THE EFFECTS OF DOMESTICATION ON CEREBELLAR MORPHOLOGY AND BRAIN COMPOSITION: COMPARING WILD RATS *(RATTUS NORVEGICUS)* TO LABORATORY RATS

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ABSTRACT

The laboratory rat, the domestic version of the wild Norway rat *(Rattus norvegicus)*, is an important model organism in scientific research. Like other domestic mammals, laboratory rats have smaller brains than their wild relatives. Little is known, however, about the effects of domestication on cerebellar morphology or the underlying changes in cellular composition responsible for such intraspecific variation in brain morphology. Here, we compared cerebellar morphology and brain composition in wild rats and two domestic laboratory strains: Long-Evans and Sprague-Dawley. In the cerebellum, we found that all strains had similar Purkinje cell numbers, but wild rats had a higher degree of foliation and larger Purkinje somas than Long-Evans rats. Next, we used isotropic fractionation to test for differences in brain composition across strains. We found that brain composition differs between laboratory and wild rats, but that the effects of domestication vary across strains and brain regions.

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LIST OF ABBREVIATIONS

μm – micrometers “microns”
ANCOVA – analysis of covariance
ANOVA – analysis of variance
CB – cerebellum
CCBN – Canadian Centre for Behavioural Neuroscience
CV – coefficient of variation
CX –cortex
DAPI – 4’,6-diamidino-2-phenylindole dihydrochloride
g – grams
F – F ratio
HSD – honest significant difference
IF – isotropic fractionator
i.p. – intraperitoneal
LE – Long-Evans
M – mean
mg – milligrams
n – number of specimen
NeuN – neuronal nuclear antigen
p – p value
OB – olfactory bulb
PBS – phosphate buffered saline
PFA – paraformaldhyde
Std – Standard deviation
ROB – rest of brain
SD – Sprague-Dawley
WWCPS – Wild Warsaw Captive Pisula-Stryjek

CHAPTER ONE: GENERAL INTRODUCTION

Domestication is the evolutionary process by which wild organisms become adapted for human use; as pets, a source of labour, for food production (e.g., meat, fat, milk) and other resources (e.g., fur, leather). In the process of creating a domestic stock, humans exert a high degree of control over both the survival and reproduction of a small isolated population of wild animals (Kruska, 1988). Typically, the process of domestication is initiated when animals colonize the "ecological niche" created by human settlements, when some individuals gain fitness advantages by tolerating human proximity and exploiting available resources (Francis, 2015; Kruska, 1988). Humans also gain adaptive advantageous by domesticating animals, especially by selectively breeding individuals for certain desirable traits, thereby increasing their utility. By imposing artificial selection humans effectively remove the natural influences of mate choice and sexual selection within the captive population, causing a gradual divergence from their wild counterparts in morphology, physiology, and behaviour. Such intraspecific variation that arises through domestication appears at an accelerated rate compared to evolution in a natural setting (Francis, 2015). Thus, domestication is an interesting evolutionary phenomenon which may grant insight into how animals adapt to diverse lifestyles.

Although species across a range of mammalian taxa (i.e., rodentia, lagomorpha, artiodactyla, perissodactyla, and carnivora) have been domesticated for diverse purposes, all domesticates share an inherited predisposition towards tameness (Belyaev, 1969; Francis, 2015; Kruska, 2005). Tameness (i.e., reduced aggression, increased docility) is fundamental to domestication as it is associated with a dampening of stress response (Künzl & Sachser, 1999) and consequently, a reduction in anxiety and fearfulness (Wilkins et al., 2014). Selection for tameness however, appears to have pleiotropic effects across seemingly unrelated traits, which produces a conserved "domestication phenotype" in mammals. For instance, domestication is

known to reduce brain size, although the degree to which brain size is reduced varies between species; with more basal species experiencing a relatively smaller decrease compared to more encephalized species (Kruska, 2005). Domestication also produces changes in the proportions of specific parts of the brain, such as the telencephalon, cerebellum, mesencephalon, medulla oblongata, and especially the neocortex (Kruska, 2005). Moreover, changes in the size of certain functional regions of the brain, such as sensory, limbic, and motor structures, are also caused by domestication (Kruska, 1988, 2005). In regards to behaviour, domesticates tend to be more docile (Belyaev, 1969), less aggressive towards conspecifics (F. Blanchard, Blanchard, 1986), less neophobic in novel and social environments (W. Blanchard, Lee, Blanchard, 1981), less athletic and less active than their wild counterparts (Francis, 2015). Such intraspecific behavioural variation is likely supported by underlying changes in the neural structures which support these functions. For example, the reduced emotional reactivity characteristic of the domestic form may reflect the diminished size of limbic structures, and consequently a higher stimulus threshold necessary to elicit such behaviours (Francis, 2015). Thus, it appears that the human environment has some consistent evolutionary effects on conserved mammalian brain homologies which yield evolutionary convergence towards development of the behavioural domestication phenotype. In addition to changes in brain and behaviour, domestication also improves reproductive efficiency in animals (Setchell, 1992). For instance, domestic animals tend to reach sexual maturity faster (at an early age), have more frequent and non-seasonal reproductive seasons and produce more offspring per litter (Setchell, 1992). This conserved set of characteristics (i.e., the domestication syndrome) might be mediated through changes in conserved developmental processes. Indeed, some authors have suggested that the disparate traits included in domestication syndrome (e.g., changes in pigmentation, skull, jaw, teeth etc.) are

primarily the result of deficits in neural crest cells, a type of embryonic stem cells which gives rise to the dorsal portion of the neural tube (Wilkins et al., 2014). Thus, domestication syndrome is likely produced when selection for tameness produces genetic changes which alter conserved developmental processes in mammals (Wilkins et al., 2014).

The laboratory rat, an important model organism in scientific research, is the result of domestication of the wild Norway rat *(Rattus norvegicus).* The laboratory rat is a unique example of mammalian domestication because unlike pet or livestock species, laboratory rats have not been bred as a source of labour, companionship, or food. Instead, laboratory rats were domesticated to serve as important model organisms for scientific research, especially in behavioural and learning experiments. Several breeding strains have been established in laboratory rats for different research purposes, and genetic similarity within strains has been accomplished through in-breeding (Francis, 2015). Like other mammalian domesticates, laboratory rats have been selected for docility, ease of handling, and captive breeding (Francis, 2015). Specifically, laboratory rats are able to breed year round whereas wild rats are seasonal breeders with seasonal estrus cycles (Francis, 2015; Kruska, 2005). Like other domesticate species, laboratory rats are more socially tolerant towards conspecifics and are less neophobic (F. Blanchard, Blanchard, 1986; W. Blanchard, Lee, Blanchard, 1981), and hence more exploratory in novel environments compared to wild rats. Additionally, laboratory rats seem to perform better at standardized spatial learning tasks than wild rats (Boice, 1970, 1972). Our knowledge about the effects of domestication on learning and memory is limited however, as most studies compare wild animals to domesticates within a captive setting. It is likely then, that the apparent advantage of domesticates in learning and memory may simply reflect their lower state of arousal within a captive setting, in part due to their reduced sensory and limbic structures

(Kruska, 2005). While wild rats will be constantly attending to their surroundings in a captive environment, laboratory rats tend to be more relaxed, allowing them greater concentration on such learning tasks (Kruska, 2005).

As in other domesticate species, laboratory rats have smaller brains than wild rats and are known to exhibit changes in the relative proportions of some brain regions (Kruska, 1988). Domestic laboratory rat strains are reported to have proportionally smaller telencephalon size, limbic structures (e.g., hippocampus), sensory structures (visual and auditory) and motor structures compared to their wild counterparts (Kruska, 1988, 2005). The one exception to these regressive trends is found in albino Wistar laboratory rat, who actually has a larger optic tract compared to wild rats (Kruska, 1988). This finding, however, is likely related to albinism and not domestication since albinism is known to produce changes in the visual regions of the brain and because pigmented laboratory rats exhibit the opposite trend (i.e., reduced optic tract size) (Kruska, 1988). Furthermore, the visual areas of the brain such as lateral geniculate nucleus, superior colliculus, and primary visual area are reduced in size in the Wistar rat, despite its albinism (Kruska, 1988). The genetic differences between albino and pigmented strains are also known to produce behavioural differences such as in motor learning (Keeley et al., 2015).

The behavioural patterns of rough-and-tumble play are also known to vary between wild and laboratory rats as well as between laboratory rat strains (Himmler, Modlinska, et al., 2014). First, domestic strains are more tolerant of proximal conspecific and are generally more playful; engaging in a higher frequency of playful attacks than their wild counterparts (Himmler, Modlinska, et al., 2014). Second, there are behavioural differences amongst domestic strains which involve the types of defense tactics used to respond to a playful attack by a conspecific (Himmler, Modlinska, et al., 2014). Long-Evans rats, for example, tend to use much less evasive

defense maneuvers compared to other rat strains (e.g., Brown Norway, Sprague Dawley, Wistar, wild-caught), whereas Sprague Dawley rats prefer evasive defense over facing defenses (Himmler, Modlinska, et al., 2014). Third, domestic strains are generally less active and display reduced acrobatic abilities in play fighting (Himmler, Modlinska, et al., 2014). Such intraspecific variability in play behaviour may reflect underlying changes in the neural mechanisms that support the coordination of related motor patterns (Himmler, Modlinska, et al., 2014).

The cerebellum (CB) is an important structure for fine-tuning and coordinating motor activity, as it receives and integrates motor inputs from the cortex, spinal cord, and other areas (Fine et al., 2002). Because play occurs primarily during the juvenile period, and the age distribution of play seems to correspond with synaptogenic modification of the CB during development, some suggest that play is an important behaviour in cerebellar development (Byers & Walker, 1995). Specifically, during the peak period of juvenile play, the CB has formed approximately 80% of its synapses, and is also engaged in synaptic pruning (Byers & Walker, 1995). Early developmental motor activity is known to affect modification of synaptogenesis in the CB, thus play may support the proper development of Purkinje synapses, producing lasting changes in motor performance (Byers & Walker, 1995). In regards to domestication, the changes in play behaviour observed between wild rats and laboratory rat strains may represent a form of early motor experience which leaves permanent effects on motor performance throughout life.

In rodents, the CB appears to occupy a relatively constant volume in proportion to total brain size, whereas the proportional volume of the cerebral cortex seems to increase with brain size (Herculano-Houzel et al., 2006). Interestingly, as brain size increases in rodents, the number of neurons in the CB increases at a faster rate than the total number of neurons in the cortex (Herculano-Houzel et al., 2006). Since cellular composition of a brain region may be indicative

of overall informational processing capacity, these findings indicate that the CB actually gains functional capacity faster than the cerebral cortex (Herculano-Houzel et al., 2006). Moreover, the increase in the relative size of the cerebral cortex as brain size increases appears to be primarily driven by the addition of non-neuronal cells in rodents (Herculano-Houzel et al., 2006). Thus, changes in the cellular composition of the CB may be implicated in the changes in play behaviour observed in domestic laboratory rat strains, and may potentially grant insight into the common evolutionary trends evident in the brains of domestic mammals.

Previous investigators have reported that laboratory rats display less spontaneous motor behaviour as well as less acrobatic play than their wild relatives (Himmler, Modlinska, et al., 2014). Therefore, laboratory rats are an ideal group in which to investigate the neural basis of these intraspecific changes in the patterns of motor behaviour. Although domestication is known to reduce total brain size, the effects of domestication on cerebellar morphology are not well understood. Because domestication also modifies play behaviour, our central hypothesis was that intraspecific variation in rat play likely reflects underlying changes in the CB, an important region for coordinating and fine-tuning motor activity. Thus, our first objective for these studies was to characterize the morphological and cellular changes that occur in the CB of domestic laboratory rats. Overall, we expected that wild rats will have a more complex CB than laboratory rats because the former exhibits relatively more aerobatic play than the latter. We investigated the effects of domestication on several aspects of cerebellar morphology including cellular composition, foliation and Purkinje soma size. A change in any of these major characteristics is presumably indicative of a change in the informational processing abilities of the CB, and may be implicated in the observed changes in play behaviour.

One indication of complexity in the CB is its relative size and surface area. Therefore, we hypothesized that the domestic laboratory rats will have smaller and/or less foliated cerebellar cortex than wild rats. A reduction in either the size or surface area of the CB could reflect a reduction in the ecological relevance of associated motor behaviours in the context of a domestic lifestyle. This prediction was based on Jerison's (1973) principle of proper mass which suggests that the mass of a neural structure is proportional to its information processing ability, and hence relates to its ecological importance. Another important parameter by which to judge the relative complexity of the CB is its cellular composition, including the total number of neurons and non-neuronal supportive cells. Thus, we also hypothesized that the cerebellar cortex of domesticated laboratory rats will contain fewer cells than wild rats. Specifically, we expected there to be a reduction in either (or both) of the two major neuron types of the CB: Purkinje cells and granule cells. Again, we expected there to be fewer neurons in laboratory rats because fewer neurons implies a reduction in computational capacity which may relate to the more simple motor behaviours evident in laboratory rats compared to wild populations. Neuronal soma size is known to increase with brain size in rodents, whereas non-neuronal cell size remains relatively fixed (Herculano-Houzel et al., 2006). Therefore, we also hypothesized that the Purkinje cell somas will be larger in wild rats than in laboratory rats because as brain size increases, neurons need to be larger (with greater arborization) to support connectivity over a larger distance.

Although domestication is known to produce changes in brain size as well as the size of brain regions (Kruska, 2005), little is known about the underlying cellular changes in cellular composition responsible for such intraspecific variation in brain morphology. Therefore, the second objective of these studies was to test for differences in the brain composition of wild rats and domestic laboratory rats. We predict that the reduction in brain size associated with

domestication will reflect smaller neuronal populations. To test our predictions, we used the isotropic fractionator (IF) method to estimate the cellular composition of the CB as well as three other regions of interest; the olfactory bulbs (OBs), cerebral cortex (CX) and the remaining tissue or 'rest of brain' (ROB) (Herculano-Houzel & Lent, 2005). Briefly, the IF method is a non-stereological means of quantifying the number of neuron and non-neuronal cells in any dissectible brain region, and it is faster than traditional stereological methods (Herculano-Houzel & Lent, 2005). After homogenization, aliquots of nuclei were incubated with the DNA-specific fluorescent label 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) and labeled immunohistochemically for neuronal nuclear antigen (NeuN) (Herculano-Houzel & Lent, 2005). Counts of all cells (DAPI) and neurons (NeuN) were then made with a hemacytometer and the number and density of total cell, neurons and non-neuronal cells were calculated (Herculano-Houzel & Lent, 2005). One limitation of this technique is that Purkinje cells do not express NeuN, and therefore will not be counted in the neuronal cell number estimate. This however, is not of major concern as Purkinje cells constitute a very small proportion of cerebellar neurons, whereas cerebellar granule cells are easily the most numerous neurons in the CB, and actually constitute about 75% of total neurons in the brain (Llinas et al., 2004).

In these studies, our primary goal was to more precisely define how domestication affects cerebellar morphology as well as the cellular composition of the brain. To assess the effects of domestication on the brain, we compared cerebellar morphology and brain composition in wild rats as well as two popular strains of laboratory rat: Long Evans (LE) and Sprague-Dawley (SD). This knowledge will allow us to better understand the evolutionary changes associated with domestication as well as how selective breeding histories and domestic environments impact our animal models.

CHAPTER TWO: THE EFFECTS OF DOMESTICATION ON CEREBELLAR MORPHOLOGY AND CELLULAR COMPOSITION: COMPARING WILD RATS (*RATTUS NORVEGICUS)* TO LABORATORY RATS

INTRODUCTION

Domestication is the process by which wild organisms become adapted for human use. In the process of creating a domestic stock, humans exert a high degree of control over both the survival and reproduction of isolated wild populations (Kruska, 1988). As a result, animals bred in captivity are subject to a unique suite of evolutionary selection pressures that are advantageous to living amongst humans. For example, artificial selection for tameness (i.e., increased docility, reduced aggression towards humans) produces pleiotropic effects across a variety of seemingly unrelated traits (Francis, 2015; Kruska, 2005). As a consequence of such selection, domestic animals have phenotypically diverged from their wild progenitors in both morphology and behaviour. For instance, domesticates are more docile (Belyaev, 1969), less aggressive towards conspecifics (Albert et al., 2008; F. Blanchard, Blanchard, 1986), less neophobic in novel and social environments (Albert et al., 2008; W. Blanchard, Lee, Blanchard, 1981), less athletic, and less active than their wild counterparts (Francis, 2015). There are also a variety of morphological changes produced by domestication. In mammals, domestication produces changes in body size, skeletal dimensions, coat colour, and tends to reduce the size of various organs (e.g., heart, intestines, liver) (S. Kruska, 1999; O'Regan & Kitchener, 2005). In domestic mammals, the suit of commonly inherited morphological, physiological and behavioural traits is known as the 'domestication syndrome', although some of these traits are also evident in domestic fish and birds (Wilkins et al., 2014).

