Demirakca T, et al. In vivo voxel based morphometry: Detection of increased hippocampal volume and decreased glutamate levels in exercising mice. NeuroImage. 2012 Jul 16;61[4]:1206–12.

Billingsley KJ, Bandres-Ciga S, Saez-Atienzar S, Singleton AB. Genetic risk factors in Parkinson’s disease. Cell Tissue Res. 2018 Jul 1;373[1]:9–20.

Blauwendraat C, Nalls MA, Singleton AB. The genetic architecture of Parkinson’s disease. The Lancet Neurology. 2020 Feb 1;19[2]:170–8.

Brené S, Bjørnebekk A, Åberg E, Mathé AA, Olson L, Werme M. Running is rewarding and antidepressive. Physiology & Behavior. 2007 Sep;92[1–2]:136–40.

Bronikowski AM, Carter PA, Swallow JG, Girard IA, Rhodes JS, Garland, Jr. T. Open-field behavior of house mice selectively bred for high voluntary wheel-running. Behav Genet. 2001 May 1;31[3]:309–16.

Bronikowski AM, Morgan TJ, Garland, Jr. T, Carter PA. Antioxidant gene expression in active and sedentary house mice [*Mus domesticus*] selected for high voluntary wheel-running behavior. Genetics. 2002;161[4]:1763–9.

Bronikowski AM, Rhodes JS, Garland, Jr. T, Prolla TA, Awad TA, Gammie SC, et al. The evolution of gene expression in mouse hippocampus in response to selective breeding for increased locomotor activity. Evolution. 2004 Sep 1;58[9]:2079–86.

Byun J, Verardo MR, Sumengen B, Lewis GP, Manjunath BS, Fisher SK. Automated tool for the detection of cell nuclei in digital microscopic images: Application to retinal images. Molecular Vision. 2006;12:949–60.

Cadney MD, Hiramatsu L, Thompson Z, Zhao M, Kay JC, Singleton JM, et al. Effects of early-life exposure to Western diet and voluntary exercise on adult activity levels, exercise physiology, and associated traits in selectively bred High Runner mice. Physiology & Behavior. 2021;234:113389.

Cahill LS, Steadman PE, Jones CE, Laliberté CL, Dazai J, Lerch JP, et al. MRI-detectable changes in mouse brain structure induced by voluntary exercise. NeuroImage. 2015 Jun;113:175–83.

Careau V, Wolak ME, Carter PA, Garland, Jr. T. Limits to behavioral evolution: The quantitative genetics of a complex trait under directional selection. Evolution. 2013 Nov 1;67[11]:3102–19.

Carter PA, Swallow JG, Davis SJ, Garland, Jr. T. Nesting behavior of house mice [*Mus domesticus*] selected for increased wheel-running activity. Behav Genet. 2000 Mar 1;30[2]:85–94.

Chao F lei, Zhang L, Zhang Y, Zhou C ni, Jiang L, Xiao Q, et al. Running exercise protects against myelin breakdown in the absence of neurogenesis in the hippocampus of AD mice. Brain Research. 2018 Apr 1;1684:50–9.

Childress JE, McDowell EJ, Dalai VVK, Bogale SR, Ramamurthy C, Jawaid A, et al. Hippocampal volumes in patients with chronic combat-related posttraumatic stress disorder: A systematic review. JNP. 2013 Jan 1;25[1]:12–25.

Colcombe SJ, Erickson KI, Scalf PE, Kim JS, Prakash R, McAuley E, et al. Aerobic exercise training increases brain volume in aging humans. The Journals of Gerontology: Series A. 2006 Nov 1;61[11]:1166–70.

Colpan ME, Slavin KV. Subthalamic and red nucleus volumes in patients with Parkinson’s disease: do they change with disease progression? Parkinsonism Relat Disord. 2010 Jul;16[6]:398–403.

Copes LE, Schutz H, Dlugosz EM, Acosta W, Chappell MA, Garland, Jr. T. Effects of voluntary exercise on spontaneous physical activity and food consumption in mice: Results from an artificial selection experiment. Physiology & Behavior. 2015 Oct 1;149:86–94.

DeCasien AR, Higham JP. Primate mosaic brain evolution reflects selection on sensory and cognitive specialization. Nat Ecol Evol. 2019 Oct;3[10]:1483–93.

Delamarre A, Meissner WG. Epidemiology, environmental risk factors and genetics of Parkinson's disease. La Presse Médicale. 2017 Mar 1;46[2, Part 1]:175–81.

Dishman RK, Berthoud HR, Booth FW, Cotman CW, Edgerton VR, Fleshner MR, et al. Neurobiology of exercise. Obesity. 2006 Mar 1;14[3]:345–56.

Dubreucq S, Durand A, Matias I, Bénard G, Richard E, Soria-Gomez E, et al. Ventral Tegmental Area Cannabinoid Type-1 Receptors Control Voluntary Exercise Performance. Biological Psychiatry. 2013 May 1;73[9]:895–903.

Eikelboom R. Human parallel to voluntary wheel running: Exercise. Animal Behaviour. 1999 Mar 1;57[3]:F11–2.

Ekkekakis P, Hall EE, Petruzzello SJ. Variation and homogeneity in affective responses to physical activity of varying intensities: An alternative perspective on dose-response based on evolutionary considerations. Journal of Sports Sciences. 2005 May 1;23[5]:477–500.

