

RESEARCH ARTICLE

Mice selectively bred for high voluntary wheel running have larger midbrains: support for the mosaic model of brain evolution

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SUMMARY

Increased brain size, relative to body mass, is a primary characteristic distinguishing the mammalian lineage. This greater encephalization has come with increased behavioral complexity and, accordingly, it has been suggested that selection on behavioral traits has been a significant factor leading to the evolution of larger whole-brain mass. In addition, brains may evolve in a mosaic fashion, with functional components having some freedom to evolve independently from other components, irrespective of, or in addition to, changes in size of the whole brain. We tested whether long-term selective breeding for high voluntary wheel running in laboratory house mice results in changes in brain size, and whether those changes have occurred in a concerted or mosaic fashion. We measured wet and dry brain mass *via* dissections and brain volume with *ex vivo* magnetic resonance imaging of brains that distinguished the caudate-putamen, hippocampus, midbrain, cerebellum and forebrain. Adjusting for body mass as a covariate, mice from the four replicate high-runner (HR) lines had statistically larger non-cerebellar wet and dry brain masses than those from four non-selected control lines, with no differences in cerebellum wet or dry mass or volume. Moreover, the midbrain volume in HR mice was ~13% larger ($P < 0.05$), while volumes of the caudate-putamen, hippocampus, cerebellum and forebrain did not differ statistically between HR and control lines. We hypothesize that the enlarged midbrain of HR mice is related to altered neurophysiological function in their dopaminergic system. To our knowledge, this is the first example in which selection for a particular mammalian behavior has been shown to result in a change in size of a specific brain region.

Key words: activity, allometry, cerebellum, dopamine, exercise behavior, locomotion, motor control.

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INTRODUCTION

Increases in relative brain size (relative to body mass) in mammals have been correlated with enhanced sensory abilities (Jerison, 1973; Catania, 2005), spatial memory (Krebs et al., 1989; Krebs, 1990; Jacobs and Liman, 1991; Jacobs and Spencer, 1994) and aspects of cognition (Jerison, 1973; Byrne and Corp, 2004; Peper et al., 2009; Mehlhorn et al., 2010). The ‘principle of proper mass’, first articulated by Jerison (Jerison, 1973), states that the size of a given neural structure reflects the complexity of the function that it subserves. This observation was applied primarily to sensory processing areas of the brain (e.g. enlarged auditory cortex in subterranean insectivores). However, there is a general trend in mammals for increased behavioral complexity with increased brain size (i.e. encephalization) (Changizi, 2003). Differences in behavioral complexity are difficult to quantify and are subject to variable interpretation (Changizi, 2003; Chittka and Niven, 2009). Both increased sensitivity to sensory stimuli (Land and Nilsson, 2002) and increased precision in motor ability (Sparks, 2002) are directly related to increases in neuron number, which, in turn, are directly related to brain size (Herculano-Houzel et al., 2006; Herculano-Houzel et al., 2007; Herculano-Houzel, 2010). Likewise,

species of mammals (Sol et al., 2008) and birds (Sol et al., 2005) with larger brains have higher survivability in novel environments, which has been attributed to an enhanced behavioral flexibility and innovation (Reader and Laland, 2002; Reader, 2003; Marino, 2005; Sol et al., 2005). Of course, changes in brain size alone obviously do not account for all behavioral evolution (e.g. see Katz, 2011).

It is unclear whether selection on a behavioral trait would result in a coordinated enlargement of the entire brain (Finlay and Darlington, 1995; Clancy et al., 2001) or size changes only in regions whose function was closely related to the behavioral trait under selection (Barton and Harvey, 2000). This latter theory, called mosaic evolution of the brain, suggests that brain systems mediating specific behavioral capacities are able to change size in response to selection (Barton and Harvey, 2000; de Winter and Oxnard, 2001). A comparative study of mammals by de Winter and Oxnard (de Winter and Oxnard, 2001) demonstrated that functionally integrated brain systems vary independently among related lineages. The authors sampled species from five mammalian orders and found relationships between the convergent evolution of lifestyle traits within orders and the size of brain structures underpinning those convergences among relatively unrelated species (e.g. locomotor

convergences within primates, spatio-sensory convergences within insectivores). Similar evidence for mosaic evolution in the avian brain has also been found and correlated to functional differences in the enlarged brain regions (Iwaniuk et al., 2006).

Cellular changes have also been observed with increased brain size. As brain volume increases, axon diameters and the fraction of myelinated axons increase, thereby reducing the delay in neural signaling between more distant regions (Wang et al., 2008). Moreover, different cellular scaling rules apply across different mammalian orders (Herculano-Houzel, 2010). For example, in rodents, neuronal cell size increases with brain size, such that neuronal density is lower in larger-brained species (albeit with a greater absolute number of neurons) (Herculano-Houzel et al., 2006). Conversely, in primates, neuronal cell size is constant and neuron density is constant, such that total neuron number is much greater with increasing brain size (Herculano-Houzel et al., 2007). Therefore, an increase in the size of a brain region could be the product of a variety of cellular changes (e.g. neuron number, neuron density, glial density, gray-to-white matter ratio, dendritic arborization).