Some of the more prominent morphological effects of domestication syndrome are changes in brain size as well as changes in the sizes of individual brain regions (Agnvall et al., 2017; Ebneter, 2016; Kruska, 2005). Specifically, domestic mammals tend to have smaller brains for their body size compared to their wild relatives (Kruska, 2005). For many domestic mammals (e.g., pig, poodle, ferret, llama), the largest change in relative brain size is observed in the telencephalon (Kruska, 2005). Thus, much of the domestication research has focused on telencephalic regions such as the cerebral cortex and hippocampus, especially regarding changes in gene expression and neurochemistry (Albert et al., 2008; Albert et al., 2012; Ruan & Zhang, 2016). The telencephalon is not, however, the only brain region that changes in size. Indeed, the cerebellum (CB) as well as thalamic and brainstem nuclei are also smaller to varying degrees in domesticates compared with their wild counterparts (Kruska, 2005).

The CB is an important structure in which to investigate the effects of domestication for several reasons. First, as mentioned above, most of the research has focused on cortical structures, so our understanding of how artificial selection affects non-cortical regions is poor. Second, there are multiple lines of evidence demonstrating that the CB is affected by domestication. For several domesticated mammalian species, the change in CB size is approximately equal to (or greater than) the changes observed in the telencephalon (10-32%, Kruska, 2005). Third, domestication produces changes in motor behaviour; in general, domesticates tend to be less active and less athletic than their wild relatives (Francis, 2015). Given this variation in motor behaviour across strains, it follows that there may be underlying neuroanatomical changes in brain regions which are involved in motor functions, such as the CB. Finally, although multiple lines of evidence demonstrate that the CB is affected in domestication, there are conflicting findings as to whether such changes are positive or negative in nature. For

example, Japanese quail (*Coturnix japonica*) artificially selected for larger egg size have relatively smaller cerebella than quail selected for smaller egg size (Ebneter, 2016). On the other hand, in red-jungle fowl (*Gallus gallus*), domestication has produced larger cerebella; birds bred for tameness had smaller brains with relatively larger cerebella than their untamed counterparts (Agnvall et al., 2017). Based on their results in Red-jungle fowl, Agnvall and colleagues (2017) have speculated that the CB does not respond to domestication in the same way as the rest of the brain appears to. Thus, it is unclear how domestication changes the CB or what is responsible for such changes on a cellular level.

To test the effects of domestication on the cellular composition and morphology of the CB, we compared wild and domestic laboratory strains of Norway rats *(Rattus norvegicus)*. The laboratory rat is a relatively unique example of mammalian domestication because unlike other mammals that were domesticated as a source of food, labour, companionship, or for other resources (e.g., fur, leather), laboratory rats were bred to serve as model organisms in scientific research (Krinke, 2000). Today, hundreds of laboratory strains have been developed for diverse purposes, especially in studies on learning and behaviour. Like other domestic mammals, laboratory rats are reported to have relatively smaller brains and cerebella compared to wild rats (Kruska, 1975, 2005). Yet, to the best of our knowledge, the only investigation into relative CB size was conducted in albino Wistar rats (Kruska, 1975), thus, the relative changes in other commonly used strains have yet to be characterized.

Given such intraspecific variation in the size of the CB, the main objective of this study was to quantitatively compare cerebellar morphology and composition in wild rats and two widely used laboratory rat strains; Long-Evans hooded (LE) and Sprague-Dawley (SD). Based on previous work by Kruska (2005) and others (Ebinger, 1995; Ebneter, 2016; Kihslinger &

Nevitt, 2006), we predict that the cerebellar morphology and cellular composition will differ between wild and domestic rats. Specifically, we predict that the CB will be relatively smaller in laboratory strains, and that this will reflect fewer neurons. To test our predictions, we assessed the effects of domestication on several aspects of cerebellar morphology, including: cerebellar mass, foliation (i.e., degree of folding) and Purkinje cell soma size. Additionally, we estimated the number of Purkinje cells using unbiased stereology and the remaining neuronal population using the isotropic fractionator (IF) method.

Purkinje cells are inhibitory (GABAergic) neurons that constitute the sole outflow of information from the cerebellar cortex. Purkinje cells send corrective feedback about ongoing muscle movement to cerebellar nuclei to ensure smooth and accurate movements. In addition to motor coordination, Purkinje cells are thought to play a key role in motor learning (Gilbert & Thach, 1977; Ito, 2002). For example, the activity of Purkinje cells produces plastic changes within the cerebellar cortex as well as in vestibular nuclei (Gilbert & Thach, 1977). Given the fundamental importance of Purkinje cells in a wide variety of motor functions, investigating Purkinje cell populations may be a critical to understanding how domestication affects cerebellar function.

MATERIALS AND METHODS

Animals

We examined a total of 67 female Norway rats *(Rattus norvegicus).* This sample included wild rats (n = 23), as well as two laboratory strains; SD (n = 22) and LE (n = 22) rats. Twenty LE rats were purchased from a commercial supplier (Charles River Laboratories, St. Constant,

Quebec) at 24 days of age and were housed in the Canadian Centre for Behavioural Neuroscience (CCBN) animal facility until approximately 70-80 days of age (University of Lethbridge, Lethbridge, Alberta). Additionally, our sample included two older LE rats which were purchased from the same supplier at 74 days of age and housed in the CCBN animal facility until 131 days of age. Pairs of LE rats were kept in standard polyethylene cages (46 cm x 25 cm x 20 cm) with corncob bedding and *ad libitum* access to food and water. The vivarium was kept at a constant temperature (21-23°C) with a 12:12 hour light-dark cycle. Animals were sacrificed via sodium pentobarbital overdose (300 ml/kg, given i.p.) and were transcardially perfused with saline (0.9%, NaCl) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS, pH = 7.4). Brains were immediately dissected from the skull, weighed, transferred into fresh 4% PFA and stored at 4°C for about 1-2 weeks. LE rats were cared for in accordance with the regulations outlines by the Canadian Council for Animal Care (CCAC).

Both SD and wild-type rats were obtained from the Mossakowski Medical Research Center at the Polish Academy of Sciences (Warsaw, Poland). The wild strain we used was derived from five independent wild colonies around the Warsaw area, but our specimens were born at the Wild Warsaw Captive Pisula-Stryjek (WWCPS) breeding colony (Stryjek & Pisula, 2008). The animals used for this study were from the F3 generation, which allows sufficient time to adapt to being raised in laboratory conditions, but not long enough for the potential early effects of domestication to begin to change the animals' phenotypes (Himmler et al., 2013; Stryjek et al., 2013). Wild rats were born at the wild breeding colony where they were housed until 82 days of age. SD rats were purchased from a commercial European supplier at 22 days of age and were housed at the Mossakowski Medical Research Center until 82 days of age. Wild and SD rats were kept in same-strain pairs in Eurostandard type IV cages (61 cm x 43.5 cm x

21.5 cm) with softwood granule bedding. As in Lethbridge, all animals were kept on a 12:12 hour light-dark cycle at a constant temperature of 21-23°C with *ad libitum* access to food and water. Adult SD and wild rats were sacrificed using isoflurane as preliminary anesthesia, followed by ketamine (Bioketan) and medetomidine. Under deep anesthesia, animals were transcardially perfused with 0.9% saline and formaldehyde. Brains were immediately dissected from the skull, weighed, placed in formaldehyde and shipped to the University of Lethbridge. The SD and wild rats were cared for according to regulations outlined by the Polish Minister of Agriculture and Rural Development on laboratory animal care (March 10, 2006).

Cerebellar Histology

After fixation, about half of the brains (11 wild, 10 LE and 10 SD) were histologically processed. To dissect cerebella from the remaining brain tissue, we carefully cut through the cerebellar peduncles. The cerebella were then cryoprotected in 30% sucrose (in 0.1 M PBS, pH = 7.4), embedded in gelatin, post-fixed in 20% sucrose (in 4% PFA) for a minimum of 2-3 hours and switched into 20% sucrose (in 0.1 M PBS, pH = 7.4) for at least 24 hours. A freezing microtome was used to section cerebella in the sagittal plane at 40 μm thickness. Every second section was mounted onto a gelatinized slide, stained for Nissl substance with thionin, dehydrated in a series of graded alcohols, cleared with Hemo-De and coverslipped with Permount.

Quantification of foliation, Purkinje cell numbers & soma size

We used a 20x lens on Olympus VS120 digital slide scanner to create 'virtual slides' of every 7th mounted CB section (i.e., every 14th section throughout the CB). The degree of

cerebellar foliation (the foliation index) was quantified from these virtual slides (Iwaniuk et al., 2006). The polyline tool in VS-ASW FL Olympus Software allowed us to trace the length of the Purkinje cell layer along the cerebellar folia in the rostral-caudal plane, as well as to measure the length of the outer perimeter of the Purkinje layer (Figure 2.1). The foliation index for each specimen was calculated by dividing the sum of the Purkinje cell layer by the sum of the outer Purkinje perimeter throughout the medio-lateral extent of the CB (Iwaniuk et al., 2006).

To quantify Purkinje soma size, we scanned three midsagittal sections per specimen using the 40x lens of the slide scanner. For each specimen, the area of 45-99 Purkinje somas (at least 15 per virtual slide) was quantified with the interpolated polygon tool. Purkinje somas were easily identifiable based on their location within the unicellular Purkinje layer as well as their prominent size (Figure 2.2). Purkinje cells were only selected if they appeared intact, which we identified as having large 'teardrop' shaped soma, prominent nucleus and the appearance of extending dendrites.

The number of Purkinje cells in each of the sectioned cerebella was quantified using unbiased stereology. Every 11th mounted section was sampled using the 40x lens on a Zeiss Axiocam Imager MRm microscope (Carl Zeiss, MicroImaging GmBH, Germany) and the optical fractionator in StereoInvestigator (Microbrightfield, Williston, VT). The optical fractionator method involves the systematic random sampling from a known fraction of a neuroanatomical structure, and in our case, we traced around the entire area of the CB sections (Gunderson et al., 1988). The counting frame size was 70 x 70 μm and the grid size was 300 x 300 μm. Again, Purkinje cells were identified based on their location with the Purkinje cell layer, their large cell body, triangular (or 'teardrop') shape, as well as the visibility of the nucleus and extending axons. Purkinje cells were counted if somas appeared entirely within the counting frame (or

overlapped the inclusion lines) but did not overlap the exclusion lines. Coefficients of error (Gunderson, m=1) for all cell counts were ≤ 0.05.

Isotropic Fractionation

With the remaining rat brains (12 wild, 12 LE and 12 SD), we used the IF method to estimate the cellular composition of the CB (Herculano-Houzel & Lent, 2005). Briefly, the IF is a non-stereological means of estimating the total number of cells, neurons, and non-neuronal cells in the entire brain, or any dissectible sub-region, such as the CB (Herculano-Houzel et al., 2015). This technique is faster compared to stereological methods and provides accurate estimates of neuron numbers, particularly for brain regions that have exceptionally high neuronal density of small cells, such as the granule cells in the cerebellar cortex (Herculano-Houzel et al., 2015).

Following tissue fixation, the cerebellar peduncles were cut to remove the CB from the remaining tissue. Cerebella were then cryoprotected in a solution of 30% sucrose (in 0.1 M PBS) until they had sunk, after which they were transferred into an antifreeze solution (30% glycerol, 30% ethylene glycol in 0.024 M phosphate buffer) and stored at 4°C (Herculano-Houzel & Lent, 2005). Once the tissue has sunk in antifreeze it was stored at -20°C until further processing.

Cerebella were then removed from antifreeze, dried off with disposable wipes and placed in tissue grinders which were filled with enough standard dissociation solution (40.4 mM sodium citrate and 1% Triton X-100) to immerse the tissue (Herculano-Houzel & Lent, 2005). Dissociation solution was used to dissolve cellular membranes while leaving nuclear membranes intact, thus the tissue was mechanically ground into a homogenous solution of suspended nuclei (Herculano-Houzel & Lent, 2005). The homogenate solution was then stained with a 1:20

dilution of the DNA-specific fluorescent label DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) which labels all cellular nuclei. The resulting solution was agitated to create a homogenous mixture of DAPI-labelled suspended nuclei, and the density of nuclei was determined using fluorescent microscopy. Several 10μL aliquots of homogenate were loaded into a Neubauer chamber (hemocytometer; mm^2 area, 0.1 mm depth), coverslipped, and counted using the 40x lens of a Zeiss Axioskop 2 MOT microscope (Carl Zeiss). At least four aliquots were counted per sample, but if the coefficient of variation (CV) between counts was greater than 15%, additional aliquots were quantified until the CV was below 15% (Herculano-Houzel et al., 2015). The total number of cells in the CB was calculated by multiplying the average nuclear density by the final volume of the homogenate solution.

Neuron counts were estimated by labeling a sample from each homogenate solution with the neuronal nuclear antigen NeuN, which labels the nucleus of most neurons (Herculano-Houzel & Lent, 2005). A 1 ml sample of homogenate was loaded into a 1.5 ml Eppendorf tube, centrifuged, and the supernatant was decanted. The pellet containing cellular nuclei was resuspended in 0.1M PBS (pH = 7.4), centrifuged, and the supernatant was decanted; this step was repeated so that the nuclei were washed with PBS at least three times. To prevent nonspecific binding, the nuclei were resuspended in 0.5 ml blocking buffer (10% Normal Goat Serum in 0.1M PBS) and incubated for one hour at room temperature. After the blocking step, the solution was centrifuged, and the supernatant was decanted. The nuclei were then immunoreacted with an Alexafluor-488 conjugated rabbit polyclonal anti-NeuN primary antibody (Millipore ABN 78C3) in a 1:100 dilution with the blocking buffer. The pellet was resuspended in blocking buffer, and the resulting solution was incubated in the dark at 4°C for 48

hours. After incubation, samples were rinsed with 0.1M PBS and stored in the dark at 4°C until they could be analyzed.

For neuron numbers, we used fluorescence microscopy to estimate the proportion of nuclei that were doubled-labeled by both NeuN and DAPI. Approximately 200-300 µL of homogenate was pipetted onto a glass slide, coverslipped, and visualized with the 40x lens of a Zeiss Axiocam Imager MRm microscope (Carl Zeiss, MicroImaging GmBH, Germany). At least 500 DAPI labeled nuclei were counted per sample, and for each DAPI-labeled nucleus, it was determined whether it was also labeled with NeuN. The proportion of double labeled nuclei was multiplied by the total number of cells (determined with the hemocytometer) to yield the number of neurons in the homogenate (Herculano-Houzel & Lent, 2005). We then subtracted the number of neurons from the total number of cells to calculate the number of non-neuronal cells. Neuron and non-neuronal cell numbers were then divided by cerebellar mass to determine cellular densities. Finally, the non-neuronal cell to neuron ratio was calculated by dividing the number of non-neuronal cells by the number of neurons.

Statistical Analysis

We used one-way analysis of variance (ANOVAs) to test for differences among strains for all the following variables: body mass, brain mass, CB mass, CB foliation, Purkinje cell counts, Purkinje soma sizes, total cell counts, neuron counts, non-neuronal cell counts, neuronal density, non-neuronal cell density and the non-neuronal cell to neuron ratio (Table 2.2). In general, brain mass is dependent on body mass (Jerison, 1973). Because there was a significant difference in body masses across strains (see below), we used analyses of covariance (ANCOVAs) to test for differences in relative measurements using brain mass as a covariate

(Table 2.3). In the analysis of relative CB size, we subtracted CB mass from brain mass (Deacon 1990). For all ANOVAs and ANCOVAs, any significant effects were then further tested with Tukey Honest Significant Difference (HSD) post-hoc tests. Prior to all statistical testing, raw data was log-transformed to improve normality as well as to be comparable with previous research (Bandeira et al., 2009; Herculano-Houzel et al., 2015).

RESULTS

Absolute Measurements

Body mass varied significantly across strains (Table 2.2); post hoc comparisons showed that LE rats were significantly larger than SD rats, which were significantly larger than wild rats. LE rats also had smaller brains and cerebella than SD and wild rats (Figure 2.4). Compared to wild rats, both domestic laboratory strains had significantly smaller foliation indexes in the CB.