Finlay BL, Darlington RB. Linked regularities in the development and evolution of mammalian brains. Science. 1995 Jun 16;268[5217]:1578–84.

Franklin K, Paxinos G. The mouse brain in stereotaxic coordinates. 3rd ed. New York, NY: Elsevier; 2008.

Franklin KBJ, Paxinos G. The Mouse Brain in Stereotaxic Coordinates. Academic Press; 1997.

Freed CR, Yamamoto BK. Regional brain dopamine metabolism: A marker for the speed, direction, and posture of moving animals. Science. 1985 Jul 5;229[4708]:62–5.

Gammie SC, Hasen NS, Rhodes JS, Girard I, Garland, Jr. T. Predatory aggression, but not maternal or intermale aggression, is associated with high voluntary wheel-running behavior in mice. Hormones and Behavior. 2003 Sep 1;44[3]:209–21.

Garland, Jr. T, Kelly SA. Phenotypic plasticity and experimental evolution. Journal of Experimental Biology. 2006 Jun 15;209[12]:2344–61.

Garland, Jr. T, Kelly SA, Malisch JL, Kolb EM, Hannon RM, Keeney BK, et al. 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. 2011a;278[1705]:574–81.

Garland, Jr. T, Morgan MT, Swallow JG, Rhodes JS, Girard I, Belter JG, et al. Evolution of a small-muscle polymorphism in lines of house mice selected for high activity levels. Evolution. 2002;56[6]:1267–75.

Garland, Jr. T, Schutz H, Chappell MA, Keeney BK, Meek TH, Copes LE, et al. 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. 2011b Jan 15;214[2]:206–29.

Garland, Jr. T, Zhao M, Saltzman W. Hormones and the evolution of complex traits: insights from artificial selection on behavior. Integrative and Comparative Biology. 2016;56:207–24.

Gilman JM, Kuster JK, Lee S, Lee MJ, Kim BW, Makris N, et al. Cannabis use is quantitatively associated with nucleus accumbens and amygdala abnormalities in young adult recreational users. J Neurosci. 2014 Apr 16;34[16]:5529–38.

Girard I, McAleer MW, Rhodes JS, Garland, Jr. T. Selection for high voluntary wheel-running increases speed and intermittency in house mice [*Mus domesticus*]. Journal of Experimental Biology. 2001 Dec 15;204[24]:4311–20.

Gonzalez-Voyer A, Kolm N. Sex, ecology and the brain: Evolutionary correlates of brain structure volumes in Tanganyikan cichlids. PLOS ONE. 2010 Dec 17;5[12]:e14355.

Greenwood BN, Fleshner M. Voluntary wheel running: A useful rodent model for investigating mechanisms of stress robustness and exercise motivation. Current Opinion in Behavioral Sciences. 2019 Aug 1;28:78–84.

Greenwood BN, Foley TE, Le TV, Strong PV, Loughridge AB, Day HEW, et al. Long-term voluntary wheel running is rewarding and produces plasticity in the mesolimbic reward pathway. Behavioural Brain Research. 2011 Mar 1;217[2]:354–62.

He Y, Madeo G, Liang Y, Zhang C, Hempel B, Liu X, et al. A red nucleus-VTA glutamate pathway underlies exercise reward and the therapeutic effect of exercise on cocaine use. Sci Adv. 2022 Sep 2;8[35]:eabo1440.

Hickmott PW, Steen PA. Large-scale changes in dendritic structure during reorganization of adult somatosensory cortex. Nat Neurosci. 2005 Feb;8[2]:140–2.

Hicks AA, Pétursson H, Jónsson T, Stefánsson H, Jóhannsdóttir HS, Sainz J, et al. A susceptibility gene for late-onset idiopathic Parkinson's disease. Annals of Neurology. 2002;52[5]:549–55.

Hillis D, Yadgary L, Weinstock GM, Pardo−Manuel de Villena G, Pomp D, Fowler A, et al. Genetic basis of aerobically supported voluntary exercise: results from a selection experiment with house mice. Genetics. 2020;216:781–804.

Hillis DA, Garland, Jr. T. Multiple solutions at the genomic level in response to selective breeding for high locomotor activity. Genetics. 2023;223[1]:iyac165.

Hiramatsu L, Garland, Jr. T. Mice selectively bred for high voluntary wheel-running behavior conserve more fat despite increased exercise. Physiology & Behavior. 2018;194:1–8.

Hiramatsu L, Kay JC, Thompson Z, Singleton JM, Claghorn GC, Albuquerque RL, et al. Maternal exposure to Western diet affects adult body composition and voluntary wheel running in a genotype-specific manner in mice. Physiology & Behavior. 2017 Oct 1;179:235–45.

Hurd YL, Suzuki M, Sedvall GC. D1 and D2 dopamine receptor mRNA expression in whole hemisphere sections of the human brain. Journal of Chemical Neuroanatomy. 2001 Jul 1;22[1]:127–37.

Hutcheon JM, Kirsch JAW, Jr TG. A comparative analysis of brain size in relation to foraging ecology and phylogeny in the Chiroptera. BBE. 2002;60[3]:165–80.

Ilango A, Kesner AJ, Keller KL, Stuber GD, Bonci A, Ikemoto S. Similar roles of substantia nigra and ventral tegmental dopamine neurons in reward and aversion. J Neurosci. 2014 Jan 15;34[3]:817–22.