A number of hypotheses have been generated to explain how selection may have driven changes in brain size (Francis, 1995; Barton and Harvey, 2000; de Winter and Oxnard, 2001; Hutcheon et al., 2002; Byrne and Corp, 2004; Lefebvre et al., 2004; Marino, 2005; Sol et al., 2005; Lefebvre and Sol, 2008; Rehkämper et al., 2008; Sol et al., 2008; Chittka and Niven, 2009; Roth and Pravosudov, 2009). Most, if not all, of these hypotheses suggest that selection is acting on behavior [e.g. behavioral flexibility – environmental change hypothesis (Sol et al., 2005; Lefebvre and Sol, 2008; Sol et al., 2008), social brain hypothesis (Byrne and Whiten, 1988; Brothers, 1990; Byrne and Corp, 2004)], rather than directly on brain size *per se* (see also Kruska, 2005). The primary question in the present study is whether selection on a particular behavioral trait (voluntary exercise), using an experimental evolution paradigm (Garland and Rose, 2009), has resulted in a change in brain size. An additional question is whether any change in brain size is concerted, involving the entire brain, or mosaic.

Voluntary wheel running is a behavioral trait that involves both motor performance (ability) and the will to engage in the activity (motivation) (Garland et al., 2011b; Novak et al., 2012). Motivation is a product of the brain, and motor planning and motor recruitment are ultimately controlled through the central nervous system. In fact, recent work in human ‘ultra-endurance’ athletes (Pearson, 2006) suggests that neurobiological attributes make a greater contribution to maximum performance ability than has previously been

acknowledged (Kayser, 2003; Baden et al., 2005; Noakes, 2007; Rose and Parfitt, 2007; Noakes, 2008). In our laboratory, selection for high voluntary wheel running in outbred laboratory house mice has been ongoing for more than 60 generations, and has resulted in numerous physiological (Girard et al., 2007; Malisch et al., 2008; Gomes et al., 2009; Meek et al., 2009), behavioral (Rhodes et al., 2001; Rhodes and Garland, 2003; Belke and Garland, 2007; Meek et al., 2010), and neurobiological (Rhodes et al., 2003a; Rhodes et al., 2003b) changes in four replicate high-runner (HR) lines of mice as compared with four non-selected control (C) lines. Moreover, a recent comparative study demonstrated a positive correlation between brain size and an index of exercise capacity, maximal oxygen consumption (Raichlen and Gordon, 2011), one of the traits that has increased in the HR lines (Rezende et al., 2006b; Kolb et al., 2010). Therefore, in this study we tested whether selective breeding for high voluntary wheel running in house mice has altered their brain size.

MATERIALS AND METHODS

Selection experiment and study animals

Analyses of brain mass and volume were conducted on independent samples of house mice (*Mus musculus* Linnaeus 1758) taken from an ongoing selection experiment for high voluntary wheel running. Succinctly, within-family selection for voluntary wheel running was performed on four independent lines of mice (HR lines), the selection criterion being the total running distance during days five and six of a 6-day trial with wheel access. Four non-selected control (C) lines were maintained under identical conditions, including wheel testing, but breeders were chosen without regard to amount of running. For details on the experimental design, see Swallow et al. (Swallow et al., 1998a). Here, it is of interest to note that, although many domesticated mammals show reductions in whole-brain size and/or the size of specific brain regions, domesticated house mice apparently do not (Kruska, 2005). All procedures conducted in this study are in accordance with the UCR Institutional Animal Care and Use Committee and US laws.

Brain mass

Three cohorts of mice were used: females from generation 34 [$N=46$; the same individuals as those studied in Rezende et al. (Rezende et al., 2006b)], retired male breeders from generation 39 ($N=138$), and retired male and female breeders from generation 52 ($N=311$). Analyses of the first cohort (females only) indicated a trend for HR mice to have larger brains (wet or dry mass), so we conducted additional sampling at later generations to increase

Table 1. Body mass analysis for each generational cohort from which brain mass data were collected

Generation	Sex	N	Mean age in days (range)	P				Least squares mean \pm s.e.m. (g)	
				Sex	Line type	Line type \times sex	Age	HR	C
34	F	46	78 (54–101)		0.0007–		<0.0001+	23.1748 \pm 0.4996	27.5314 \pm 0.4783
39	M	138	91 (76–97)		0.0158–		0.3070+	32.6852 \pm 0.9780	37.3369 \pm 0.9969
52	M/F	311	165 (126–186)	0.0055–	0.0420–	0.7362	Not used	F: 31.4427 \pm 1.3944 M: 34.1602 \pm 1.3910	F: 36.5357 \pm 1.5054 M: 38.8347 \pm 1.5046

A nested ANCOVA with line type as the main effect and age as a covariate was conducted on generations 34 and 39.

A nested ANCOVA with mini-muscle phenotype, line type, sex and the line type \times sex interaction was conducted on generation 52. Mini-muscle individuals tended to be smaller in body mass ($F=3.58$, d.f.=1,207, two-tailed $P=0.0599$; least squares mean \pm s.e.m.=36.4 \pm 0.95 and 34.1 \pm 1.37, respectively, for normal and mini-muscle individuals).

Age was not used as a covariate in generation 52 because age was confounded with sex (due to the breeding schedule, males were sampled first and females second).

Positive signs following P -values indicate HR>C, females>males or positive effect of body mass, and *vice versa* for negative signs.

P -values in bold were considered statistically significant ($P<0.05$), and all statistical tests were two-tailed.