In terms of the cellular composition of the CB, LE rats had fewer cells and fewer neurons than both SD and wild rats. Yet, there was no significant difference across strains in the number of non-neuronal cells or in the number of Purkinje neurons. Despite similar Purkinje cell numbers, LE rats had smaller Purkinje somas than the other two strains. Finally, the neuron and non-neuronal cell densities, as well as the ratio of non-neuronal cells to neurons did not vary significantly across strains.

Relative Measurements

Brain mass varied significantly with both strain and body mass, but no strain-body mass interaction effect was detected. Post-hoc analyses indicated that LE rats had relatively smaller brains than the other two strains (Figure 2.3). CB mass varied by brain mass, but not by strain,

and no interaction was detected. CB foliation varied by strain but not brain mass, and there was a significant strain-brain mass interaction effect. Thus, the strains varied in the degree to which their CB foliation changed with brain size. Post-hoc tests revealed that wild rats had a significantly higher CB foliation index than LE rats (Figure 2.5).

In terms of the cellular composition of the CB, the total number of cells increased with brain size, but did not vary by strain and the interaction term was not significant (Table 2.3). Unlike the total number of cells, the number of neurons did vary by strain; LE rats had relatively fewer CB neurons compared to wild rats. The number of CB neurons also increased with brain mass, and we did not detect a significant interaction effect. Although there was a trend, neuron density did not vary significantly by strain or by brain mass, and no significant interaction effect was detected.

As with the neuron counts, there were significant effects of both strain and brain mass on the number of non-neuronal cells and no significant interaction was found. Specifically, LE rats had relatively more non-neuronal cells than wild rats. Likewise, LE rats had a higher non-neuronal cell density than wild rats. However, there was no significant effect of brain mass or of a strain-brain interaction on non-neuronal cell density. LE rats also had more non-neuronal cells per neuron than SD and wild rats. The non-neuronal cell to neuron ratio was not affected, however, by brain mass or by any strain-brain mass interaction.

Finally, there was no significant effect of strain, brain mass or their interaction on the number of Purkinje cells. Average Purkinje soma size, however, was significantly smaller in LE rats than in the other two strains, and there was no effect of brain size nor any strain-brain mass interaction.

DISCUSSION

The main finding of the present study is that in both terms of morphology and cellular composition, the CB of adult female LE rats is different from SD and wild rats. All strains had relatively similar sized cerebella in relative terms, yet LE rats had cerebella with significantly less foliation compared to the other two strains. Compared to wild rats, LE rats also had relatively fewer neurons, more non-neuronal cells and a greater ratio of non-neuronal cells to neurons in their CB. Moreover, all three strains had similar numbers of Purkinje cells, but LE rats had significantly smaller Purkinje somas than SD and wild rats. Some differences between the two laboratory strains were also detected, but the differences between the LE and wild rats were far greater in magnitude. Overall, we did not find consistent differences between the cerebella of domesticated laboratory and wild rats. Instead, our findings suggest that cerebellar anatomy varies significantly across strains, with LE rats being different to both SD and wild rats.

One limitation of the current study is that the housing conditions between institutions differed; SD and wild rats were kept in cages with more than twice the floor space than LE rats. More specifically, the LE rats were housed in 46 cm x 25 cm x 20 cm cages whereas the SD and wild rats were housed in 61 cm x 43.5 cm x 21.5 cm cages. This difference in housing conditions was compounded by the fact that LE rats had the largest body mass of all three strains, resulting in even less space per unit body size compared to SD and wild rats. Thus, it is possible that these differences in housing conditions resulted in LE rats having different motor experiences and/or physical exercise opportunities compared to the other two strains.

Previous behavioural studies on play and acrobatics of LE and SD rats showed that strain differences were the same whether they were housed in similar or dissimilar cages (Himmler, Lewis, et al., 2014). That is, the behaviour is consistently different across these rearing

conditions. Since it was the behavioural differences that led to the study and the predicted patterns, then if dissimilar caging leads to same behaviour but differences in brain, then it cannot be argued that behavioural changes are reflected in brain changes that reflect on those functions (see Introduction). Most critically, SD and wild rats reared in the same cage types are the most divergent in acrobatic behaviour, yet they showed the least differences in CB.

There are multiple lines of evidence that suggest environmental enrichment and motor experience can alter morphology and cellular composition in the CB. In steelhead fish (*Oncorhynchus mykiss*), for example, enrichment of the captive environment produced significantly larger relative cerebellar volumes as well as associated changes in motor activity (Kihslinger & Nevitt, 2006). Moreover, rats reared in enriched conditions with diverse sensory and motor experiences have Purkinje cells with more spines than those reared in standard laboratory conditions (Greenough et al., 1986). Exercise also promotes Purkinje cell survival (Larsen et al., 2000), increases synaptogenesis in Purkinje cells (Black, 1990), and increases synaptic activity (Vissing et al., 1996), while reducing the dendritic loss associated with ageing (Greenough et al., 1986). Furthermore, rats who exercise engage in more spontaneous motor activity have more Purkinje cells ($4.1x10^5$) than sedentary ($3.7x10^5$) rats (Larsen et al., 2000). Likewise, various forms of motor activity promote angiogenesis in the cerebellar cortex (Black, 1990). Thus, rearing environments can produce changes in the CB, which may be mistakenly attributed to selective breeding (Kihslinger & Nevitt, 2006).

Regardless of whether the differences between LE rats and the other two strains are due to domestication, housing or a combination of the two, our results still have important implications for understanding the effects of domestication on brain size and cerebellar anatomy. For example, despite having the largest bodies of all three strains, LE rats had the smallest

brains. This reduction in relative brain size is consistent with previous reports of reduced relative brain size in other domesticates (Kruska, 2005), including in albino Wistar rats compared with wild rats (Kruska, 1975). In contrast, the relative brain size of SD rats was similar to wild rats. To our knowledge this is the first report of a domestic laboratory rat strain that does not differ from wild rats in relative brain size. Our finding that all three strains had similar relative CB sizes was also incongruent with prior investigations that concluded domestic (Wistar) rats had relatively smaller cerebella compared to wild rats (Kruska, 1975, 2005). However, our results are somewhat consistent with research indicating that domestic strains (Wistar and LE) have similar sized cerebella (Yanai, 1979). Comparing our results directly to previous findings, however, is complicated by differences in sex and age. Whereas we strictly controlled for sex and age to limit the potentially confounding effects of both on our quantitative measurements, previous studies mixed sex and strain of laboratory rats (primarily Wistar rats, but also some SD) to wild rats of mixed sex, age and body size (Ebinger, 1972; Kruska, 1975). Thus, the differences among studies could reflect variations in sex, age and other variables and not domestication alone.

Prior investigations found that Wistar rats have a less foliated cerebella compared to SD rats (Heinsen & Heinsen, 1984). Here, we did not observe a difference in the relative degree of cerebellar foliation across domestic strains. Instead we found that LE rats had relatively less folded cerebella than wild rats. This reduction in foliation paralleled a similar decrease in Purkinje cell soma size in LE rats. Purkinje cells send inhibitory (GABAergic) projections to cerebellar nuclei and are the only neurons to send output from the cerebellar cortex. As such, Purkinje cells are critical for modulating motor activity (Albus, 1971) as well as motor learning (Gilbert & Thach, 1977; Ito, 2002), so changes in Purkinje soma size may be indicative of the functional capacity of the CB for motor behaviours. Indeed, differences in soma size can reflect

differences in cellular transcription, metabolism and/or conduction velocity (Muma et al., 1991). Because cerebellar foliation is associated with a reduction in the unicellular Purkinje layer (Heinsen & Heinsen, 1984), we suggest that the less folded cerebella of LE rats arise from a reduction in Purkinje soma size.

Although Yanai (1979) reported that LE rats have more Purkinje cells with greater arborization compared to other domestic (Wistar) strains, we found no differences in the number of Purkinje cells across domestic and wild-type rats. On average, our specimens had 5.59-5.81 x 10^5 Purkinje cells (Table 2.1), which falls within the range of previous Purkinje cell numbers in laboratory rats 2.1x 10^5 to 6.1x 10^5 (Harvey & Napper, 1988; Hillman & Chen, 1981; Inukai, 1928; Korbo et al., 1993; Mayhew, 1991). The variation across studies can be attributed to a range of factors including differences in strain, age, sex, histological techniques, sampling regimes, and stereological parameters and is typical of reported intraspecific variation in neuron counts (e.g., Herculano-Houzel et al., 2015; Sherwood, 2005)

In contrast to the number of Purkinje cells, the total number of CB neurons differed across strains, as estimated with the IF method. Specifically, LE rats had approximately 27% fewer cerebellar neurons than both SD and wild rats. NeuN does not label Purkinje cells (Herculano-Houzel et al., 2015; Mullen et al., 1992), but these comprise a relatively small proportion of the total number of neurons in the CB (< 1% based in the current study). Cerebellar granule cells are the most numerous neurons in the central nervous system (Bilimoria & Bonni, 2008) and therefore, the vast majority of our neuronal estimates likely reflects granule cell populations rather than basket, stellate, Golgi and other cerebellar neuron types. Our neuronal estimates are highly comparable to what has been reported in the Wistar rat CB using the same technique (Bandeira et al., 2009; Herculano-Houzel et al., 2006). LE rats had relatively more

non-neuronal cells for their brain size than wild rats, and the estimated number of non-neuronal cells across our strains was comparable with data in Wistar rats (Bandeira et al., 2009; Herculano-Houzel et al., 2006). The combination of fewer neurons and more non-neuronal cells lead to a higher ratio of non-neuronal cells to neurons in LE rats, but these values were within the range of previous reports (Bandeira et al., 2009; Ghandour, 1980; Herculano-Houzel et al., 2006).

At present, we can only speculate as to the functional changes associated with such strain-specific variation in cerebellar composition and morphology. However, there are some differences in the motor behaviour of domestic and wild rats. In general, laboratory rats are more sedentary and engage in less spontaneous motor activity compared to wild rats (Stryjek & Pisula, 2008). Wild rats are also better at digging than domestic (Wistar) rats, and create more complex tunnel systems (Stryjek et al., 2012). Similarly, wild rats do not require early climbing experience to be proficient adult climbers, whereas domestic (LE) rats only climb if given early climbing experience in development (Huck & Price, 1976). In other words, LE rats reared in standard laboratory cages do not climb at all, whereas wild rats reared in laboratory cages do climb, but at a reduced rate than if reared in an enriched environment (Huck & Price, 1976). Similarly, wild rats will jump whether reared in an enriched or standard laboratory environment, whereas LE rats do not jump under either condition (Huck & Price, 1975). The diminishment of these naturalistic behaviours seen in domestic rats may reflect a variety of factors; such as their generally reduced activity level, increased fat composition or larger body mass (Keeler, 1947; Price, 1999). Rough-and-tumble play fighting is another motor behaviour affected by domestication; laboratory rat strains (LE, SD, Brown-Norway and Wistar) engage in a higher frequency of playful attacks than their wild counterparts but are generally less active, and have

reduced acrobatic abilities (Himmler, Modlinska, et al., 2014; Himmler et al., 2013). There are also differences amongst laboratory strains in their preferred playful defense tactics maneuvers; LE rats, for example, use much less evasive defense maneuvers compared to other strains, whereas SD rats prefer evasive defense over facing defenses (Himmler, Modlinska, et al., 2014). In addition to qualitative differences in play behaviour, laboratory strains also differ across other motor abilities; for instance, LE rats tend to perform better at motor tasks than Wistar rats (Yanai, 1979) and are better at skilled reaching than both SD and Fischer-344 rats (VandenBerg et al., 2002; Whishaw et al., 2003). Whether these behavioural differences lead to (or are a consequence of) underlying changes in cerebellar anatomy, however, remains unknown.

In conclusion, the present investigation indicates that domestication has strain-specific effects on cerebellar morphology and cellular composition. Since not all strains are equal, considering such anatomical differences may be relevant to selecting optimal animal models in scientific research, including in behavioural neuroscience. The CB, however, is not a homogeneous structure; for example, there are regional differences in sizes and densities of both Purkinje and granule cells (Cerminara et al., 2015). Thus, a potential future direction would be to characterize regional differences (which may correspond to functionally diverse regions) across strains.

Table 2.1– Descriptive statistics for 12 measurements of mass and cerebellar anatomy across three rat strains: Long-Evans, Sprague-Dawley and wild. The three mass measurements were body mass (grams), brain mass (grams) and cerebellum (CB) mass (milligrams). The nine measurements of cerebellar anatomy were foliation index, Purkinje soma size (micrometers squared), number of Purkinje cells, total number of cells, number of neurons, number of non-neuronal cells, neuronal density (neurons per milligram), non-neuronal cell density (non-neuronal cells per milligram) and the number of non-neuronal cells per neuron. All values are reported as strain means (M) ± standard deviation (Std) for (n) number of individuals analyzed.

Measurements	Long-Evans			Sprague-Dawley			Wild		
	n	M	Std	n	M	Std	n	M	Std
Body mass (g)	18	276	75.5	22	229	14.8	20	165	17.9
Brain mass (g)	22	1.84	0.153	22	2.07	0.0708	23	2.03	162
CB mass (mg)	12	231	25.4	12	292	12.7	12	293	22.4
Foliation index	10	2.99	0.103	10	3.05	0.061	11	3.15	0.0831
Purkinje soma size (μm^2)	10	244	18.4	10	328	22.4	10	336	15.9
Purkinje cells	10	5.62×10^5	4.73×10^4	8	5.59×10^5	1.74×10^4	11	5.81×10^5	5.26×10^4
Total cells	12	1.51×10^8	2.19×10^7	12	2.01×10^8	1.39×10^7	12	1.98×10^8	1.58×10^7
Neurons	12	1.21×10^8	1.47×10^7	12	1.64×10^8	1.18×10^7	12	1.66×10^8	1.36×10^7
Non-neuronal cells	12	3.00×10^7	9.81×10^6	12	3.69×10^7	6.37×10^6	12	3.19×10^7	5.82×10^6
Neuron density	12	5.27×10^5	5.90×10^4	12	5.62×10^5	3.68×10^4	12	5.65×10^5	3.06×10^4
Non-neuronal cell density	12	1.28×10^5	3.44×10^4	12	1.26×10^5	1.78×10^4	12	1.09×10^5	1.86×10^4
Non-neuronal cell : neuron	12	0.246	0.0692	12	0.226	0.0405	12	0.193	0.0359

Table 2.2 – Results of one-way analyses of variance (ANOVAs) comparing 12 absolute measurements of mass and cerebellar anatomy across three rat strains; Long-Evans (LE), Sprague-Dawley (SD) and wild. In addition to analyzing body, brain and cerebellum (CB) mass, we also tested for differences in the following CB measurements: foliation index, Purkinje soma size (micrometers squared), number of Purkinje cells, total number of cells, number of neurons, number of non-neuronal cells, neuronal density (neurons per milligram), non-neuronal cell density (non-neuronal cells per milligram) and the number of non-neuronal cells per neuron. ANOVA results are reported as F-ratios (F), degrees of freedom (df) and p-values (p), where (*) denotes a significant difference between strains. For all significant p-values ($p < 0.05$) effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. In the post-hoc column, significant differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	F	df	p	Tukey HSD
Body mass	46.43	2, 57	**<0.01***	LE > SD > Wild
Brain mass	16.82	2, 64	**<0.01***	SD, Wild > LE
CB mass	32.56	2, 33	<0.01*	SD, Wild > LE
Foliation index	9.59	2, 28	**<0.01***	Wild > LE, SD
Purkinje soma size	79.30	2, 27	**<0.01***	Wild, SD > LE
Purkinje cells	0.75	2, 26	0.48	
Total cells	28.01	2, 33	**<0.01***	SD, Wild > LE
Neurons	41.49	2, 33	**<0.01***	Wild, SD > LE
Non-neuronal cells	2.96	2, 33	0.07	
Neuron density	2.73	2, 33	0.08	
Non-neuronal cell density	2.02	2, 33	0.15	
Non-neuronal cell : neuron	3.02	2, 33	0.06	