Jellinger K, Reynolds GP, Riederer P. Dopamine and noradrenaline in the magno- and parvocellular portions of the red nucleus. In: Riederer P, Usdin E, editors. Transmitter Biochemistry of Human Brain Tissue [Internet]. Palgrave Macmillan, London; 1981 [cited 2021 Nov 22]. p. 115–25. Available from: https://doi.org/10.1007/978-1-349-05932-4_11

Johnson RA, Rhodes JS, Jeffrey SL, Garland, Jr. T, Mitchell GS. Hippocampal brain-derived neurotrophic factor but not neurotrophin-3 increases more in mice selected for increased voluntary wheel running. Neuroscience. 2003 Sep 26;121[1]:1–7.

Jónás I, Schubert KA, Reijne AC, Scholte J, Garland, Jr. T, Gerkema MP, et al. Behavioral traits are affected by selective breeding for increased wheel-running behavior in mice. Behav Genet. 2010 Jul 1;40[4]:542–50.

Keeney BK, Meek TH, Middleton KM, Holness LF, Garland, Jr. T. Sex differences in cannabinoid receptor-1 (CB1) pharmacology in mice selectively bred for high voluntary wheel-running behavior. Pharmacology Biochemistry and Behavior. 2012 Jun 1;101[4]:528–37.

Keeney BK, Raichlen DA, Meek TH, Wijeratne RS, Middleton KM, Gerdeman GL, et al. 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. 2008 Dec;19[8]:812.

Kelly SA, Bell TA, Selitsky SR, Buus RJ, Hua K, Weinstock GM, et al. 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. 2013 Dec 1;195[4]:1385–95.

Kelly SA, Gomes FR, Kolb EM, Malisch JL, Garland, Jr. T. Effects of activity, genetic selection and their interaction on muscle metabolic capacities and organ masses in mice. Journal of Experimental Biology. 2017 Mar 15;220[6]:1038–47.

Kieburtz K, Wunderle KB. Parkinson's disease: Evidence for environmental risk factors. Movement Disorders. 2013;28[1]:8–13.

Klein MO, Battagello DS, Cardoso AR, Hauser DN, Bittencourt JC, Correa RG. Dopamine: functions, signaling, and association with neurological diseases. Cell Mol Neurobiol. 2019 Jan;39[1]:31–59.

Kolb EM, Kelly SA, Garland, Jr. T. Mice from lines selectively bred for high voluntary wheel running exhibit lower blood pressure during withdrawal from wheel access. Physiology & Behavior. 2013a Mar;112–113:49–55.

Kolb EM, Kelly SA, Garland, Jr. T. Mice from lines selectively bred for high voluntary wheel running exhibit lower blood pressure during withdrawal from wheel access. Physiology & Behavior. 2013b Mar 15;112–113:49–55.

Kolb EM, Kelly SA, Middleton KM, Sermsakdi LS, Chappell MA, Garland, Jr. T. Erythropoietin elevates VO_2max but not voluntary wheel running in mice. J Exp Biol. 2010 Feb 1;213[3]:510–9.

Kolb EM, Rezende EL, Holness L, Radtke A, Lee SK, Obenaus A, et al. Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution. Journal of Experimental Biology. 2013c Feb 1;216[3]:515–23.

Kolpakwar S, Arora AJ, Pavan S, Kandadai RM, Alugolu R, Saradhi MV, et al. Volumetric analysis of subthalamic nucleus and red nucleus in patients of advanced Parkinson's disease using SWI sequences. Surg Neurol Int. 2021 Jul 27;12:377.

Krebs JR, Sherry DF, Healy SD, Perry VH, Vaccarino AL. Hippocampal specialization of food-storing birds. PNAS. 1989 Feb 1;86[4]:1388–92.

LaDage LD, Riggs BJ, Sinervo B, Pravosudov VV. Dorsal cortex volume in male side-blotched lizards, *Uta stansburiana*, is associated with different space use strategies. Animal Behaviour. 2009 Jul;78[1]:91–6.

Latchney SE, Cadney MD, Hopkins A, Garland, Jr. T. DNA methylation analysis of imprinted genes in the cortex and hippocampus of cross-fostered mice selectively bred for increased voluntary wheel-running. Behavior Genetics. 2022;52:281–97.

Latchney SE, Cadney MD, Hopkins A, Garland, Jr. T. Maternal upbringing and selective breeding for elevated voluntary exercise modify DNA methylation profiles and expression of genes involved in converging signaling pathways in the brain. Unpublished results;

Lee SC, Amir A, Headley DB, Haufler D, Pare D. Basolateral amygdala nucleus responses to appetitive conditioned stimuli correlate with variations in conditioned behaviour. Nat Commun. 2016 Jul 22;7[1]:12275.

LeGates TA, Kvarta MD, Tooley JR, Francis TC, Lobo MK, Creed MC, et al. Reward behaviour is regulated by the strength of hippocampus–nucleus accumbens synapses. Nature. 2018 Dec;564[7735]:258–62.

Lerch JP, Yiu AP, Martinez-Canabal A, Pekar T, Bohbot VD, Frankland PW, et al. Maze training in mice induces MRI-detectable brain shape changes specific to the type of learning. NeuroImage. 2011 Feb 1;54[3]:2086–95.