HR, high runner; C, control.

sample size, include both sexes, separate cerebellar from non-cerebellar brain (which can be accomplished easily *via* gross dissections) and obtain dry brain masses. All mice were sexually mature adults (>7 weeks old) at the time of measurement (Table 1); during this life stage the brain exhibits little growth in laboratory house mice, resulting in a negligible allometric slope (see Martin and Harvey, 1985). All mice had previously undergone 6 days of wheel testing in the usual selection protocol. The brains were harvested in slightly different ways for each cohort, but results were quite consistent across generations (see Results). For generation 34, mice were frozen following euthanasia, and then whole brains were dissected and weighed at a later date. For generation 39, the whole brain was removed and weighed wet immediately after euthanasia, and then the cerebellum was removed and weighed separately. For generation 52, the cerebellum was removed prior to weighing, and the cerebellum and non-cerebellar brain were weighed separately. After wet masses were obtained, samples from each cohort were placed in a drying oven at 60°C for 7 days (to constant mass), and then weighed to obtain a dry mass.

Brain volume

Motivated by our observations on brain mass at generations 34 and 39, we decided to study volumes of key brain regions. Retired female breeders from generation 41 were re-housed four per cage after the weaning and separation of their first litters. All had previously undergone the standard 6 days of wheel testing. A total of 48 (six per line) animals were used in this experiment. The 48 individuals were separated into 12 housing cages with two HR and two C line animals in each cage. At all times during this 2-week housing period, mice were given food (Teklad Rodent Diet 8604, Madison, WI, USA) and water *ad libitum*. At the time of perfusion, mice were anesthetized (4% isoflurane) and then maintained [ketamine (90 mg kg⁻¹) and xylazine (20 mg kg⁻¹), i.p. injection] in deep anesthesia for the duration of the procedure. Mice were perfused transcardially with 4% paraformaldehyde (PFA) at a rate of 4 ml min⁻¹ for 10–15 min. The brain was removed and submerged in 4% PFA for 1 h with gentle shaking, and then rinsed in 0.12 mmol l⁻¹ Millonig's buffer for 3×30 min. Brains were stored in 4% PFA at 2°C until magnetic resonance imaging (MRI) (McRobbie and Moore, 2003).

Imaging for the brain volume analyses was conducted on a Bruker Advance 11.7-Tesla MR imager (8.9-cm bore) with a 3.0 cm (internal diameter) volume radio-frequency coil (Bruker Biospin, Billerica, MA, USA). Two data sets were collected, coronal and axial T2-weighted magnetic resonance images (T2WI MRI) and a volumetric 3-D rapid acquisition with relaxation enhancement (3-D RARE) data set. Scout images were obtained in axial and coronal planes to accurately position and align the brain in the MRI. The following imaging parameters were used: (1) T2WI MRI were

obtained using an echo time (TE)/time to repetition (TR) of 4600/10.2 ms, a 256×256 matrix, a 2-cm field of view (FOV) and two averages for an acquisition time of 40 min; (2) the 3-D RARE data were acquired using a TR/TE of 1000/31.8 ms, a 256×256 matrix, a 2-cm FOV and four averages for an acquisition time of 2 h 20 min. The 3-D RARE provides true volumetric acquisition such that the data can be formatted in multiple orientations (coronal, axial and sagittal).

Regional brain volumes were delineated manually from the coronal views of the 3-D RARE sequence and formatted to a slice thickness of 0.75 mm with a slice interval of 1.25 mm. Therefore, the resulting 3-D reconstructions do not include the 0.5 mm of tissue that lies between the end of one image slice and the interval to the next image slice. Image analysis was conducted using Amira imaging software (Amira 5.2, Visage Imaging, San Diego, CA, USA).

Volumes were measured for five brain regions: caudate-putamen, hippocampus, midbrain, cerebellum and remaining forebrain (i.e. the forebrain area not containing the hippocampus or caudate-putamen) (Fig. 1). Three of the brain regions selected for analysis (i.e. caudate-putamen, midbrain and cerebellum) were gross morphological structures that contain systems involved in motor-sensory processing and that could be consistently delineated in the MRI scans. Hippocampus was also included based on previous work that demonstrated correlations between wheel running and activation of hippocampal neurons in HR and C mice (Rhodes et al., 2003b). The remaining forebrain regions were aggregated together and termed 'other forebrain' in the volumetric analyses. Each of the regions was delineated using major anatomical landmarks and cross-referenced to *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2003).

On the MRI images, the caudate-putamen was defined as the ovoid area bound dorsolaterally by the corpus callosum and ventrally by the anterior commissures. The hippocampus was defined as the area bound by the cingulum dorsally, and the lateral and third ventricles laterally and medially. These boundaries provided a strong contrasting outline (cingulum: dark; ventricles: bright) surrounding the hippocampus. Given the difficulty of discerning specific nuclei and landmarks within the midbrain, the anterior boundary was uniformly set as the first image slice in which the hippocampus merged with the ventral surface of the forebrain. This boundary occurs around bregma minus 2.75 mm (Paxinos and Franklin, 2003), which coincides with the presence of various midbrain nuclei (e.g. subgenulate nucleus, substantia nigra). The midbrain region was bound dorsolaterally by the hippocampus. The boundary of the midbrain and cerebellum was defined as the most anterior coronal image slice in which the dorsal surface of the brain displays the characteristic double arches of the inferior colliculus and the anterior lobes of the cerebellum become visible (~bregma minus 5.09 mm). Caudal to the midbrain boundary, the spinal cord (i.e.