Table 2.3 – Results of one-way analysis of covariance (ANCOVAs) of strain, brain mass (co-variate) and their interaction on ten measurements of the cerebellum (CB): mass, foliation index, Purkinje soma size, number of Purkinje cells, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. The three strains examined were Long-Evans (LE), Sprague-Dawley (SD), and wild rats. ANCOVA results are reported as F-ratios (F), degrees of freedom (df) and p-values (p), where (*) denotes a significant effect. For all significant p-values of strain (i.e., p-value < 0.05) effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	Strain				Brain mass			Interaction		
	F	df	p	Tukey HSD	F	df	p	F	df	p
CB mass	0.63	2, 30	**0.54**		42.67	1, 30	**<0.01***	0.51	2, 30	0.61
Foliation index	8.88	2, 25	**<0.01***	Wild > LE	2.86	1, 25	0.10	5.34	2, 25	**0.01***
Purkinje soma size	61.86	2, 24	**<0.01***	Wild, SD > LE	0.49	1, 24	0.49	0.73	2, 24	0.49
Purkinje cells	0.49	2, 23	0.62		0.29	1, 23	0.59	1.05	2, 23	0.37
Total cells	0.82	2, 30	0.45		12.06	1, 30	**<0.01***	0.17	2, 30	0.85
Neurons	4.68	2, 30	**0.02***	Wild > LE	7.55	1, 30	**0.01***	0.37	2, 30	0.69
Non-neuronal cells	3.50	2, 30	**0.04***	LE > Wild	7.16	1, 30	**0.01***	2.12	2, 30	0.14
Neuron density	3.26	2, 30	0.052		0.30	1, 30	0.59	0.77	2, 30	0.47
Non-neuronal cell density	5.32	2, 30	**0.01***	LE > Wild	2.31	1, 30	0.14	2.11	2, 30	0.14
Non-neuronal cell : neuron	9.73	2, 30	**<0.01***	LE > Wild, SD	3.52	1,30	0.07	3.52	2,30	**0.04***

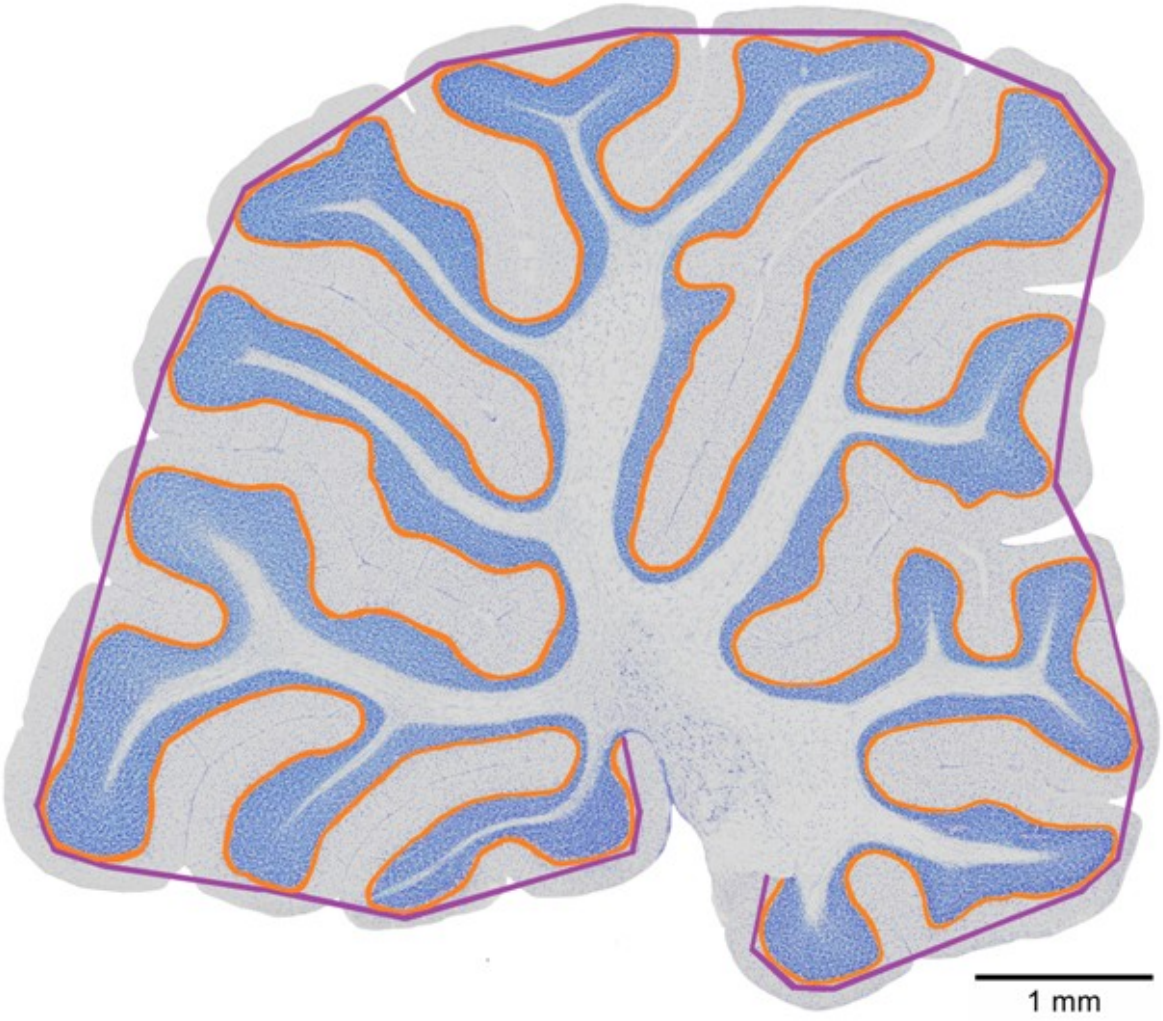

Figure 2.1– Method to quantify the foliation index from 'virtual slides'. Virtual slides were created using the 20x lens of an Olympus slide scanner to scan sagittal, nissl-stained cerebellum (CB) sections. We measured the length of the inner contour of the Purkinje cell layer (orange) in the rostral-caudal plane as well as the length of outer contour perimeter of the Purkinje cell layer (purple). Foliation index for each specimen was calculated by dividing the sum of the inner Purkinje layer (orange) by the sum of the outer Purkinje perimeter throughout the medio-lateral extent of the CB (Iwaniuk et al., 2006). Scale bar = 1 millimeter.

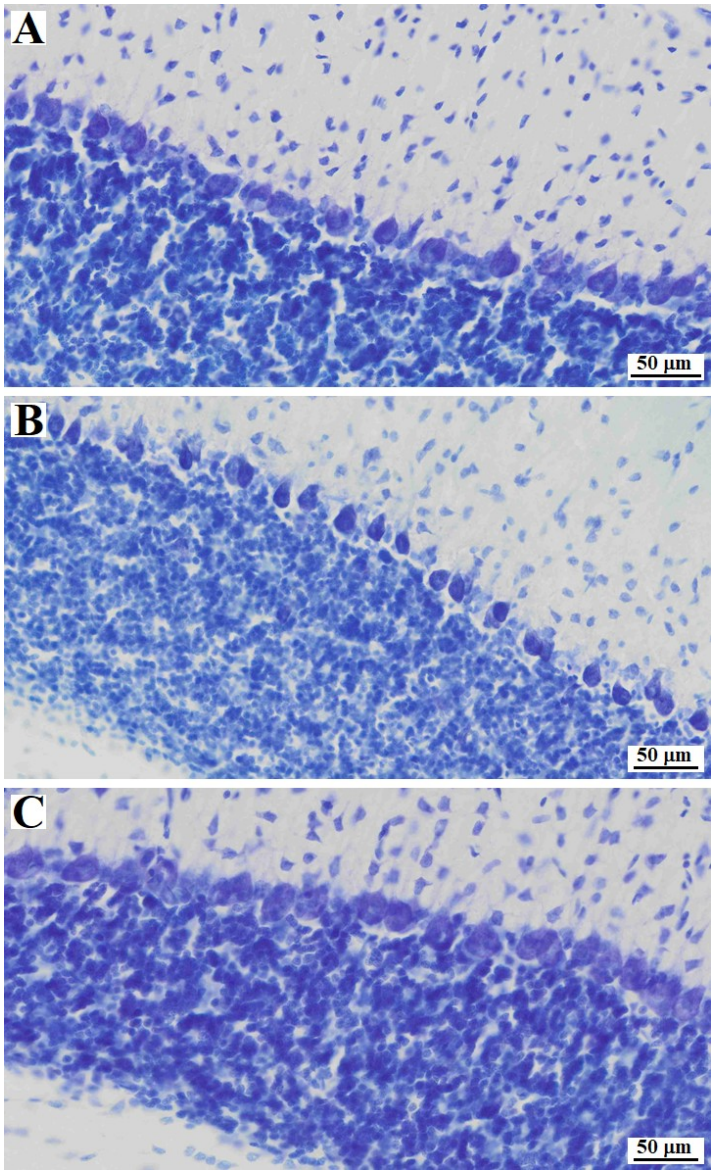

Figure 2.2 – Photomicrograph of the Purkinje cell layer in a (a) wild rat, (b) Long-Evans (LE) rat and (c) Sprague-Dawley (SD) rat. Images were created using the 40x lens of an Olympus slide scanner to scan sagittal, nissl-stained cerebellum (CB) sections. In the cerebellar cortex, the Purkinje cell layer is easily identified by the prominent, unimolecular arrangement of large, triangular (or teardrop) shaped Purkinje somas, as well as by its location in between the densely packed granule layer and the sparsely populated molecular layer. We found that wild and SD rats had significantly larger Purkinje cell somas compared to LE rats. Scale bar = 50 µm.

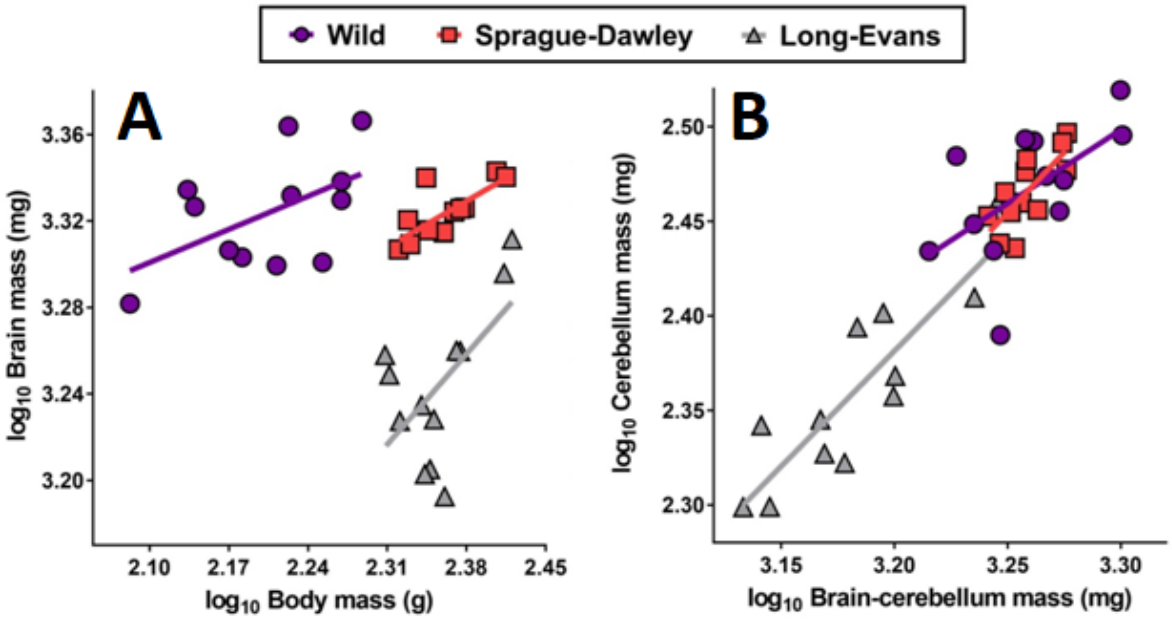

Figure 2.3 – Double log scatterplots showing the intraspecific relation of (a) brain mass (grams) to body mass (grams), and (b) cerebellum mass (milligrams) to brain mass minus CB mass (milligrams). As per the legend, wild rats = purple circles, Sprague-Dawley rats = orange triangles and Long-Evans rats = grey triangles. The regression line for each group represents the relationship between the x and y variables. We found that Spargue-Dawley and wild rats had larger brains than Long-Evans rats, relative to body mass. However, the size of the cerebellum relative to brain mass was similar across all three strains.

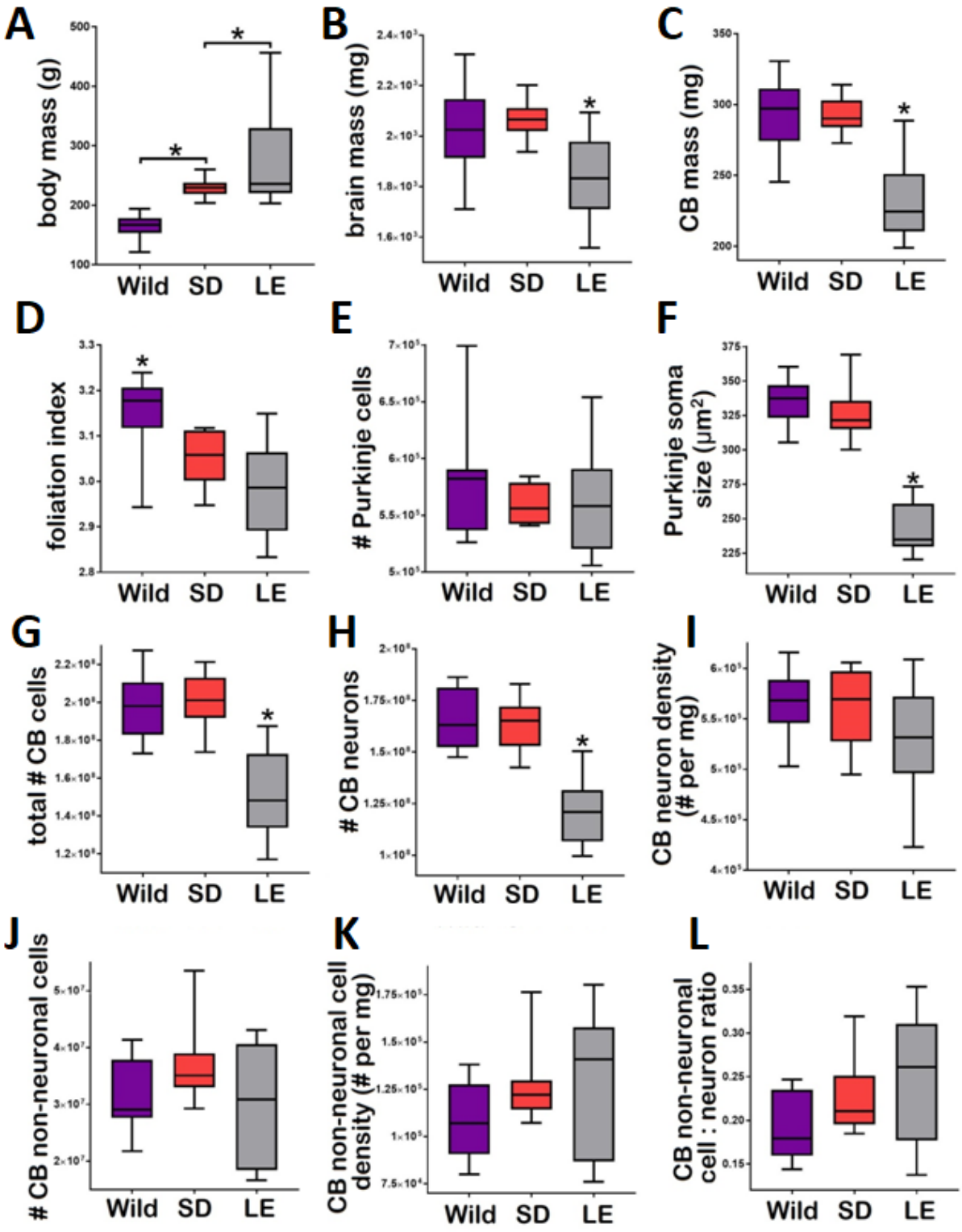

Figure 2.4 – Boxplots of 12 absolute measurements of mass and cerebellar anatomy across three rat strains; wild (purple), Sprague-Dawley (SD = orange) and Long-Evans (LE = grey). Mass measurements were (a) body mass (grams), (b) brain mass (milligrams) and (c) cerebellum (CB)

mass (milligrams). CB measurements were (d) foliation index, (e) number of Purkinje cells, (f) Purkinje soma size (micrometers squared), (g) total number of cells, (h) number of neurons, (i) number of non-neuronal cells, (j) neuronal density (neurons per milligram), (k) non-neuronal cell density (number of non-neuronal cells per milligram) and the (l) number of non-neuronal cells per neuron. Horizontal lines within the box represent the mean for each group and error bars represent the minimum and maximum values within each group. *denotes a significant difference between indicated strains. We found that LE rats had smaller brains and cerebella than SD and wild rats, despite having the largest body size of all three strains. In the cerebellum, we found that all three strains had similar numbers of Purkinje cells, but LE rats had smaller Purkinje somas than SD and wild rats. Furthermore, the other two strains also had more cells and neurons in the CB than LE rats.