Li G, Rhodes JS, Girard I, Gammie SC, Garland, Jr. T. Opioid-mediated pain sensitivity in mice bred for high voluntary wheel running. Physiology & Behavior. 2004 Dec 15;83[3]:515–24.

Li M yi, Huang M mao, Li S zhen, Tao J, Zheng G hua, Chen L dian. The effects of aerobic exercise on the structure and function of DMN-related brain regions: A systematic review. International Journal of Neuroscience. 2017 Jul 3;127[7]:634–49.

Liao WB, Lou SL, Zeng Y, Merilä J. Evolution of anuran brains: Disentangling ecological and phylogenetic sources of variation. Journal of Evolutionary Biology. 2015;28[11]:1986–96.

Lin TW, Chen SJ, Huang TY, Chang CY, Chuang JI, Wu FS, et al. Different types of exercise induce differential effects on neuronal adaptations and memory performance. Neurobiology of Learning and Memory. 2012 Jan 1;97[1]:140–7.

Maguire EA, Gadian DG, Johnsrude IS, Good CD, Ashburner J, Frackowiak RSJ, et al. Navigation-related structural change in the hippocampi of taxi drivers. PNAS. 2000 Apr 11;97[8]:4398–403.

Makris N, Oscar-Berman M, Jaffin SK, Hodge SM, Kennedy DN, Caviness VS, et al. Decreased volume of the brain reward system in alcoholism. Biological Psychiatry. 2008 Aug 1;64[3]:192–202.

Malaeb SN, Davis JM, Pinz IM, Newman JL, Dammann O, Rios M. Effect of sustained postnatal systemic inflammation on hippocampal volume and function in mice. Pediatr Res. 2014 Oct;76[4]:363–9.

Malisch JL, Breuner CW, Kolb EM, Wada H, Hannon RM, Chappell MA, et al. Behavioral despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations. Behav Genet. 2009a Mar 1;39[2]:192–201.

Malisch JL, Breuner CW, Kolb EM, Wada H, Hannon RM, Chappell MA, et al. Behavioral despair and home-cage activity in mice with chronically elevated baseline corticosterone concentrations. Behavior Genetics. 2009b;39[2]:192–201.

Malisch JL, Saltzman W, Gomes FR, Rezende EL, Jeske DR, Garland, Jr. T. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. Physiological and Biochemical Zoology. 2007 Jan 1;80[1]:146–56.

Martin RD, Harvey PH. Brain size allometry ontogeny and phylogeny. In: Size and Scaling in Primate Biology [Internet]. Springer, Boston, MA; 1985 [cited 2018 May 10]. p. 147–73. [Advances in Primatology]. Available from: https://link.springer.com/chapter/10.1007/978-1-4899-3647-9_8

Mathes WF, Nehrenberg DL, Gordon R, Hua K, Garland, Jr. T, Pomp D. Dopaminergic dysregulation in mice selectively bred for excessive exercise or obesity. Behavioural Brain Research. 2010 Jul 11;210[2]:155–63.

Meek TH, Lonquich BP, Hannon RM, Garland, Jr. T. Endurance capacity of mice selectively bred for high voluntary wheel running. Journal of Experimental Biology. 2009 Sep 15;212[18]:2908–17.

Meijer JH, Robbers Y. Wheel running in the wild. Proc R Soc B. 2014 Jul 7;281[1786]:20140210.

Mercuri NB, Calabresi P, Bernardi G. The electrophysiological actions of dopamine and dopaminergic drugs on neurons of the substantia nigra pars compacta and ventral tegmental area. Life Sciences. 1992 Jan 1;51[10]:711–8.

Mull CG, Yopak KE, Dulvy NK. Maternal investment, ecological lifestyle, and brain evolution in sharks and rays. The American Naturalist. 2020 Jun;195[6]:1056–69.

Muller AS, Montgomery SH. Co-evolution of cerebral and cerebellar expansion in cetaceans. Journal of Evolutionary Biology. 2019;32[12]:1418–31.

Nguyen QAT, Hillis D, Katada S, Harris T, Pontrello C, Garland, Jr. T, et al. Coadaptation of the chemosensory system with voluntary exercise behavior in mice. PLoS ONE. 2020;15[11]:e0241758.

Novak CM, Burghardt PR, Levine JA. The use of a running wheel to measure activity in rodents: Relationship to energy balance, general activity, and reward. Neuroscience & Biobehavioral Reviews. 2012 Mar 1;36[3]:1001–14.

Patzke N, Spocter MA, Karlsson KÆ, Bertelsen MF, Haagensen M, Chawana R, et al. In contrast to many other mammals, cetaceans have relatively small hippocampi that appear to lack adult neurogenesis. Brain Structure and Function. 2015;220:361–83.

van Praag H, Kempermann G, Gage FH. Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus. Nat Neurosci. 1999 Mar;2[3]:266–70.

Prado WA, Raghubir R, Roberts MHT. Long duration antinociception induced by red nucleus stimulation in the rat. Pain. 1984;18:S329.

Qiu LR, Germann J, Spring S, Alm C, Vousden DA, Palmert MR, et al. Hippocampal volumes differ across the mouse estrous cycle, can change within 24 hours, and associate with cognitive strategies. NeuroImage. 2013 Dec 1;83:593–8.

Raichlen DA, Alexander GE. Why Your Brain Needs Exercise. Scientific American. 2020 Jan 1;322[1]:26–31.