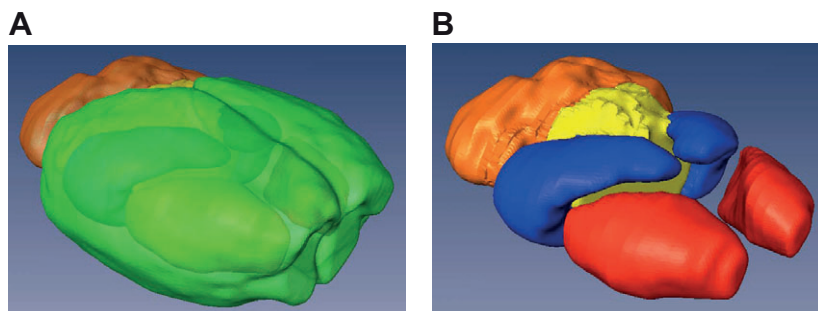


Fig. 1. Mouse brain 3-D reconstructions based on magnetic resonance imaging. The images are oriented as follows: rostral, right side; caudal, left side. (A) A whole mouse brain (oblique angle) with forebrain (green), caudate (red), hippocampus (blue), midbrain (yellow) and cerebellum (orange). The forebrain region (green) has been made partially transparent to show the underlying regions (i.e. caudate and hippocampus). (B) The same brain but with the forebrain region removed (with the exception of the caudate and hippocampus). The midbrain (yellow) and cerebellum (orange) are more easily viewed in this panel. This brain is from a control mouse (control line 1; mouse ID 441007).

the region below the fourth ventricle) was excluded from the analysis. Given that the spinal cord was cut during brain dissection, the variation in the dissection of that structure could have obscured differences in total brain volume if it had been included. Additionally, the paraflocculus of the cerebellum was also excluded because of variations in its presence after dissection. Therefore, cerebellum volume will be slightly underestimated using this methodology.

The forebrain region that was manually delineated during the MRI slice analysis (labeled as ‘other forebrain’ in the Results) excluded the volumes of the caudate-putamen and the hippocampus (see Fig. 1). Therefore, a separate variable called ‘total forebrain’ was calculated as the sum of forebrain, caudate-putamen and hippocampus. Additionally, a variable called ‘whole brain’ was calculated as the sum of all the measured brain regions. The olfactory bulbs were removed at dissection and were not included in the analysis of forebrain volume.

Statistical analyses

Nested analyses of covariance (ANCOVA) models were used for all analyses. These are mixed models, with line type (HR *versus* C) as a fixed effect and line as a random effect nested within line type. Degrees of freedom for testing the effect of line type were always 1 and 6. Covariates were included as appropriate for different analyses [age, body mass and/or brain volume (minus the volume of the region under analysis)]. Note that when body mass is used as a covariate in the analysis of brain mass or volume, then the statistical model is being applied to relative brain size.

Body mass was analyzed separately for all three generational cohorts that provided data on brain masses. For analysis of brain masses from generations 34 and 39 (females and retired male breeders, respectively), one-way ANCOVAs with line type as the main effect were used. Body mass and age were used as covariates in both of these analyses. For analysis of brain masses in generation 52 (retired male and female breeders), a two-way ANCOVA with line type and sex as the main effects was used. Mini-muscle status (see next paragraph) was included as an additional factor (Garland et al., 2002). Degrees of freedom for testing the effects of line type, sex and the line type × sex interaction were always 1 and 6. Body mass was used as a covariate in these analyses, but age was not because retired male breeders were sampled earlier than female breeders, thus confounding age and sex.

An unexpected finding of the selection experiment has been the discovery and increase in frequency, within two of the four HR lines, of individuals bearing the so-called mini-muscle phenotype (the result of a Mendelian recessive allele), recognized primarily by a reduction of ~50% in hindlimb muscle mass (Garland et al., 2002; Houle-Leroy et al., 2003; Syme et al., 2005; Wong et al., 2009). Mini-muscle individuals also differ from wild-type individuals in the size of some internal organs, including the heart, liver, spleen, lungs and kidneys [adjusting for variation in body size (e.g. Garland et al., 2002; Meek et al., 2009; Kolb et al., 2010; Downs et al., 2012)]. Therefore, it seemed reasonable to expect that mini-muscle individuals might also differ in relative brain size.

For generation 52, which had the largest sample size, we also tested for differences among the four replicate HR lines and among the four C lines, analyzing the HR and C lines separately with line treated as a fixed effect (e.g. Garland et al., 2011a). Body mass was again used as a covariate.

Brain volumes were analyzed in a one-way ANCOVA with body mass and age as covariates. Additionally, brain volumes were analyzed without the covariates of body mass and age.