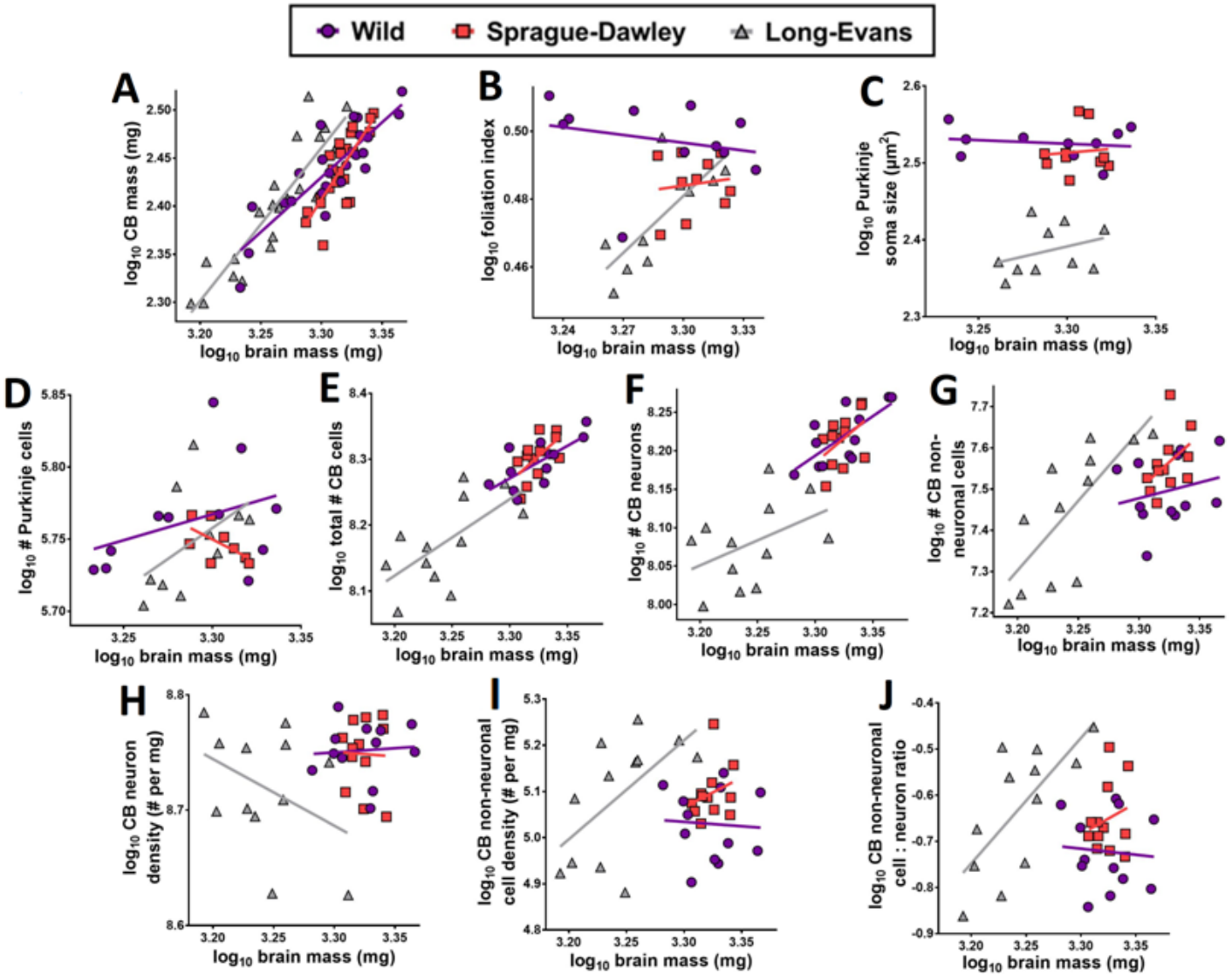

Figure 2.5 – Double log scatterplots showing the intraspecific relation of nine cerebellum (CB) measurements to brain mass (milligrams), including: (a) CB mass (milligrams), (b) foliation index, (c) Purkinje soma size (micrometers squared), (d) number of

Purkinje cells, (e) total number of cells, (f) number of neurons, (g) number of non-neuronal cells, (h) neuronal density (neurons per milligram), (i) non-neuronal cell density (non-neuronal cells per milligram) and the (j) number of non-neuronal cells per neuron. As per the legend, wild rats = purple circles, Sprague-Dawley (SD) rats = orange triangles; Long-Evans (LE) rats = grey triangles. The regression line for each group represents the relationship between each CB measurement and brain mass. Relative to brain size, we found that LE rats had larger cerebella, but smaller Purkinje somas than wild and SD rats. Yet, all three strains had similar total cell and Purkinje cell numbers. Wild rats had more foliation and more neurons for their brain size than LE rats. On the other hand, LE rats had relatively more non-neuronal cells, a higher non-neuronal cell density and more non-neuronal cells per neuron than wild rats.

CHAPTER THREE: USING THE ISOTROPIC FRACTIONATOR TECHNIQUE TO ASSESS THE EFFECTS OF DOMESTICATION ON NEURONAL AND NON-NEURONAL CELL NUMBERS IN THE RAT *(RATTUS NORVEGICUS)*

INTRODUCTION

Domestication is the evolutionary process by which populations of wild animals become adapted to living in captivity amongst humans. In domestication, humans often employ selective breeding programs to increase the frequency of certain desirable traits in animals, thus exerting a high degree of control over their reproduction and survival (Kruska, 1988). Over time, the unique selection pressures of the captive environment and selective breeding regimes have caused domesticates to phenotypically diverge from their wild counterparts, in both morphology and behaviour (Francis, 2015; Kruska, 2005).

Mammalian species have been domesticated for diverse purposes, including as sources of food, labour, companionship or for other resources (e.g., fur, leather). Despite their diverse domestication histories and purposes, a commonality amongst domesticates is a predisposition to be docile and relatively non-aggressive towards humans (Francis, 2015). The process of selecting for tameness in mammals however, appears to produce pleiotropic affects across a range of seemingly unrelated traits (Francis, 2015; Kruska, 2005). Indeed, across a range of tamed mammals, domestication has inadvertently produced changes in body size, fur pigmentation (e.g., decreased pigmentation, increased piebald colouring), shortened limbs, shortened faces, floppy ears, curly tails, and has reduced organ sizes (e.g., heart, liver, adrenals, brain) (Albert et al., 2008; Francis, 2015; Kruska, 2005; Trut et al., 2009). In addition to these morphological changes, many behavioural differences also exist between domesticates and their wild ancestors.

Domesticates, for instance, tend to be less athletic, more tolerant of conspecifics, less neophobic in novel environments and less anxious than their wild counterparts (F. Blanchard, Blanchard, 1986; W. Blanchard, Lee, Blanchard, 1981; Francis, 2015).

One of the most prominent morphological features of domestication is that domesticates tend to have relatively smaller brains for their body size than wild animals (Kruska, 2005). In addition to reducing overall brain size, domestication also affects the sizes of specific brain regions and brain systems (Kruska, 2005). For instance, domestication generally leads to a reduction in the size of motor (e.g., cerebellum) and limbic structures (e.g., hippocampus) within the brain (Kruska, 2005). There are also reductions within the sensory systems of domestic mammals, as they tend to have smaller sensory organs (e.g., eyes, ears) than their wild ancestors, and these sensory organs may also contain fewer sensory receptors (Kruska, 2005). Such changes in the sensory organs of domesticates are paralleled by decreases in the sizes of brain regions dedicated to vision (e.g., striate cortex, superior colliculus), olfaction (e.g., olfactory bulbs) and audition (e.g., auditory cortex, medial geniculate nucleus) (Kruska, 2005). Interestingly, although the overall size of neural sensory systems tends to be diminished in domestication, brain regions involved in 'higher-level' sensory processing (such as the cerebral cortex) appear to undergo the largest size reduction compared to sub-cortical regions (Kruska, 2005).

Decreases in brain size and/or the proportional size of specific brain regions may indicate a reduction in the neural processing capacity of domesticates. Yet, most of the research on the neural effects of domestication has focused on volumetric changes, with little attention to the underlying cellular changes that are responsible for decreases in size. Brain regions primarily vary in size due to changes in either neuron size or number of

neurons, but there are no data for either of these measurements across wild and domesticated strains of the same species. Additionally, brain regions can also change in size in relation to vasculature, water content and neuropil (i.e., glial cell processes, microvascular, axons and dendrites), although none of these are frequently examined. As a first step towards addressing this knowledge gap, we used the isotropic fractionator (IF) method (Herculano-Houzel & Lent, 2005) to estimate neuron numbers in three brain regions; the cortex (CX), olfactory bulbs (OB) and 'rest of brain' (ROB) (for cerebellum see Chapter 2) in wild rats *(Rattus norvegicus)* and two domestic laboratory rat strains: Long-Evans hooded (LE) and Sprague-Dawley (SD). Based on previous work by Kruska (2005), we predicted that the size and cellular composition of the CX, OB and ROB would differ between wild and domestic rats. Specifically, we expected that the size of these regions will be relatively smaller in laboratory strains, and that this will reflect fewer neurons.

MATERIALS AND METHODS

Animals

We examined a total of 36 female Norway rats *(Rattus norvegicus).* This sample included 12 wild rats, as well as two laboratory strains: 12 SD and 12 LE. Ten LE rats were purchased from a commercial supplier (Charles River Laboratories, St. Constant, Quebec) at 24 days of age and were housed in the Canadian Centre for Behavioural Neuroscience (CCBN) animal facility (University of Lethbridge, Lethbridge, Alberta). Additionally, our sample of LE rats included two older adults which were purchased from the same supplier at 74 days of age and housed in the CCBN animal facility until 131

days of age. Pairs of LE rats were kept in standard polyethylene cages (46 cm x 25 cm x 20 cm) with corncob bedding and *ad libitum* access to food and water. The vivarium was kept at a constant temperature of 21-23°C with a 12:12 hour light-dark cycle. Animals were sacrificed via sodium pentobarbital overdose (300 ml/kg, i.p.) and transcardially perfused with saline (0.9%, NaCl) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline (PBS, pH = 7.4). Brains were immediately dissected from the skull, weighed, transferred into fresh 4% PFA and stored at 4°C for about 1-2 weeks. LE rats were cared for in accordance with the regulations outlines by the Canadian Council for Animal Care (CCAC).

Both SD and wild-type rats were obtained from the Mossakowski Medical Research Center at the Polish Academy of Sciences (Warsaw, Poland). The wild rat strain (i.e., the Wild Warsaw Captive Pisula-Stryjek – WWCPS strain) was derived from five independent wild colonies found around the Warsaw area. The subjects were captive born and derived from breeders within the first five generations of captivity (Stryjek & Pisula, 2008). Wild rats were born at the wild breeding colony where they were housed until 82 days of age. SD rats were purchased from a commercial European supplier at 22 days of age and were housed at the Mossakowski Medical Research Center until 82 days of age. Wild and SD rats were kept in same-strain pairs in Eurostandard type IV cages (61 cm x 43.5 cm x 21.5 cm) with softwood granule bedding. As in Lethbridge, all animals were kept on a 12:12 hour light-dark cycle at a constant temperature of 21-23°C, and had *ad libitum* access to food and water. Adult SD and wild rats (approximately 70-80 days of age) were sacrificed using isoflurane as preliminary anesthesia, followed by ketamine (Bioketan) and medetomidine. Under deep anesthesia, animals were transcardially perfused with saline (0.9%) and formaldehyde. Brains were immediately dissected from

the skull, weighed, placed in formaldehyde and shipped to the University of Lethbridge. SD and wild rats were cared for according to regulates outlined by the Polish Minister of Agriculture and Rural Development on laboratory animal care (March 10, 2006).

Isotropic Fractionation

We used the IF method to estimate the cellular composition of the three main regions of interest: the CX, OB, and the remaining tissue excluding the cerebellum (ROB) (Herculano-Houzel & Lent, 2005). Following tissue fixation, brains were dissected into the CX (i.e., the neocortex, entorhinal cortex and hippocampus), OB (i.e., the main and accessory olfactory bulbs) and ROB (i.e., the diencephalon, mesencephalon, medulla and pons) following the method of Herculano-Houzel et al. (2005). The dissected regions were then cryoprotected in a solution of 30% sucrose (in 0.1 M PBS) until they had sunk, after which they were transferred into antifreeze (30% glycerol, 30% ethylene glycol in 0.024 M phosphate buffer) and stored at 4°C (Herculano-Houzel & Lent, 2005). After the tissue sank in antifreeze, it was stored at -20°C until further processing.

Tissue grinders were used to mechanically grind the region of interest into a homogenous mixture with a standard dissociation solution (40.4 mM sodium citrate and 1% Triton X-100), which destroys cellular membranes while leaving the nuclear membranes intact (Herculano-Houzel & Lent, 2005). The solution was then stained with a 1:20 dilution of the DNA-specific fluorescent label DAPI (4', 6-diamidino-2-phenylindole dihydrochloride) that labels all cellular nuclei. The resulting solution was agitated to create a homogenous mixture of DAPI-labeled suspended nuclei, and the density of nuclei was determined using fluorescent microscopy. Several 10µL aliquots of homogenate were loaded into a Neubauer chamber (hemocytometer; mm^2 area, 0.1 mm

depth), coverslipped, and counted using the 40x lens of a Zeiss Axioskop 2 MOT microscope (Carl Zeiss). Following Herculano-Houzel et al. (2005), a minimum of four aliquots were counted per sample, but if the coefficient of variation between counts was greater than 15%, additional aliquots were quantified (Herculano-Houzel et al., 2015). The total number of cells in each region was calculated by multiplying the average nuclear density by the final volume of the homogenate solution.

Neuron counts were estimated by labeling a sample from each homogenate solution with the neuronal nuclear antigen NeuN, which labels the nucleus of most neuron types (Herculano-Houzel & Lent, 2005). A 1 ml sample of homogenate was loaded into a 1.5 ml Eppendorf tube, centrifuged, and the supernatant was decanted. The pellet containing cellular nuclei was resuspended with 0.1M PBS (pH = 7.4), centrifuged, and the supernatant was decanted; this step was repeated so that the nuclei were washed with PBS at least three times. To prevent nonspecific binding, the nuclei were resuspended in 0.5 ml blocking buffer (10% Normal Goat Serum in 0.1M PBS) and incubated for one hour at room temperature. After the blocking step, the solution was centrifuged and the supernatant was decanted. The nuclei were then immunoreacted with the Alexafluor-488 conjugated rabbit polyclonal anti-NeuN primary antibody (Millipore ABN 78C3) in a 1:100 dilution with the blocking buffer. The pellet was resuspended and the resulting solution was incubated in the dark at 4°C for 48 hours. After incubation, samples were rinsed with 0.1M PBS and stored in the dark at 4°C until they could be analyzed.

For neuron numbers, we used fluorescence microscopy to estimate the proportion of nuclei that were double-labeled with both NeuN and DAPI. A small amount of homogenate (approximately 200-300μL) was pipetted onto a glass slide, coverslipped, and visualized with the 40x lens of a Zeiss Axiocam Imager MRm microscope (Carl

Zeiss, MicroImaging GmBH, Germany). At least 500 DAPI labeled nuclei were counted per sample, and for each DAPI-labeled nucleus, it was determined whether it was also labeled with NeuN. The proportion of double labeled nuclei was multiplied by the number of total cells (determined with the hemocytometer) to yield the number of neurons in the homogenate. Once the neuron number was determined we subtracting it from the number of cells to calculate the number of non-neuronal cells. Neuronal and non-neuronal cell densities were calculated by dividing the number of neurons (or non-neuronal cells) by the mass of the region of interest. Finally, the non-neuronal cell to neuron ratio was calculated by dividing the number of non-neuronal cells by the number of neurons.

Statistical Analysis

We used one-way ANOVAs to test for differences among strains for all the following variables: body mass, brain mass, region of interest mass, total cell counts, neuron counts, non-neuronal cell counts, neuron density, non-neuronal cell density and the non-neuronal cell to neuron ratio. In general, brain mass is dependent on body mass (Jerison, 1973). Because there was a significant difference in body masses across strains (see below), we used analyses of covariance (ANCOVAs) to test for differences in relative measurements using brain mass as a covariate. For all ANOVAs and ANCOVAs, any significant effects were then further tested with Tukey Honest Significant Difference (HSD) post-hoc tests. Prior to statistical testing, all raw data was log-transformed to improve normality as well as to be comparable to other IF literature (Bandeira et al., 2009; Herculano-Houzel et al., 2015).