Raichlen DA, Gordon AD. Relationship between exercise capacity and brain size in mammals. PLOS ONE. 2011 Jun 22;6[6]:e20601.

Rapuano KM, Zieselman AL, Kelley WM, Sargent JD, Heatherton TF, Gilbert-Diamond D. Genetic risk for obesity predicts nucleus accumbens size and responsivity to real-world food cues. PNAS. 2017 Jan 3;114[1]:160–5.

Rendeiro C, Rhodes JS. A new perspective of the hippocampus in the origin of exercise–brain interactions. Brain Structure and Function [Internet]. 2018 [cited 2018 May 10]; Available from: https://link.springer.com/article/10.1007/s00429-018-1665-6

Rezende EL, Garland, Jr. T, Chappell MA, Malisch JL, Gomes FR. Maximum aerobic performance in lines of *Mus* selected for high wheel-running activity: Effects of selection, oxygen availability and the mini-muscle phenotype. Journal of Experimental Biology. 2006 Jan 1;209[1]:115–27.

Rhodes J, Hosack G, Girard I, Kelley A, Mitchell G, Garland, Jr. T. Differential sensitivity to acute administration of cocaine, GBR 12909, and fluoxetine in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology. 2001 Nov 1;158[2]:120–31.

Rhodes JS, Gammie SC, Garland, Jr. T. Neurobiology of mice selected for high voluntary wheel-running activity. Integrative and Comparative Biology. 2005;45[3]:438–55.

Rhodes JS, Garland, Jr. T. Differential sensitivity to acute administration of Ritalin, apomorphine, SCH 23390, but not raclopride in mice selectively bred for hyperactive wheel-running behavior. Psychopharmacology. 2003 May 1;167[3]:242–50.

Rhodes JS, Garland, Jr. T, Gammie SC. Patterns of brain activity associated with variation in voluntary wheel-running behavior. Behavioral Neuroscience. 2003a Dec;117[6]:1243–56.

Rhodes JS, Kawecki TJ. Behavior and neurobiology. In: Garland, Jr. T, Rose MR, editors. Experimental evolution: concepts, methods, and applications of selection experiments. Berkeley: University of California Press; 2009. p. 263–300.

Rhodes JS, van Praag H, Jeffrey S, Girard I, Mitchell GS, Garland, Jr. T, et al. Exercise increases hippocampal neurogenesis to high levels but does not improve spatial learning in mice bred for increased voluntary wheel running. Behavioral Neuroscience. 2003b;117[5]:1006–16.

Roderick TH, Wimer RE, Wimer CC. Genetic manipulation of neuroanatomical traits. In: Petrinovich L, McGaugh L, editors. Knowing, thinking, and believing. New York, NY: Plenum; 1976. p. 143–78.

Roth TC, Brodin A, Smulders TV, LaDage LD, Pravosudov VV. Is bigger always better? A critical appraisal of the use of volumetric analysis in the study of the hippocampus. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010 Feb 15;365[1542]:915–31.

Ruiz-Tejada A, Neisewander J, Katsanos CS. Regulation of voluntary physical activity behavior: A review of evidence involving dopaminergic pathways in the brain. Brain Sciences. 2022 Mar;12[3]:333.

Saul M, Majdak P, Perez S, Reilly M, Garland, Jr. T, Rhodes JS. High motivation for exercise is associated with altered chromatin regulators of monoamine receptor gene expression in the striatum of selectively bred mice. Genes, Brain and Behavior. 2017;16:328–41.

Schieber MH, Baker JF. Control of Movement. In: Squire LR, Berg D, Bloom FE, du Lac S, Ghosh A, Spitzer NC, editors. Fundamental Neuroscience (Fourth Edition) [Internet]. San Diego, CA: Academic Press; 2013 [cited 2019 Jan 11]. p. 631–51. Available from: http://www.sciencedirect.com/science/article/pii/B9780123858702000299

Schmill MP, Thompson Z, Argueta DA, DiPatrizio NV, Garland, Jr. T. Effects of selective breeding, voluntary exercise, and sex on endocannabinoid levels in the mouse small-intestinal epithelium. Physiology & Behavior. 2022 Mar 1;245:113675.

Scholz J, Allemang-Grand R, Dazai J, Lerch JP. Environmental enrichment is associated with rapid volumetric brain changes in adult mice. NeuroImage. 2015a Apr 1;109:190–8.

Scholz J, Niibori Y, W Frankland P, P Lerch J. Rotarod training in mice is associated with changes in brain structure observable with multimodal MRI. NeuroImage. 2015b Feb 15;107:182–9.

Schultz W. Predictive reward signal of dopamine neurons. Journal of Neurophysiology. 1998 Jul 1;80[1]:1–27.

Seifert CL, Magon S, Sprenger T, Lang UE, Huber CG, Denier N, et al. Reduced volume of the nucleus accumbens in heroin addiction. Eur Arch Psychiatry Clin Neurosci. 2015 Dec 1;265[8]:637–45.

Sharma A, Couture J. A review of the pathophysiology, etiology, and treatment of attention-deficit hyperactivity disorder (ADHD). Ann Pharmacother. 2014 Feb;48[2]:209–25.

Sher L. The endogenous euphoric reward system that reinforces physical training: A mechanism for mankind's survival. Medical Hypotheses. 1998 Dec 1;51[6]:449–50.