Table 2. Brain mass analysis for three separate generations of mice

Generation	Sex	N	Mean age in days (range)	Brain mass, wet or dry	Sex	Line type		Line type × sex		Age	Least squares mean ± s.e.m. (g)	
						Line type	Sex	Body mass	Body mass × sex		HR	C
34	F	46	78 (54–101)	Whole, wet	0.0380+	0.0768+	0.0103+	0.8377+	0.4345±0.01290	0.3910±0.01218		
				Whole, dry		0.0650+	0.0241+	0.5074+	0.09708±0.002719	0.08741±0.002567		
				Whole, wet		0.2462+	0.0239+	0.6071+	0.3786±0.009818	0.3597±0.01042		
39	M	138	91 (76–97)	Whole, dry	0.0345+	0.1727+	0.0144+	0.3888+	0.08278±0.002244	0.07758±0.002372		
				Non-cereb., wet		0.0452+	0.0087+	0.7565–	0.3279±0.004378	0.3102±0.004989		
				Non-cereb., dry		0.0341+	0.0044+	0.8807+	0.07091±0.001071	0.06629±0.001197		
52 ^a	Both	310	165 (126–186)	Cerebellum, wet	0.0380+	0.9190+	0.4031+	0.1077+	0.05060±0.006129	0.04964±0.006371		
				Cerebellum, dry		0.7836+	0.3451+	0.0892+	0.01187±0.001320	0.01131±0.001374		
				Non-cereb., wet		0.0345+	0.5590+	Not used	F: 0.3535±0.003521	F: 0.3407±0.003982		
				Non-cereb., dry	0.0347+	0.8166	0.1718+	M: 0.3497±0.003380	M: 0.3364±0.004039			
				Cerebellum, wet	0.0091+	0.7492+	0.0030+	F: 0.07582±0.000865	F: 0.07323±0.000971			
				Cerebellum, dry	0.0051+	0.3845+	<0.0001+	M: 0.07221±0.000984	M: 0.07221±0.000984			
								F: 0.1263±0.002937	F: 0.1236±0.003230			
								M: 0.1177±0.002821	M: 0.1179±0.003278			
								F: 0.02838±0.000650	F: 0.02734±0.000722			
								M: 0.02636±0.000618	M: 0.02584±0.000737			

^aMini-muscle status was used as an additional factor for generation 52. Mini-muscle individuals tended to have larger values in all cases, but the effect was never statistically significant (wet non-cerebellar brain, $P=0.6446$; dry non-cerebellar brain, $P=0.5386$; wet cerebellum, $P=0.2471$; dry cerebellum, $P=0.1076$). Positive signs following P -values indicate HR>C, females>males or positive effect of body mass. P -values in bold were considered statistically significant ($P<0.05$), and all statistical tests were two-tailed. HR, high runner; C, control.

Analyses were run in the SAS 9.1 statistical software package (SAS Institute, Cary, NC, USA) using Proc Mixed. Values are reported as least squares means and associated standard errors. Statistical tests were two-tailed and significance was defined as a P -value <0.05 .

RESULTS

Brain mass

In all analyses, HR mice had smaller body mass than C mice (Table 1). In generation 34, HR females were 15.8% smaller. In generation 39, HR males were 12.5% smaller. In generation 52, HR females and males were 13.9 and 12.0% smaller, respectively.

Whole brain mass did not significantly differ between HR and C lines in generation 34 females (after accounting for body mass differences), although the HR lines tended to have heavier brains (Table 2). In generation 39 males, HR lines had significantly larger non-cerebellar brain mass (5.7% greater wet mass, 7.0% greater dry mass), but did not differ statistically in cerebellum mass. In generation 52, non-cerebellar brain mass was again significantly greater in HR lines, for both sexes (dry mass, females=5.2%, males=4.9%; Fig. 2, Table 2). Females had significantly greater masses for both non-cerebellar and cerebellar components than males, with no line type \times sex interaction (Table 2).

For generation 52, Fig. 3 displays variation in dry non-cerebellar mass among the replicate lines within the C and HR line types. Analyzed separately, the four C lines differed significantly from each other in dry non-cerebellar mass ($P=0.0020$), females had larger values than males ($P=0.0145$), there was no line type \times sex interaction ($P=0.0867$), but there was a positive effect of body mass ($P=0.0137$). The four HR lines also differed significantly in dry non-cerebellar mass ($P=0.0317$), but females did not have statistically larger values than males ($P=0.1102$), there was no line type \times sex interaction ($P=0.5931$), no effect of the mini-muscle phenotype ($P=0.9799$) and no effect of body mass ($P=0.7053$). For dry cerebellar mass, the four C lines differed significantly ($P=0.0025$), females had larger values than males ($P=0.0095$), there was no line type \times sex interaction ($P=0.3651$) but there was a positive effect of body mass ($P=0.0255$). The four HR lines did not differ significantly in dry cerebellar mass ($P=0.0608$), but females again had larger values than males ($P=0.0002$), with no line type \times sex interaction ($P=0.1613$), no effect of the mini-muscle phenotype ($P=0.2216$), but a positive effect of body mass ($P=0.0110$).

Brain volume

Controlling for variation in body mass and age, HR females from generation 41 had midbrain volumes that averaged 13.4% larger than