RESULTS

Absolute Measurements

Body mass varied across strains ($F = 46.43$, $df = 2, 57$, $p < 0.01$), LE rats were significantly larger than SD rats which were significantly larger than wild rats (Figure 3.1). Absolute brain mass also varied across strains ($F = 16.82$, $df = 2.64$, $p < 0.01$). Despite having the largest bodies, LE rats had smaller brains compared to the other two strains.

Cortical mass varied significantly across strains; LE rats had a smaller cortex than both SD and wild rats (Table 3.1). In contrast to cortical mass, all three strains had similar numbers of cortical cells, neurons, non-neuronal cells, as well as similar neuronal and non-neuronal cell densities. The ratio of non-neuronal cells to neurons in the CX was also consistent across strains (Figure 3.2).

Unlike the CX, the size of the OBs varied significantly by strain and the specific differences among the strains varied across the seven measurements (Table 3.2). Wild rats had significantly smaller OBs with a greater non-neuronal cell density than the two domestic laboratory strains (Figure 3.3). Wild rats also had significantly fewer non-neuronal cells and a lower non-neuronal cell to neuron ratio than SD and LE rats, respectively. Both the total number of cells and number of neurons differed between the domestic strains, with the wild rats differing from only one of the domestic strains. More specifically, the total number of OB cells was significantly higher in SD rats compared to LE and wild rats, but the total number of neurons was significantly higher in both wild and SD rats compared to LE rats. In addition to having fewer neurons than the other two strains, LE rats also had a lower neuronal density in the OBs than both SD and wild rats.

As in the OB, the ROB mass was significantly different across strains; ROB was significantly smaller in LE rats than in wild rats (Table 3.3). Interestingly, SD rats had significantly fewer ROB neurons and a lower ROB neuronal density compared to wild rats, despite having similar ROB masses (Figure 3.4). But, no other significant differences across strains were detected for any of the other measurements.

Relative Measurements

We found a significant effect of both strain ($F = 31.20$, $df = 2, 54$, $p < 0.01$) and body mass ($F = 7.91$, $df = 1, 54$, $p < 0.01$) on brain mass, but no significant interaction effect was detected ($F = 0.38$, $df = 2, 54$, $p = 0.69$). Post-hoc analysis revealed that LE rats had significantly smaller brain mass than the other two strains, independent of body mass.

There was a significant effect of brain mass, but not strain on the relative mass of the CX, and no interaction was detected (Table 3.4). There were no significant effects of brain mass on any of the other measurements and no effect of strain on relative numbers or densities of cells in the CX, despite a trend in the plots for LE rats to be at the lower end of the spectrum for all six measurements (Figure 3.5).

There were no significant effects of brain mass or the interaction between brain mass and strain on any of the OB measurements, including OB mass (Table 3.5). The total number of cells, number of non-neuronal cells and non-neuronal cell density also did not vary significantly across strains. Four measurements did, however, vary across strains, all of which reflected differences between LE rats and the other two strains. First, OB mass is significantly larger in LE than wild rats. Second, LE rats had significantly fewer neurons than SD rats. Third, LE rats had a significantly lower neuronal density than

wild rats. Last, LE rats had significantly higher non-neuronal cell to neuron ratios than wild rats

ROB mass varied significantly with strain and with brain mass, but there was no significant interaction effect (Table 3.6). This was due to LE rats having significantly larger ROB than SD and wild rats relative to brain mass. There were not, however, any effects of brain mass or strain on total number of cells, number of non-neuronal cells, non-neuronal cell density or non-neuronal cell to neuron ratio. Neuron estimates in ROB also did not vary by strain or brain mass, but there was a significant strain-brain mass interaction; LE rats had fewer ROB neurons than SD rats. Last, there were significant effects of both strain and brain mass on neuronal density in ROB, but no significant interaction was detected. Post-hoc analysis revealed that LE rats had a significantly lower neuron density compared to wild rats.

DISCUSSION

The results indicate that the adult females from two domestic rat strains differ in different ways relative to wild rats with regard to their relative brain size, as well as in the size and cellular composition of specific brain regions. LE rats had relatively larger ROB than the other two stains, but this did not correspond to strain differences in ROB cellular composition. There were no significant differences in relative CX size across strains or in any of the six cortical cellular composition measurements. Strains did differ, however, in relative OB size and cellular composition. Specifically, LE rats had relatively larger OBs with more non-neuronal cells per neuron compared to wild rats. The composition of the OBs also differed between domestic strains; LE rats had fewer OB neurons than SD rats despite no significant differences in relative OB size.

One limitation of the current study is that the rats experienced different housing conditions; LE rats were kept in smaller 46 cm x 25 cm x 20 cm cages compared to the 61cm x 43.5 cm x 21.5 cm cages in which the SD and wild rats were housed. Consequently, SD and wild rats had more than double the floor space in their cages than LE rats. Furthermore, this potential issue is compounded by the fact that LEs rats had the largest bodies of all three strains, resulting in even less space per unit body size. This difference in housing may constitute some form of environmental enrichment for wild and SD rats as they may have had greater motor and exercise opportunities. Environmental complexity alters the properties of many types of brain cells (e.g., neurons, astrocytes, endothelial cells), therefore, it is possible that the differences in the cellular compositions of the examined brain regions across strains is at least partially due to differences in living conditions as opposed to differential selective breeding histories (Greenough & Benefiel, 2004). Although we cannot discount the effects of housing entirely on our results, if environmental enrichment was solely responsible for observed differences in cellular composition across rat strains, the LE rats would likely have differed from the other strains across a wider range of measurements. The fact that LE and SD rats differed from one another across different measurements and both had different similarities to wild rats, suggests that cage size cannot account for all of these differences. Furthermore, these seemingly inconsistent strain differences were much more variable compared to our results in the cerebellum (see Chapter 2). Whereas SD and wild rats were similar to each other (and were dissimilar to LE rats) across a range of measurements in the cerebellum, in the OBs strains did not differ from each other in predictable ways.

Regardless of whether the differences between LE rats and the other two strains are due to domestication, housing or a combination of the two, our results still have important implications for understanding the effects of domestication on the cellular composition of the brain. In comparison with previous cell and neuron counts using the IF, our strains had similar cortical neuron and non-neuronal cell numbers, neuron densities, non-neuronal cell densities as well as a similar non-neuronal cell to neuron ratio to Wistar rats (Table 3.7). In the ROB, our non-neuronal cell counts and neuron densities were highly comparable to those reported in Wistar rats, yet, our strains had fewer ROB neurons, a higher non-neuronal cell density and a greater non-neuronal cell to neuron ratio. In the OBs, our specimen had fewer neurons compared to Wistar rats, but appeared to be similar across all other cellular measures. Many factors can influence cell numbers, including age, sex, housing, developmental conditions and strain, which can result in differences across studies that use the same quantitative methods (Flood & Coleman, 1988; Kempermann et al., 1997). Here, we only examined females of a specific age to limit these potentially confounding effects – studies using mixed sexes or ages could generate different results.

The largest difference we observed among strains was relative brain size; relative to body size, LE rats had significantly smaller brains than SD and wild rats (Figure 3.1). On average, LE rats have brains that are 24-35% smaller, relative to body mass, than the other two strains. Although we predicted that LE rats would have smaller brains than wild rats, we did not expect that our other domestic strain (SD rats) would be similar to wild rats. While previous investigators found that domestic (Wistar) rats had relatively smaller brains compared to wild rats (Kruska, 1975), to our knowledge this is the first report of a domestic rat strain not undergoing a reduction in relative brain size due to domestication.

This difference in relative brain size does not, however, extend to differences across all brain regions (see Chapter 2 and below).

Unlike relative brain size, we found no effects of strain on cell numbers or densities in CX (Tables 3.7). If environmental enrichment was having an effect in the cortex (see above), then SD and wild rats should have relatively larger cortices with more neurons (Diamond et al., 1964; Kempermann et al., 1997), yet we found no differences among strains in these or any other relative measurements of cellular composition in the CX (Table 3.7). Thus, housing does not appear to have an effect on cortical neuron or non-neuronal cell numbers. Our results were also in contrast to previous work indicating that Wistar rats have smaller cortices than wild rats (Kruska, 1975). As discussed above, this could have been due to mixed ages and sexes in Kruska's (1975) study, the inclusion of several regions and not just neocortex in our CX cell counts (Bandeira et al., 2009) or the Wistar rat strain may differ from both LE and SD rats in CX size.

Although no significant differences in cortical cell counts were detected, LE rats tend to have fewer cortical neurons and cells than the other strains, but this is largely due to them having smaller brains (Figure 3.5). This effect could be related to housing or could reflect some of the reported behavioural differences among strains. For example, LE rats require early climbing experience to be proficient adult climbers whereas wild rats will climb regardless of early rearing conditions (Huck & Price, 1976). Similarly, wild rats will jump whether reared in an enriched or standard laboratory environment, whereas LE rats do not jump under either condition (Huck & Price, 1975). The diminishment of these naturalistic behaviours in LE rats may reflect a variety of factors such as their reduced activity level, increased fat composition or larger body mass compared to wild rats (Keeler, 1947; Price, 1999), but also may be related to absolute

differences in the number of cells (or neurons) in cortical regions involved in these behaviours, such as motor cortex or premotor regions. Rough and tumble play is yet another behaviour that differs between strains; LE rats tend to prefer the more complex facing defense in response to a playful attack, whereas SD rats prefer evasive defenses (Himmler, Lewis, et al., 2014). Moreover, these differences in play behaviour are consistent whether SD rats are housed in European or North American cages (Himmler, Lewis, et al., 2014).

There are also strain differences in neophobia and exploratory behaviours. For example, compared to Wistar rats, wild rats are much more neophobic toward novel objects (Stryjek et al., 2012). Stryjek and colleagues (2012) found that when exploring a baited trap, wild rats tend to implore a wider array of cautionary behaviours and are therefore much less likely to get trapped than Wistar rats. While wild rats explore the area surrounding the trap and adopted body postures that facilitated immediate retreat, Wistar rats tend to enter the trap almost immediately and are promptly captured (Stryjek et al., 2012). In subsequent behavioural studies of food neophobia, wild rats also displayed more behavioural symptoms of stress compared to LE, SD and Brown Norway rats (Modlinska et al., 2015).

Besides motor behaviours and neophobia, rat strains also differ in cognitive abilities (Andrews, 1996). LE rats, for example, learn to develop strong and more persistent taste aversions in classical conditioning compared to SD rats (Dragoin, 1971). LE rats also appear to be superior to SD rats in the Morris Water Maze task which is a measure of spatial navigation (Lindner & Schallert, 1988). Based on these behavioural results one might expect LE rats to have relatively more cortical neurons than SD rats. Our results, however, did not support this conclusion as all three strains had similar

relative cortical neuron numbers. Thus, the link between neuron numbers and cognitive abilities across strains is uncertain and may be an interesting area for future research.

LE rats had an absolutely smaller ROB than wild rats, but a relatively larger ROB compared to the other two strains; this may indicate that the majority of the reduction in absolute brain size is due to the shrinkage of other structures. Compared to wild rats, the absolute masses of the cerebellum (see chapter 2) and CX was 27% and 14% smaller in LE rats respectively, but absolute ROB size was only reduced by 7%. In other words, most brain structures get smaller in LE rats, but the differences are greater for some structures than others. This difference in ROB size however did not translate into any strain differences in relative cellular composition. Because the relative cellular composition did not differ between strains, it is possible that the relatively larger ROB mass observed in LE rats may also be due to differences in cell sizes, connectivity, dendritic branching or neuropil. The ROB is comprised of many diverse brain regions; thus, it is difficult to determine what having a relatively larger ROB means for ROB functionality in LE rats. A more detailed analysis of neuron numbers focusing on small regions within the ROB could reveal more specific differences, but this is not possible using isotropic fractionation.

There were many differences in the size and cellular composition of the OBs across strains. First, LE rats had relatively larger OBs compared to wild rats. To the best of our knowledge, the only other report of a domestic strain having a larger brain part than wild rats was found in the optic tract of albino Wistar strain (Kruska, 1988). However, Kruska (1988) concluded that this difference was likely related to albinism and not domestication because albinism is associated with changes across visual regions of the brain, including smaller lateral geniculate nucleus, superior colliculus and primary

visual cortex, and pigmented laboratory rats exhibit the opposite trend (i.e., reduced optic tract size relative to wild rats). Our finding that all three strains had similar numbers of cells, despite differences in OB sizes, is consistent with the notion that due to adult neurogenesis, the relationship between cell numbers and tissue volume is more plastic in the OBs than in other brain regions (Ribeiro et al., 2014). That is, since adult neurogenesis affects both tissue volume and cell turnover rate (Díaz et al., 2017), OB volume does not necessarily scale with cell numbers across strains.

Compared to the other two strains, LE rats also had OBs with fewer neurons, lower non-neuronal cell densities and higher non-neuronal cell to neuron ratios. Once more, if housing was having the primary effect on brain region masses and cellular composition, then LE rats should have smaller OBs with fewer neurons compared to both SD and wild rats. However, effects of housing may not correspond to differences in OB neurons, as there is some evidence to suggest that neither exercise nor environmental enrichment is sufficient to increase adult OB neurogenesis in mice (Brown, 2003). It is important to note that it is difficult to determine what these strains differences might mean in terms of behaviour and sensory abilities. There is some evidence, however, that strains differ in their production and/or detection of specific odorants. For example, Wistar rats are less reactive (i.e., display less freezing behaviour) in response to the synthetic predator odor 2,4,5-trimethylthiazoline (TMT) compared to SD and LE rats (Rosen et al., 2006). Wistar rats are also less reactive (and appear to produce less of) the so-called “alarm substance” released by conspecifics in the forced swim tasks compared to SD rats (Abel, 1992). These strain differences in reactivity towards specific odorants alongside our finding that the composition of the OBs varies across strains seem to suggest that domestication has produced functional changes in olfaction in a strain-

specific manner. To better understand differences in olfaction across strains, it would be advantageous to characterize the behavioural responses to a variety of odorants as well as to quantify specific cell types in the OB.

Mitral cells, the major output neurons of the OB, are one of the few neuron types that do not express NeuN, and thus are misclassified as non-neuronal cells using the IF method (Mullen et al., 1992). Mitral cells encompass a relatively small number of neurons compared with granule cells in the OBs (Meisami, 1989; Royet et al., 1998) but are an important metric for assessing olfactory sensitivity in the olfactory system (Meisami, 1989; van Drongelen et al., 1978). The other important measurement needed to estimate olfactory sensitivity is the number of glomeruli, which also cannot be quantified using isotropic fractionation. Given the lack of information on strain differences in olfactory abilities and sufficient quantitative neuroanatomical measurements, we cannot predict how our results might translate to strain differences in olfaction, but this would be an important area for future research.

Overall, our results indicate that the cellular composition of brain regions (especially the OBs) varies across rat strains, but that there are no consistent differences between domesticated strains and wild rats. In other words, the effects of domestication are variable across strains and brain regions. Thus, based on our findings, some of the observed decreases in brain region sizes (Kruska) may be due to changes in neuron or non-neuronal cell numbers, but at least in rats, these differences are strain specific. To fully address how domestication affects the quantitative neuroanatomy of the brain, it will be necessary to compare a wider range of domesticated strains as well as to quantify specific cell types and their morphology.