Sherry DF, Hoshooley JS. Seasonal hippocampal plasticity in food-storing birds. Philos Trans R Soc Lond B Biol Sci. 2010 Mar 27;365[1542]:933–43.

Sherwin CM. Voluntary wheel running: A review and novel interpretation. Animal Behaviour. 1998 Jul 1;56[1]:11–27.

Singleton J, Garland, Jr. T. Influence of corticosterone on growth, home-cage activity, wheel running, and maximal oxygen consumption in replicate lines of house mice selectively bred for high voluntary wheel-running behavior. Physiology & Behavior. 2019;198:27–41.

Smith KS, Tindell AJ, Aldridge JW, Berridge KC. Ventral pallidum roles in reward and motivation. Behavioural Brain Research. 2009 Jan 23;196[2]:155–67.

Stuber GD, Sparta DR, Stamatakis AM, van Leeuwen WA, Hardjoprajitno JE, Cho S, et al. Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. Nature. 2011 Jul;475[7356]:377–80.

Sumiyoshi A, Taki Y, Nonaka H, Takeuchi H, Kawashima R. Regional gray matter volume increases following 7 days of voluntary wheel running exercise: A longitudinal VBM study in rats. NeuroImage. 2014 Sep 1;98:82–90.

Swallow JG, Carter PA, Garland, Jr. T. Artificial selection for increased wheel-running behavior in house mice. Behavior Genetics. 1998 May;28[3]:227–37.

Swallow JG, Koteja P, Carter PA, Garland, Jr. T. Artificial selection for increased wheel-running activity in house mice results in decreased body mass at maturity. Journal of Experimental Biology. 1999 Sep 15;202[18]:2513–20.

Swallow JG, Rhodes JS, Garland, Jr. T. Phenotypic and evolutionary plasticity of organ masses in response to voluntary exercise in house mice. Integrative and Comparative Biology. 2005 Jun 1;45[3]:426–37.

Swallow JG, Wroblewska AK, Waters RP, Renner KJ, Britton SL, Koch LG. Phenotypic and evolutionary plasticity of body composition in rats selectively bred for high endurance capacity. Journal of Applied Physiology. 2010 Sep;109[3]:778–85.

Swanson EM, Holekamp KE, Lundrigan BL, Arsznov BM, Sakai ST. Multiple determinants of whole and regional brain volume among terrestrial carnivorans. PLOS ONE. 2012 Jun 13;7[6]:e38447.

Thacker EL, Chen H, Patel AV, McCullough ML, Calle EE, Thun MJ, et al. Recreational physical activity and risk of Parkinson's disease. Movement Disorders. 2008;23[1]:69–74.

Thompson Z, Argueta D, Garland, Jr. T, DiPatrizio N. 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. Physiology & Behavior. 2017 Mar 1;170:141–50.

Vargas-Pérez H, Borrelli E, Díaz JL. Wheel running use in dopamine D2L receptor knockout mice. Neuroscience Letters. 2004 Aug 12;366[2]:172–5.

Waters RP, Pringle RB, Forster GL, Renner KJ, Malisch JL, Garland, Jr. T, et al. Selection for increased voluntary wheel-running affects behavior and brain monoamines in mice. Brain Research. 2013 May 1;1508:9–22.

Weisberg SM, Newcombe NS, Chatterjee A. Everyday taxi drivers: Do better navigators have larger hippocampi? Cortex. 2019 Jun 1;115:280–93.

Yamamoto M, Wada-Isoe K, Yamashita F, Nakashita S, Kishi M, Tanaka K, et al. Association between exercise habits and subcortical gray matter volumes in healthy elderly people: A population-based study in Japan. eNeurologicalSci. 2017 Jun 1;7:1–6.

Yang Y, Wang JZ. From structure to behavior in basolateral amygdala-hippocampus circuits. Front Neural Circuits. 2017;11:86.

Zhu X, Ottenheimer D, DiLeone RJ. Activity of D1/2 receptor expressing neurons in the nucleus accumbens regulates running, locomotion, and food intake. Front Behav Neurosci. 2016;10:66.

Table 1. Significance levels for wheel running and home-cage activity during each week. P values ≤ 0.05 are in **bold**. Degrees of freedom for testing the linetype, wheel access, and interaction effects were all 1 and 6. Signs after P values indicate direction of effect: linetype + indicates HR higher; mini-muscle + indicates mice with the mini-muscle phenotype higher. Signs are not shown for HCA because the interaction was always significant (see Fig. 2).

Trait	**N**	**P_{linetype}**	**$P_{\text{wheelaccess}}$**	**$P_{\text{interaction}}$**	**P_{mini}**
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+

Table 2. Statistical analyses for body mass, whole brain mass, and brain region volumes. Values for mixed models comparing linetype (HR vs. C lines), groups with or without 10-13 weeks of wheel access (WhlAcc), and their interaction. Mini-muscle mice were identified at dissection (see Methods). Age was included as a covariate in all analyses (results not shown). For brain regional volumes, time spent in PFA prior to sectioning and $\log_{10}$ time spent in the freezer before Nissl staining were included as covariates in all analyses (results not shown). Analyses were also conducted with body mass as a covariate **(top row), brain mass as a covariate (middle row) or neither (bottom row)**. For main effects, P values $\leq$ 0.05 are considered statistically significant, whereas for interactions P $\leq$ 0.1 are considered significant (see Methods), and both are in **bold**. **Signs after main effects and covariates indicate direction of effect: + indicates HR lines > C; + indicates wheel access > sedentary; + indicates mini-muscle > normal.** See Figures 3-5 for graphical representations of least squares means (adjusted for covariates).