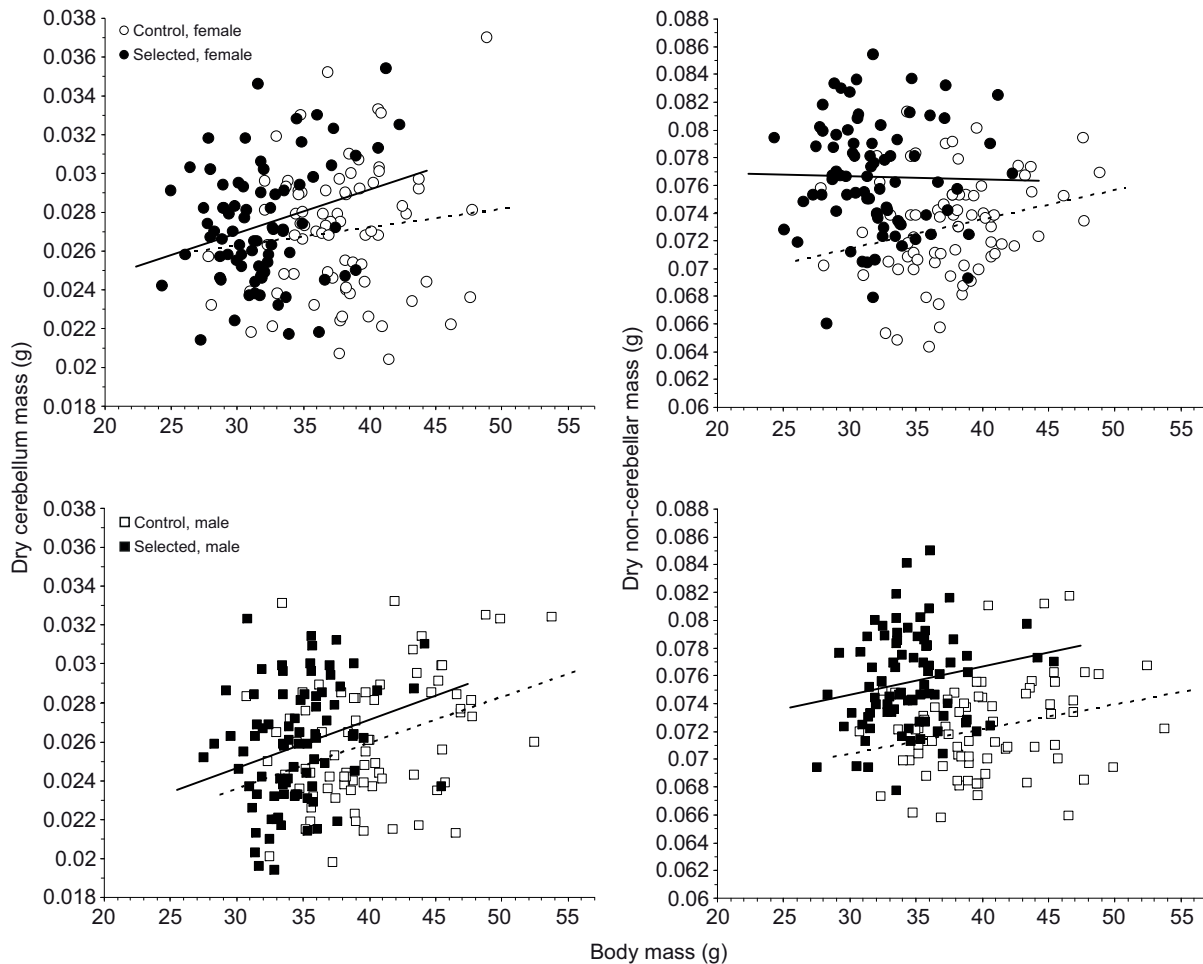


Fig. 2. Dry mass of cerebellum (left panels) and non-cerebellar brain (right panels) plotted in relation to body mass for mice sampled from generation 52. Mice from the selectively bred high-runner lines have statistically larger non-cerebellar brain mass (see Table 2). As a heuristic, simple least-squares linear regression lines are shown for each subgroup; for statistical comparisons, body mass was included as a covariate, thus constraining the slopes to be parallel for the groups being compared (Table 2).

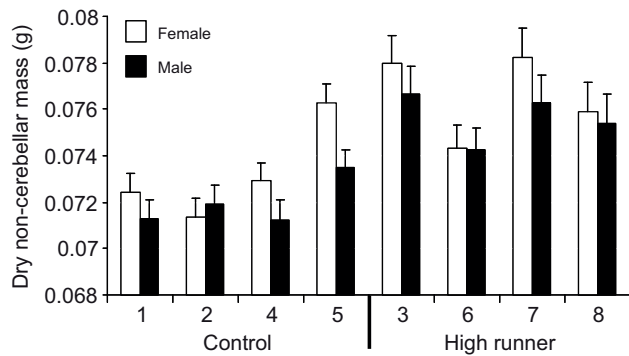


Fig. 3. Dry mass of non-cerebellar brain (g), separated by sex, replicate line and line type [control (C) versus high runner (HR)]. Values are least squares means and associated standard errors from SAS Proc Mixed, with body mass as a covariate, and separate analyses performed on the C and HR lines (see Materials and methods). Both line types showed statistically significant differences among the replicate lines. The C lines also showed a significant sex effect, but the HR lines did not (see Results). The laboratory designations of the lines are nos 1, 2, 4, 5 for C and 3, 6, 7, 8 for HR, presented in that order. Note that, as a group, the HR lines had significantly larger values than the C lines (Table 2, Fig. 2).

those of C mice (from least squares means with body mass and age as covariates; Table 3). However, use of brain volume (minus midbrain volume) as a covariate increased the P -values to >0.05 . There were no statistical differences for either total brain volume or the remaining constituent regions, irrespective of the covariates used.

DISCUSSION

Compared with four non-selected C lines, mice from the four selectively bred HR lines had significantly greater midbrain volume (Table 3) as well as larger non-cerebellar brain mass, but not larger cerebellar or total brain mass (Table 2, Fig. 2). We also documented statistically significant differences among the four C lines and among the four HR lines with respect to brain dry mass (e.g. Fig. 3), indicating the importance of random genetic processes [e.g. founder effects, drift, unique mutations (Garland et al., 2002; Kane et al., 2008)] as well as possible ‘multiple solutions’ in the HR lines in response to the selective breeding regimen (Garland et al., 2011a). Nonetheless, these differences among the replicate lines were not so great as to obscure differences between the sets of HR and C lines.