Table 3.1 – Results of one-way analyses of variance (ANOVAs) examining the effects of strain (Long-Evans = LE, Sprague-Dawley = SD and wild rats) on seven cortex (CX) measurements: mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. ANOVA results are reported as F-values (F), degrees of freedom (df), and p-values (p). For all ANOVAs, any significant effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. *denotes a significant difference between indicated strains. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	F	df	p	Tukey HSD
CX mass	19.78	2, 33	**<0.01***	LE < Wild, SD
Total CX cells	2.60	2, 32	0.09	
CX neurons	1.03	2, 32	0.37	
CX non-neuronal cells	1.89	2, 32	0.17	
CX neuron density	1.44	2, 32	0.25	
CX non-neuronal cell density	0.17	2, 32	0.85	
CX non-neuronal cell : neuron	0.73	2, 32	0.49	

Table 3.2 – Results of one-way analyses of variance (ANOVAs) examining the effects of strain (Long-Evans = LE, Sprague-Dawley = SD and wild rats) on seven olfactory bulb (OB) measurements: mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. ANOVA results are reported as F-values (F), degrees of freedom (df), and p-values (p). For all ANOVAs, any significant effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. *denotes a significant difference between indicated strains. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	F	df	p	Tukey HSD
OB mass	11.41	2, 32	**<0.01***	Wild < LE, SD
Total OB cells	10.90	2, 28	**<0.01***	LE, Wild < SD
OB neurons	11.24	2, 28	**<0.01***	LE < Wild, SD
OB non-neuronal cells	6.65	2, 28	**<0.01***	Wild < SD
OB neuron density	15.87	2, 27	**<0.01***	LE < Wild, SD
OB non-neuronal cell density	13.44	2, 27	**<0.01***	LE, SD < Wild
OB non-neuronal cell : neuron	6.64	2, 28	**<0.01***	Wild < LE

Table 3.3 – Results of one-way analyses of variance (ANOVAs) examining the effects of strain (Long-Evans = LE, Sprague-Dawley = SD and wild rats) on seven 'rest of brain' (ROB) measurements: mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. ANOVA results are reported as F-values (F), degrees of freedom (df), and p-values (p). For all ANOVAs, any significant effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. *denotes a significant difference between indicated strains. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	F	df	p	Tukey HSD
ROB mass	4.10	2, 33	**0.03***	LE < Wild
Total ROB cells	1.59	2, 32	0.22	
ROB neurons	4.42	2, 32	**0.02***	SD < Wild
ROB non-neuronal cells	0.89	2, 32	0.42	
ROB neuron density	4.42	2, 32	**0.02***	SD < Wild
ROB non-neuronal cell density	0.09	2, 32	0.91	
ROB non-neuronal cell : neuron	1.52	2, 32	0.23	

Table 3.4 – Results of one-way analysis of covariance (ANCOVAs) of strain, brain mass (covariate) and their interaction on seven measurements of cortex (CX): mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. The three strains examined were Long-Evans (LE), Sprague-Dawley (SD) and wild rats. ANCOVA results are reported as F-ratios (F), degrees of freedom (df) and p-values (p), where (*) denotes a significant effect. For all significant p-values of strain (i.e., p-value < 0.05) effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	Strain				Brain mass			Interaction		
	F	df	p	Tukey HSD	F	df	p	F	df	p
CX mass	0.99	2, 30	0.38		37.55	1, 30	**<0.01***	0.24	2, 30	0.79
Total CX cells	0.87	2, 29	0.43		0.01	1, 29	0.91	1.56	2, 29	0.23
CX neurons	0.04	2, 29	0.96		0.08	1, 29	0.78	0.34	2, 29	0.71
CX non-neuronal cells	1.13	2, 29	0.34		0.01	1, 29	0.91	1.69	2, 29	0.20
CX neuron density	0.06	2, 29	0.94		2.56	1, 29	0.12	0.46	2, 29	0.64
CX non-neuronal cell density	1.83	2, 29	0.18		1.82	1, 29	0.19	2.02	2, 29	0.19
CX non-neuronal cell : neuron	0.55	2, 29	0.58		0.07	1, 29	0.79	0.47	2, 29	0.77

Table 3.5 – Results of one-way analysis of covariance (ANCOVAs) of strain, brain mass (covariate) and their interaction on seven measurements of the olfactory bulbs (OBs): mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. The three strains examined were Long-Evans (LE), Sprague-Dawley (SD) and wild rats. ANCOVA results are reported as F-ratios (F), degrees of freedom (df) and p-values (p), where (*) denotes a significant effect. For all significant p-values of strain (i.e., p-value < 0.05) effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	Strain				Brain mass			Interaction		
	F	df	p	Tukey HSD	F	df	p	F	df	p
OB mass	6.78	2, 29	**<0.01***	Wild < LE	1.40	1, 29	0.25	0.31	2, 29	0.74
Total OB cells	2.41	2, 25	0.11		0.001	1, 25	0.98	0.07	2, 25	0.93
OB neurons	3.68	2, 25	**0.04***	LE < SD	0.05	1, 25	0.83	0.12	2, 25	0.89
OB non-neuronal cells	2.33	2, 25	0.12		0.16	1, 25	0.69	0.35	2, 25	0.71
OB neuron density	17.27	2, 24	**<0.01***	LE < Wild, SD	6.50	1, 24	**0.02***	1.23	2, 24	0.29
OB non-neuronal cell density	11.53	2, 24	**<0.01***	LE < Wild	11.04	1, 24	**<0.01***	1.46	2, 24	0.25
OB non-neuronal cell : neuron	4.12	2, 25	**0.03***	Wild < LE	0.23	1, 25	0.63	0.46	2, 25	0.64

Table 3.6 – Results of one-way analysis of covariance (ANCOVAs) of strain, brain mass (covariate) and their interaction on seven measurements of 'rest of brain' (ROB): mass, total number of cells, number of neurons, number of non-neuronal cells, neuronal density, non-neuronal cell density and the number of non-neuronal cells per neuron. The three strains examined were Long-Evans (LE), Sprague-Dawley (SD) and wild rats. ANCOVA results are reported as F-ratios (F), degrees of freedom (df) and p-values (p), where (*) denotes a significant effect. For all significant p-values of strain (i.e., p-value < 0.05) effects were further tested with Tukey Honest Significant Difference (HSD) post-hoc analyses. In the post-hoc column, differences between strains are indicated using greater than (>) or less than (<) signs.

Measurements	Strain				Brain mass			Interaction		
	F	df	p	Tukey HSD	F	df	p	F	df	p
ROB mass	14.14	2, 30	**<0.01***	LE > SD, Wild	41.42	1, 30	**<0.01***	3.14	2, 30	0.06
Total ROB cells	0.27	2, 29	0.77		0.003	1, 29	0.95	1.13	2, 29	0.34
ROB neurons	1.08	2, 29	0.35		0.10	1, 29	0.76	4.17	2, 29	**0.03***
ROB non-neuronal cells	0.20	2, 29	0.82		0.02	1, 29	0.88	0.53	2, 29	0.60
ROB neuron density	1.08	2, 29	0.35		0.10	1, 29	0.76	4.17	2, 29	**0.03***
ROB non-neuronal cell density	0.85	2, 29	0.44		2.14	1, 29	0.15	0.07	2, 29	0.93
ROB non-neuronal cell : neuron	0.56	2, 29	0.56		0.13	1, 29	0.73	1.14	2, 29	0.33

Table 3.7 – Descriptive statistics comparing the results of the current study comparing three rat strains (Long-Evans (LE), Sprague-Dawley (SD) and wild) with previous findings in mixed-sex and male Wistar rats using the same method (i.e., isotropic fractionation). For each strain, mass and cellular composition was measured in three regions of interest; the cortex (CX), olfactory bulbs (OBs) and the 'rest of brain' (ROB). There were six cellular measurements in each region; the total number of cells, number of neurons, number of non-neuronal cells, neuronal density (neurons per milligram), non-neuronal cell density (non-neuronal cells per milligrams) and the number of non-neuronal cells per neuron. Values for LE, SD and wild rats are reported as ranges (i.e., minimums and maximums) whereas values for Wistar rats are reported as means. In the current study we found a large degree of variation within each strain. Yet, the ranges of LE, SD and wild rats usually overlap one another and tend to include the means reported in Wistar rats.
[1]Cortex values are the sum of hippocampal and cortex numbers from Banderia et al., 2009. Mixed sex Wistar rats were 90 days old.
[2]Estimates were based on 4 adult male Wistar rats as reported in Herculano-Houzel & Lent 2005.

		Wild	Sprague-Dawley	Long-Evans	Mixed sex Wistar[1]	Male Wistar[2]
CX	**Mass**	1013–1181	1023–1170	882–1078	690	770
	Total cells	8.88–12.4 x 10^7	8.84–11.7 x 10^7	8.75–10.9 x 10^7	7.06 x 10^7	7.67 x 10^7
	Neurons	3.10–4.63 x 10^7	2.53–4.10 x 10^7	2.85–4.25 x 10^7	2.31 x 10^7	3.10 x 10^7
	Non-neuronal cells	5.54–8.48 x 10^7	4.88–8.34 x 10^7	5.03–6.91 x 10^7	4.75 x 10^7	4.57 x 10^7
	Neuron density	2.77–4.32 x 10^4	2.17–4.01 x 10^4	3.13–4.59 x 10^4	3.35 x 10^4	4.11 x 10^4
	Non-neuronal cell density	5.03–7.57 x 10^4	4.66–7.59 x 10^4	5.31–7.59 x 10^4	6.88 x 10^4	5.93 x 10^4
	Non-neuronal cell : neuron	1.43–2.73	1.19–2.82	1.18–2.42	2.06	1.47
OBs	**Mass**	48–87	82–124	82–109	75	77
	Total cells	2.41–3.26 x 10^7	2.87–3.63 x 10^7	2.23–3.22 x 10^7	2.25 x 10^7	2.09 x 10^7
	Neurons	1.42–2.02 x 10^7	1.64–2.26 x 10^7	1.12–1.80 x 10^7	1.15 x 10^7	1.13 x 10^7
	Non-neuronal cells	0.90–1.43 x 10^7	1.21–1.83 x 10^7	1.05–1.58 x 10^7	1.10 x 10^7	9.61 x 10^6
	Neuron density	1.63–3.95 x 10^5	1.51–2.65 x 10^5	1.27–1.94 x 10^5	1.53 x 10^5	1.47 x 10^5
	Non-neuronal cell density	1.06–2.79 x 10^5	1.11–1.86 x 10^5	1.15–1.62 x 10^5	1.47 x 10^5	1.25 x 10^5
	Non-neuronal cell : neuron	0.52–0.88	0.59–1.06	0.66–1.22	0.96	0.85
ROB	**Mass**	452–593	489–532	433–563	673	683
	Total cells	5.56–7.29 x 10^7	5.24–6.75 x 10^7	5.07–6.75 x 10^7	6.99 x 10^7	6.59 x 10^7
	Neurons	1.17–1.57x 10^7	1.06–1.47 x 10^7	1.02–1.49 x 10^7	2.01 x 10^7	1.87 x 10^7
	Non-neuronal cells	4.29–5.96 x 10^7	4.12–5.39 x 10^7	3.80–5.48 x 10^7	4.98 x 10^7	4.72 x 10^7
	Neuron density	2.33–3.23 x 10^4	2.05–2.83 x 10^4	1.92–3.42 x 10^4	2.99 x 10^4	2.78 x 10^4

	Non-neuronal cell density	0.83–1.19 x 10^5	0.81–1.07 x 10^5	0.73–1.22 x 10^5	7.40 x 10^4	6.91 x 10^4
	Non-neuronal cell : neuron	2.82–4.49	3.00–4.49	2.55–4.43	2.48	2.528

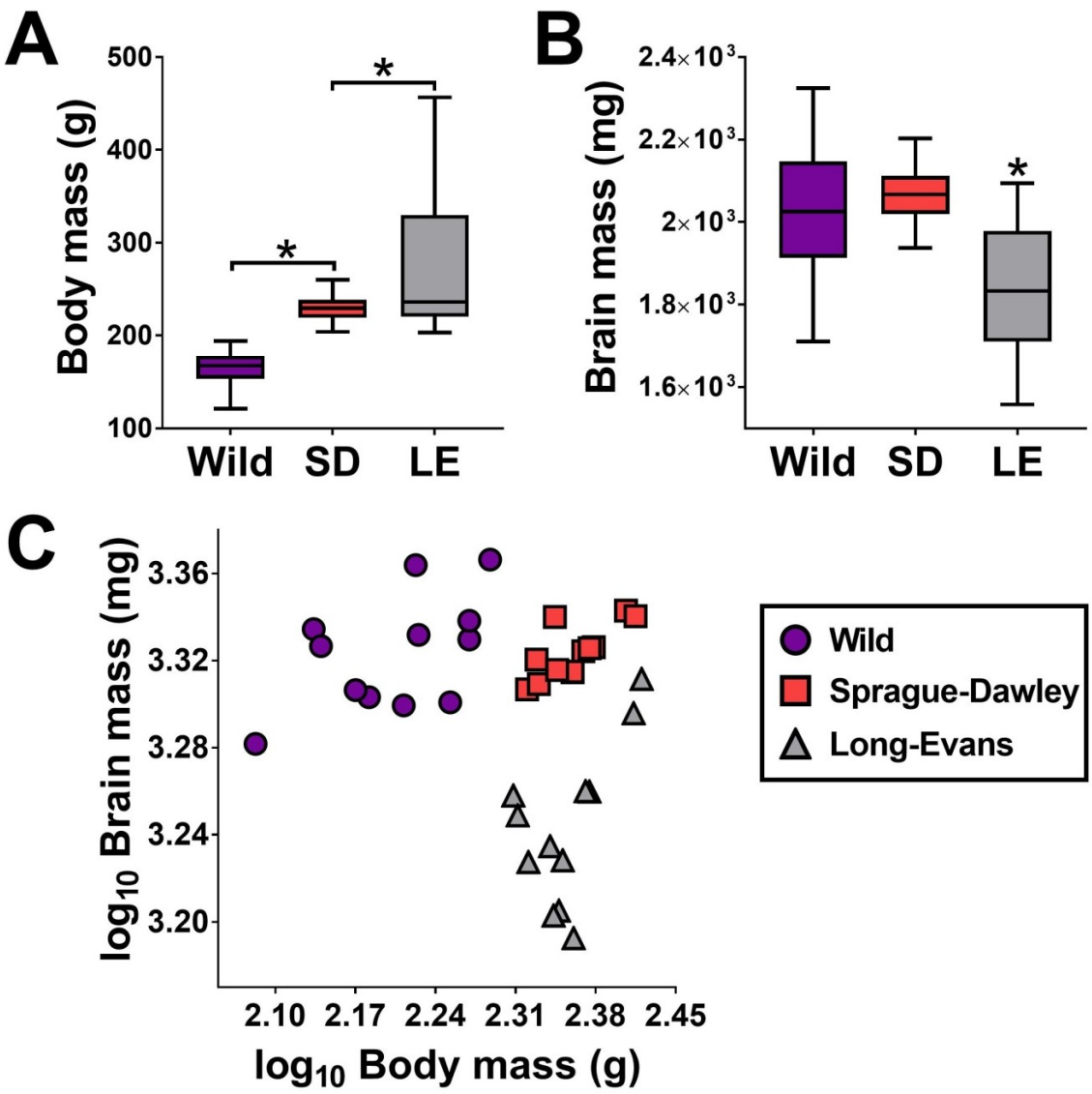

Figure 3.1 – Boxplots of (a) absolute body mass (grams) and (b) absolute brain mass (milligrams) across three rat strains; wild (purple), Sprague-Dawley (SD = orange) and Long-Evans (LE = grey). Horizontal lines within the box represent the mean for each group and error bars represent the minimum and maximum values within each group. *denotes a significant difference between indicated strains. LE rats had the largest body mass of all three strains but wild rats and Sprague-Dawley rats had absolutely larger brains. *denotes a significant difference between indicated strains. (c) Shows a double log scatterplot of intraspecific brain mass (milligrams) to body mass (grams) relation. As per the legend, wild rats = purple circles, SD rats = orange triangles; LE rats = grey triangles. LE rats had relatively smaller brains for their body mass than the other two strains.