	Body Mass *(n = 100)*			Brain Mass *(n = 93)*			Whole Midbrain *(n=64)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	4.04	0.0912-	1,6	4.96	0.0676+	1,6	4.79	0.0713+
								1.92	0.2150+
					2.10	0.1973+		3.21	0.1235+
Wheel Access	1,6	2.67	0.1533-	1,6	12.33	**0.0127+**	1,6	0.29	0.6070+
								0.11	0.7512+
					8.51	**0.0267+**		0.34	0.5828+
Linetype*WhlAcc	1,6	1.39	0.2825	1,6	0.64	0.4535	1,6	0.07	0.8002
								0.07	0.8068
					1.24	0.3086		0.12	0.7444
Mini-muscle	1,82	0.16	0.6895+	1,47	1.40	0.2410+	1,43	0.31	0.5785-
							1,43	0.25	0.6163-
				1,48	1.78	0.1861+	1,44	0.20	0.6531-
Body Mass				1,47	8.16	**0.0056+**	1,43	0.87	0.3561+
Brain Mass							1,43	0.97	0.3314+

	Substantia Nigra *(n = 78)*			Ventral Tegmental Area *(n = 72)*			Red Nucleus *(n = 68)*			Periaqueductal Gray *(n = 76)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	0.01	0.9321+	1,6	0.92	0.3743+	1,6	11.34	**0.0151+**	1,6	0.08	0.7860+
		0.02	0.8935-		1.71	0.2381+		3.81	0.0988+		0.69	0.4388-
		0.00	0.9839+		4.35	0.0820+		8.65	**0.0259+**		0.09	0.7737+
Wheel Access	1,6	0.06	0.8080-	1,6	1.28	0.3012-	1,6	0.71	0.4324-	1,6	0.14	0.7243+
		0.19	0.6775-		1.46	0.2725-		2.44	0.1692-		0.08	0.7894-
		0.07	0.7865-		0.76	0.4180-		1.33	0.2935-		0.13	0.7264+
Linetype*WhlAcc	1,6	1.00	0.3563	1,6	0.02	0.9002	1,6	0.02	0.8930	1,6	8.75	**0.0253**
		0.92	0.3742		0.26	0.6265		0.08	0.7837		6.63	**0.0421**
		1.15	0.3251		0.06	0.8184		0.00	0.9823		9.12	**0.0234**
Mini-muscle	1,57	0.21	0.6506-	1,51	0.57	0.4533-	1,46	0.05	0.8276-	1,55	1.97	0.1658-

	1,57	0.21	0.6457-	1,51	1.42	0.2390-	1,46	0.00	0.9474-	1,55	2.44	0.1237-
	1,58	0.20	0.6575-	1,52	1.13	0.2919-	1,47	0.00	0.9815+	1,56	2.00	0.1630-
Body Mass	1,57	0.08	0.7765+	1,51	3.05	0.0866-	1,46	2.49	0.1212+	1,55	0.00	0.9880+
Brain Mass	1,57	0.52	0.4734+	1,51	3.81	0.0564+	1,46	4.76	**0.0343+**	1,55	9.32	**0.0035+**

	Hippocampus *(n = 68)*			Basolateral Amygdala *(n = 68)*			Nucleus Accumbens *(n = 57)*			Ventral Pallidum *(n = 63)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	11.99	**0.0134+**	1,6	2.86	0.1417+	1,6	0.91	0.3773+	1,6	2.65	0.1546+
		4.16	0.0876+		0.28	0.6165+		0.39	0.5567+		1.00	0.3548+
		8.42	**0.0273+**		1.43	0.2773		0.81	0.4015+		4.00	0.0924+
Wheel Access	1,6	0.01	0.9318+	1,6	0.07	0.8072+	1,6	2.67	0.1535-	1,6	0.68	0.4398-
		0.12	0.7386-		0.37	0.5638-		3.30	0.1990-		1.53	0.2617-
		0.00	0.9966-		0.00	0.9564		2.87	0.1412-		0.56	0.4843-
Linetype*WhlAcc	1,6	0.34	0.5811	1,6	0.85	0.3921	1,6	0.30	0.6054	1,6	0.09	0.7699
		0.53	0.4925		0.33	0.5871		0.20	0.6723		0.40	0.5495
		0.17	0.6952		1.17	0.3212		0.29	0.6126		0.14	0.7170
Mini-muscle	1,47	0.05	0.8305-	1,47	0.78	0.3819+	1,36	0.38	0.5402-	1,42	0.16	0.6902-
	1,47	0.02	0.8964-	1,48	0.70	0.4062+	1,36	0.33	0.5720-	1,42	0.44	0.5103-
	1,48	0.01	0.9290-		0.94	0.3365	1,37	0.41	0.5236-	1,43	0.23	0.6349-
Body Mass	1,47	1.29	0.2623+	1,47	2.91	0.0946+	1,36	0.09	0.7604+	1,42	0.44	0.5087-
Brain Mass	1,47	5.49	**0.0235+**	1,47	5.55	**0.0227+**	1,36	0.90	0.3503+	1.42	4.38	**0.0425+**