Mice from the HR lines run voluntarily on wheels for longer distances, at higher speeds and in a more intermittent fashion than mice from non-selected C lines (Girard et al., 2001; Garland et al., 2011a). When deprived of wheels, HR mice are more active in their home cages than C mice (Malisch et al., 2009). As compared with C mice, HR mice exhibit various anatomical (Garland and Freeman, 2005; Kelly et al., 2006) and physiological differences (Rezende et al., 2006a; Rezende et al., 2006b; Gomes et al., 2009; Meek et al., 2009) that appear to support their 2.5- to 3-fold greater daily wheel-running distances. Although previous studies have revealed physiological (Dumke et al., 2001; Rezende et al., 2006a; Rezende et al., 2006b; Gomes et al., 2009; Meek et al., 2009; Kolb et al., 2010), morphological (Garland and Freeman, 2005; Kelly et al., 2006) and motivational (Rhodes et al., 2005; Belke and Garland, 2007) components underlying this HR phenotype, the present findings are the first evidence of neuroanatomical changes in the HR lines. [A previous study that quantified volume of the dentate gyrus of the hippocampus found no statistical difference between the HR and C lines (Rhodes et al., 2003a).] Additionally, to our

knowledge, this is the first time that intraspecific changes in brain mass or volume have been associated with selection for increased locomotor activity in any mammal.

In our analyses of brain mass and volume, we used body mass as a covariate because brain size generally scales allometrically with body size, both within and among species of eutherian mammals (see Kruska, 2005), and because the HR mice from three of the four generations sampled had significantly smaller body masses than the control lines (Table 1). Lower body mass in HR lines has been a consistent finding in the selection experiment since generation 14 (13.6% smaller at 79 days old) (Swallow et al., 1999), and a trend for reduced body mass was observed at generation 10 (e.g. Swallow et al., 1998b). The difference in body mass between HR and C lines makes it important to include body mass as a covariate in statistical comparisons of organ size, including brain mass and volume (see Tables 2, 3).

Whole brain mass (corrected for body mass) was not statistically different between the line types in our initial cohort of females from generation 34, although a trend for HR $>$ C was apparent (Table 2), and this stimulated our more detailed measurements. Therefore, in the subsequent cohorts, the cerebellum was separated from the rest of the brain and this exposed underlying mass differences in the non-cerebellar brain (HR $>$ C; Fig. 2). Volumetric MRI analyses suggest that these differences were partly associated with an enlarged midbrain in HR lines (Table 3). Conversely, the size of the cerebellum appears to have remained unaltered by selective breeding (Fig. 2, Tables 2, 3).

MRI technology provides a non-invasive modality for studying *ex vivo* organ volumetrics without the potentially confounding problem of tissue artifacts often seen in histological sectioning. Additionally, MRI of *ex vivo* samples allows for direct comparison with subsequent longitudinal assessments, should *in vivo* volumetric analyses of brain regions be warranted in future studies. Nevertheless, using MRI restricts the ability to evaluate brain regions that are distinct because of cell morphology, neurochemistry, etc., and that is a limitation of the present study. Therefore, it is important to note that the intent of our analysis by MRI was to obtain a measure of volumetric changes within gross brain structures (i.e. midbrain, etc.). Future *ex vivo* studies can be undertaken to assess the volumetric and neuroanatomical changes within the midbrain in finer detail, similar to our published studies (e.g. DeFazio et al., 2012) and those by other groups (Johnson et al., 2012; Ullmann et al., 2012). In addition, a significant advantage of MRI is the ability to collect longitudinal data on a cohort of animals. Although we did not employ such sampling in the present study, we plan to do so in the future.

The midbrain contains a variety of sensory and motor nuclei. These include the corpora quadrigemina, red nuclei, substantia nigra (SN) and ventral tegmental area (VTA), as well as various efferent projections from the cortex to brainstem and spinal cord (Waxman, 2010). Without further information on the relative size of each of these nuclei, it is impossible to say which (if not all) might be contributing to the overall enlargement of the midbrain region that we observed from our MRI-derived volumes. Two of these nuclei (SN, VTA) are of particular interest because they participate in two central dopaminergic axes in the mammalian brain: the nigrostriatal and the mesocorticolimbic pathways, respectively (Waxman, 2010).

The SN is involved in ‘smoothing’ of motor impulses, and when it is damaged (most notably in Parkinson’s disease), a bradykinesia or akinesia results (reviewed in Poewe, 2009). Previous work has shown that blocking wheel access after 6 days of wheel running elevates neuronal activity in the SN in mice (Rhodes et al., 2003b).

Table 3. Volume analysis of brain regions from female mice from post-selection generation 41