Table 3. Statistical analyses for brain region cell densities. Values for mixed models comparing linetype (HR vs. C lines), groups with or without 10-13 weeks of wheel access (WhlAcc), and their interaction. Mini-muscle mice were identified at dissection (see Methods). Age, time spent in PFA prior to sectioning, and $\log_{10}$ time spent in the freezer before Nissl staining were included as covariates in all analyses (results not shown). For main effects, P values ≤ 0.05 are considered statistically significant, whereas for interactions P ≤ 0.1 are considered significant (see Methods), and both are in **bold**. **Signs after main effects and covariates indicate direction of effect: + indicates HR lines > C; + indicates wheel access > sedentary; + indicates mini-muscle > normal.**

	Whole Midbrain *(n = 80)*		
Effect	d.f.	F	P
Linetype	1,6	0.15	0.7122+
Wheel Access	1,6	1.19	0.3165+
Linetype*WhlAcc	1,6	0.67	0.4454
Mini-muscle	1,60	1.29	0.2613+

	Substantia Nigra *(n = 83)*			Ventral Tegmental Area *(n = 83)*			Red Nucleus *(n = 68)*			Periaqueductal Gray *(n = 81)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	0.05	0.8270+	1,6	0.04	0.8525+	1,6	2.11	0.1963+	1,6	0.26	0.6278-
Wheel Access	1,6	0.82	0.3997+	1,6	0.68	0.4421+	1,6	1.13	0.3293+	1,6	3.45	0.1127+
Linetype*WhlAcc	1,6	1.37	0.2868	1,6	0.16	0.7056	1,6	1.00	0.3549	1,6	0.72	0.4282
Mini-muscle	1,63	0.12	0.7323+	1,63	0.00	0.9590-	1,48	0.64	0.4265-	1,61	0.64	0.4281+

	Hippocampus *(n = 68)*			Basolateral Amygdala *(n = 68)*			Nucleus Accumbens *(n = 54)*			Ventral Pallidum *(n = 63)*		
Effect	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
Linetype	1,6	0.07	0.7950-	1,6	0.06	0.8124-	1,6	0.09	0.7694+	1,6	0.02	0.8806-
Wheel Access	1,6	0.22	0.6589+	1,6	0.27	0.6188-	1,6	0.34	0.5802+	1,6	0.00	0.9538+
Linetype*WhlAcc	1,6	1.45	0.2736	1,6	1.73	0.2369	1,6	3.97	**0.0935**	1,6	2.21	0.1878
Mini-muscle	1,48	0.72	0.3988-	1,48	1.14	0.2912+	1,34	0.48	0.4912-	1,43	0.80	0.3756-

Figure Legends

Fig 1. Average wheel revolutions run per week (simple means) during the course of the experiment. Significance levels from comparisons of the HR and C lines are presented in Table 1.

Fig. 2. Average home-cage activity per week (LS Means from SAS Procedure Mixed) over the course of the experiment. Mini-muscle status was included in the model, but never had a significant effect (Table 1).

Fig. 3. Body mass (A), whole brain mass (B), and whole brain mass, analyzed with body mass as a covariate (C). Value are LS means and SEs from SAS Procedure Mixed. HR mice tended to be smaller (A, linetype F = 4.04, d.f. = 1,6, P = 0.0912), but have relatively heavier brains than C mice (C, linetype F = 4.96, d.f. = 1,6, P = 0.0676), and mice with wheel access for 10-13 weeks had relatively heavier brains than those without wheels (F = 12.33, d.f. = 1,6, P = 0.0127), with no interaction (F = 0.64, d.f. = 1,6, P = 0.4535) (Table 2).

Fig. 4. Whole midbrain total volume in cubic millimeters (LS means and SEs from SAS Procedure Mixed), with mini-muscle status, body mass, and age included in the model (Table 2). The volume of the midbrain tended to be larger in HR mice (F = 4.79, d.f. = 1,6, P = 0.0713), with no effect of wheel access, no interaction, and no mini-muscle effect.

Fig. 5. Regional brain volumes in cubic millimeters. Values are LS means and SEs from SAS Procedure Mixed. Body mass (except for VTA), age, time spent in PFA prior to sectioning, and $\log_{10}$ time spent in the freezer before Nissl staining were included as covariates in all analyses (results not shown). See Table 2 for full statistical results.

(A) Ventral tegmental area. HR mice tended to have a larger VTAs (P = 0.0820), with no statistical effect of wheel access and no interaction or mini-muscle effect.

(B) Red nucleus. HR mice had a significantly larger red nucleus (P = 0.0151), with no significant effect of wheel access, no interaction, and no mini-muscle effect.

(C) Periaqueductal grey. 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, with no effect of mini-muscle.

(D) Hippocampus. HR mice had a significantly larger hippocampus (P = 0.0138), with no significant effect of wheel access, no interaction, and no mini-muscle effect.

Fig. 6. Illustration of direct versus indirect genetic effects on brain size. The larger brain size (or differences in brain region volumes) of HR mice as compared with C mice may be attributable to direct genetic effects and/or indirect genetic effects mediated through the intermediate phenotype of higher physical activity by HR mice (Figures 1 and 2). Body size also "affects" brain size, or at least is correlated with it in some cases, and body size can be affected by physical activity acting across development and ontogeny.