N	Mean age in days (range)	Trait	P			Least squares mean \pm s.e.m. (cm ³)		
			Line type ^a	Body mass or remainder brain volume ^a	Age	HR	C	HR/C
29	162 (145–174)	Whole brain	0.1392+ (0.1757+)	0.5146+	0.1870+	0.4094 \pm 0.00873	0.3880 \pm 0.00841	1.05
30	162 (145–174)	Total forebrain	0.1224+ (0.1765+) [0.4825+] {0.4759+}	0.3719+ <0.0001+ {<0.0001+}	0.1725+ [0.4706+]	0.2894 \pm 0.00614	0.2732 \pm 0.00614	1.06
31	162 (145–174)	Other forebrain	0.1573+ (0.1489+) [0.5033+] {0.4972+}	0.5969+ <0.0001+ {<0.0001+}	0.4175+ [0.6189–]	0.2284 \pm 0.00522	0.2162 \pm 0.00507	1.06
32	162 (145–174)	Caudate	0.9695– (0.7061–) [0.2187–] {0.1967–}	0.4865+ <0.0001+ {<0.0001+}	0.0505+ [0.1053+]	0.03001 \pm 0.001002	0.03006 \pm 0.001002	1.00
33	161 (145–174)	Hippocampus	0.1328+ (0.3549+) [0.4990+] {0.5582}	0.0656+ [0.1247] {0.0667+}	0.0163+ [0.2218]	0.03012 \pm 0.001319	0.02683 \pm 0.001299	1.12
29	161 (145–174)	Midbrain	0.0479+ (0.0317+) [0.0732+] {0.0673+}	0.6213– [0.0048+] {0.0029+}	0.4670+ [0.9018–]	0.04420 \pm 0.001497	0.03898 \pm 0.001402	1.13
32	161 (145–174)	Cerebellum	0.8273– (0.8535–) [0.3900–] {0.3887–}	0.8740– [0.0046+] {0.0029+}	0.2580+ [0.9736+]	0.07444 \pm 0.002467	0.07526 \pm 0.002474	0.99

One-way ANOVAs with line type as the main effect were conducted in SAS using Proc Mixed.

^aAnalyses were also conducted: (1) without body mass and age as covariates (*P*-values in parentheses); (2) with brain volume, minus the region under analysis (rather than body mass), and age as covariates (*P*-values in square brackets); and (3) with brain volume, minus the region under analysis (instead of body mass), as a covariate (*P*-values in curly brackets).

For analysis of body mass (*N*=33), *P*=0.2035 for line type, *P*=0.2555 for age (least squares mean \pm s.e.m.=32.1533 \pm 1.0789 for HR, 34.3193 \pm 1.0677 for C). Excluding age as a covariate, *P*=0.2180 for line type (least squares mean \pm s.e.m.=32.1691 \pm 1.1089 for HR, 34.3163 \pm 1.0981 for C).

Positive signs following *P*-values indicates HR>C, or positive effects of body mass or age, and *vice versa* for negative signs.

P-values in bold were considered statistically significant (*P*<0.05), and all statistical tests were two-tailed.

HR, high runner; C, control.

The same study showed a trend for the distance run (accumulated over an approximate 5-h period prior to sampling) to be positively correlated with neuronal activation (*via* expression of the transcription factor Fos-IR) in the SN [$F_{1,14}=4.1$, *P*=0.06; *p*.1251 of Rhodes et al. (Rhodes et al., 2003b)]. The enhanced activity of the SN in response to wheel running coupled with its high concentrations of dopaminergic neurons make this region worthy of future study.

The VTA is an integral part of the reward circuit that motivates and reinforces behaviors (Wise and Bozarth, 1987; Fibiger and Phillips, 1986; Koob, 1992; Pierce and Kumaresan, 2006; Ikemoto, 2007; Ikemoto, 2010). Previous behavioral work (Belke and Garland 2007) suggests that HR lines have alterations in aspects of their reward system. Moreover, a general alteration of function in dopaminergic D1 receptor-mediated signaling exists in HR lines (Rhodes et al., 2001; Rhodes and Garland, 2003), and recent work in one of the HR lines demonstrated elevated dopamine concentrations in both the nigrostriatal and the mesocorticolimbic pathways (Mathes et al., 2010). Both the SN and VTA contain high densities of dopaminergic neurons, and a modification in either one could be related to the functional changes already observed in the HR mice.

Should a modification in function necessarily lead to an expansion in the size of a brain region? The hippocampus is important in spatial

orientation, and examples exist of enlargement of the hippocampus in both birds (Krebs et al., 1989; Krebs, 1990; Jacobs and Liman, 1991) and mammals (Sherry et al., 1992; Jacobs and Spencer, 1994; Lavenex et al., 2000) involved in seasonal caching behavior, as well as sex differences in space use (related to breeding system) in some small mammals (Jacobs and Spencer, 1994; Sherry et al., 1996; Lavenex et al., 2000). Moreover, among species of primates, enlargement of the neocortex has been correlated with the likelihood of using deception as a strategy for social advancement (Byrne and Corp, 2004). Additionally, recent work has found an enlargement in associative cortical regions (but not sensory and motor regions) in tool-making New Caledonian crows (*Corvus moneduloides*) (Mehlhorn et al., 2010).

Our initial question was whether selection on a behavioral trait would result in a change in brain size and, if so, whether that change would be concerted or mosaic. The finding that HR lines of mice, bred for high levels of voluntary exercise, have an enlarged non-cerebellar brain mass and an enlarged volume of the midbrain, but do not show a statistically significant increase in overall brain mass or volume, supports the mosaic theory of brain evolution. Whether this change in midbrain size is primarily being driven by alterations in motor ability or aspects of behavioral reward is an open question, and represents an important direction for future study.

LIST OF ABBREVIATIONS

C	non-selected control lines of mice that were bred without regard to amount of wheel running
FOV	field of view of the MRI image slice
HR	high-runner lines of laboratory house mice, selectively bred for high voluntary wheel running
MRI	magnetic resonance imaging
PFA	paraformaldehyde
RARE	rapid acquisition with relaxation enhancement MRI scan
SN	substantia nigra; a midbrain nucleus
TE	echo time within an MRI sequence
TR	time to repetition within an MRI sequence
VTA	ventral tegmental area; a midbrain nucleus

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