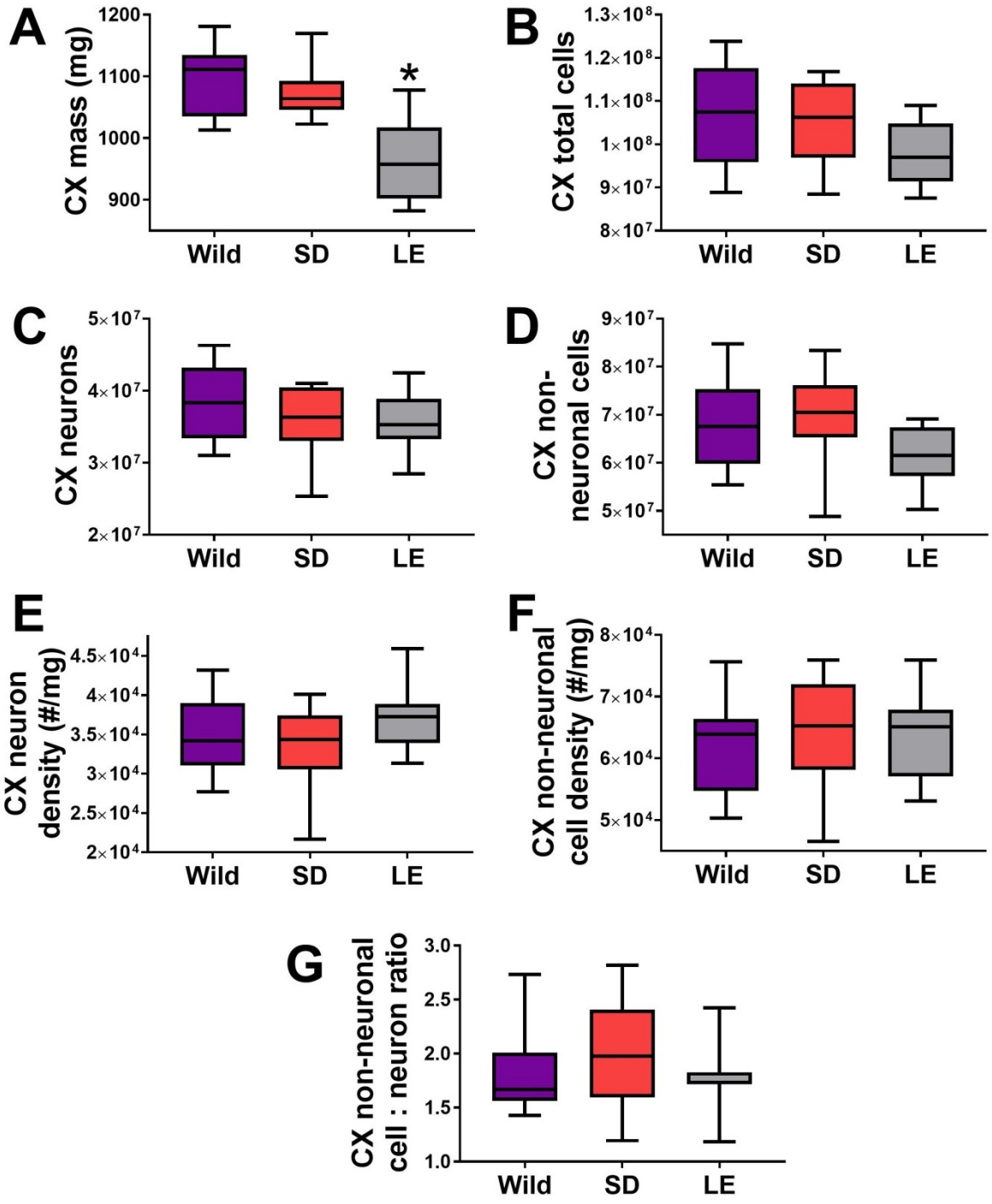

Figure 3.2 – Boxplots of seven absolute cortex (CX) measurements across three strains of rats; wild (purple), Sprague-Dawley (SD = orange) and Long-Evans (LE = grey). In the CX we measured: mass (milligrams), (b) total number of cells, (c) number of neurons, (d) number of non-neuronal cells, (e) neuronal density (neurons per milligram), (f) non-neuronal cell density (non-neuronal cells per milligram) and the (g) number of non-neuronal cells per neuron. Horizontal lines within the box represent the mean for each group and error bars represent the minimum and maximum values within each group. *denotes a significant difference between

indicated strains. We found that LE rats had a smaller CX than both SD and wild rats, but there were no differences across strains in any cellular CX measurements.

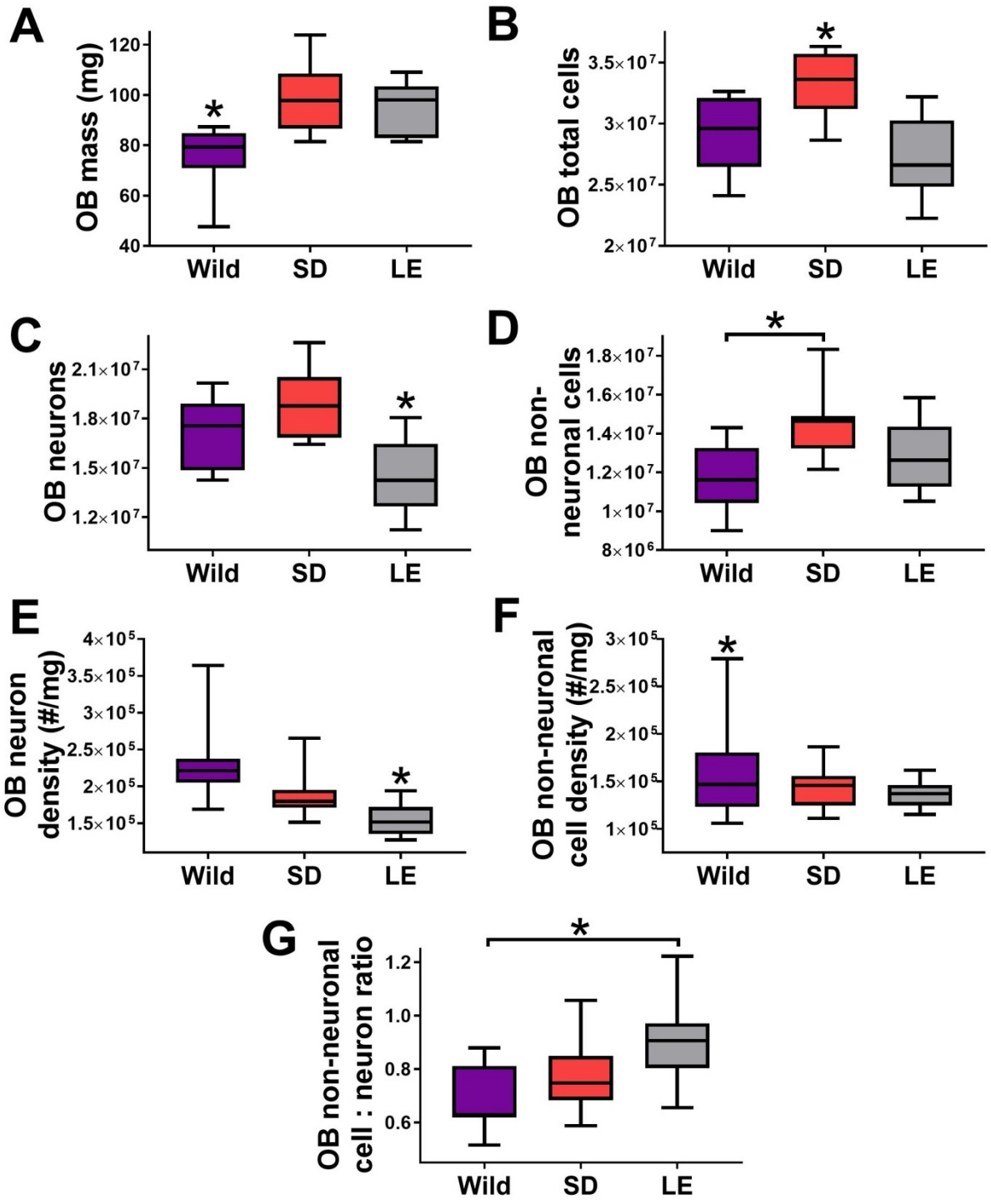

Figure 3.3 – Boxplots of seven absolute olfactory bulb (OB) measurements across three strains of rats; wild (purple), Sprague-Dawley (SD = orange) and Long-Evans (LE = grey). In the OBs we measured: mass (milligrams), (b) total number of cells, (c) number of neurons, (d) number of non-neuronal cells, (e) neuronal density (neurons per milligram), (f) non-neuronal cell density (non-neuronal cells per milligram) and the (g) number of non-neuronal cells per neuron. Horizontal lines within the box represent the mean for each group and error bars represent the

minimum and maximum values within each group. *denotes a significant difference between indicated strains. We found that strains differed across all absolute measures in the OB, but not in a consistent manner.

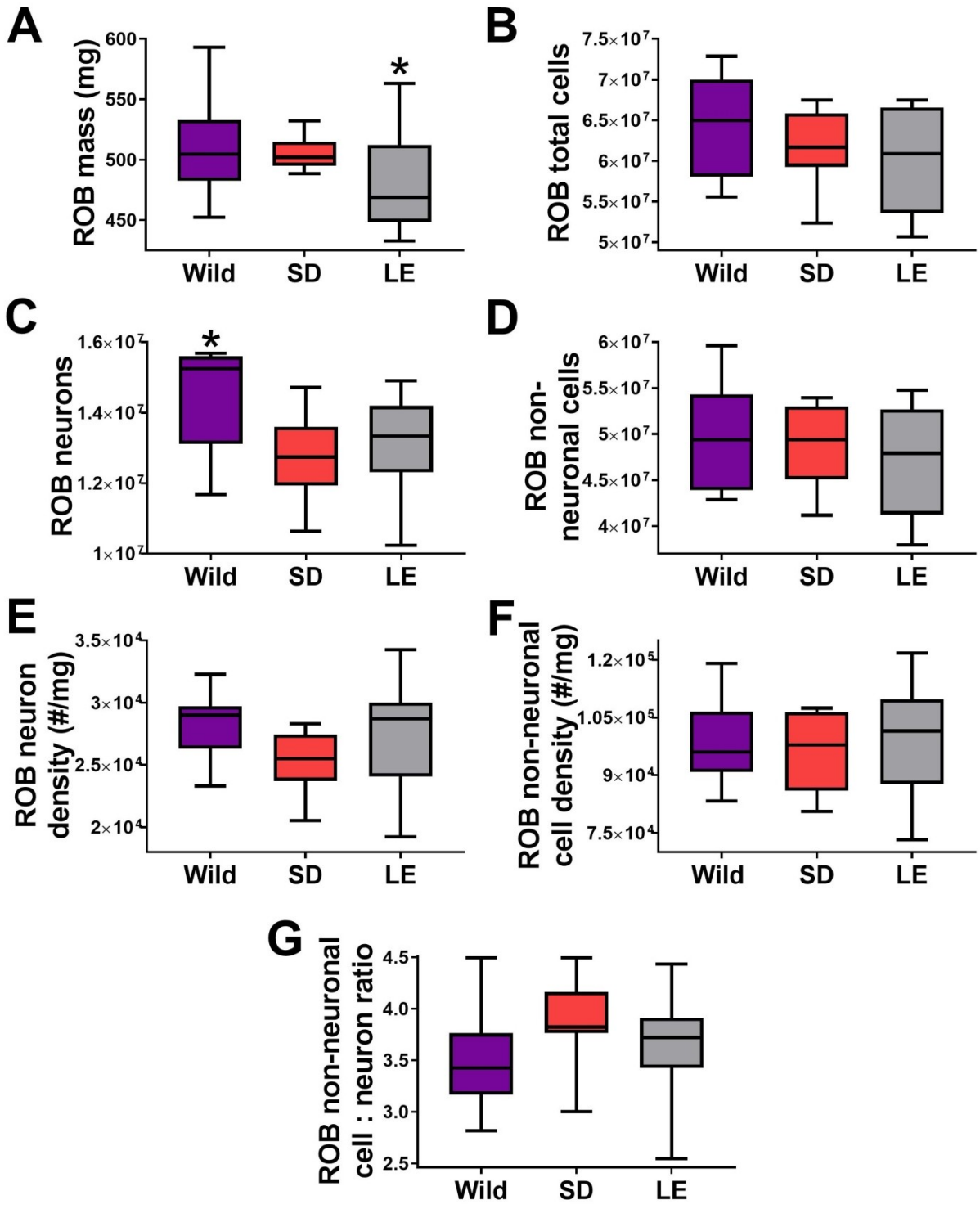

Figure 3.4 – Boxplots of seven absolute rest of brain (ROB) measurements across three strains of rats; wild (purple), Sprague-Dawley (SD = orange) and Long-Evans (LE = grey). In ROB we measured: mass (milligrams), (b) total number of cells, (c) number of neurons, (d) number of non-neuronal cells, (e) neuronal density (neurons per milligram), (f) non-neuronal cell density (non-neuronal cells per milligram) and the (g) number of non-neuronal cells per neuron. Horizontal lines within the box represent the mean for each group and error bars represent the

minimum and maximum values within each group. *denotes a significant difference between indicated strains. We found that wild and SD rats had a larger ROB than LE rats. Wild rats also had more ROB neurons than the domestic strains.

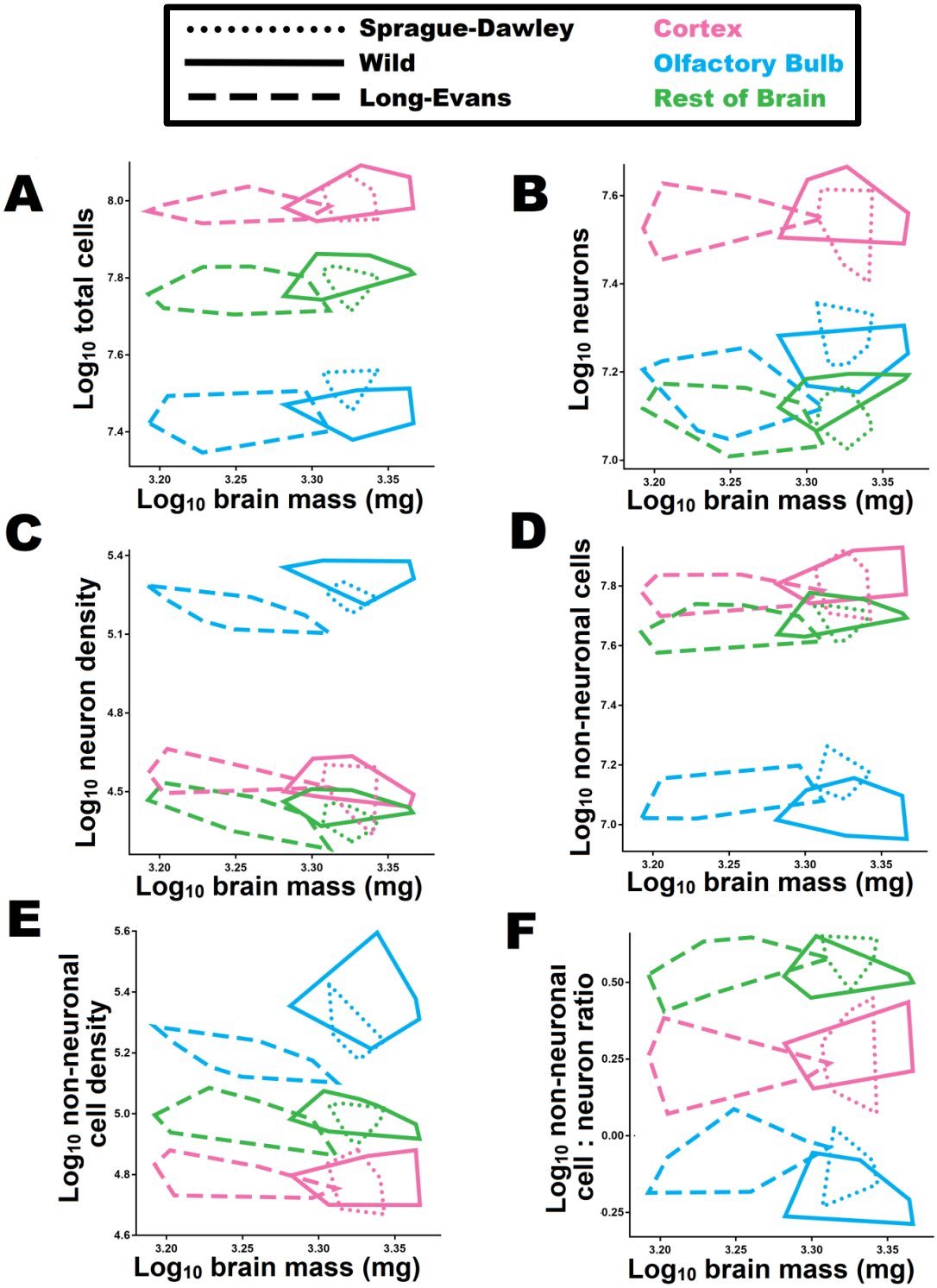

Figure 3.5 – Double log polygon plots of six cellular measurements in three regions of interest, across three rat strains, relative to brain mass (milligrams). The three brain regions of interest are the cortex (CX) in pink, olfactory bulbs (OBs) in blue and rest of brain (ROB) in green. For each region of interest, these polygon plots depict the (a) total number of cells, (b) number of neurons, (c) number of non-neuronal cells, (d) neuronal density (neurons per milligram), (e) non-neuronal

cell density (non-neuronal cells per milligram) and the (f) number of non-neuronal cells per neuron. As per the legend, the data for Sprague-Dawley (SD) rats are grouped by a dotted line, wild rats by a solid line and Long-Evans (LE) rats by a dashed line. The polygon plots were created by drawing lines around the outermost data points for each strain so that data from all individuals in a group are contained in the polygon area. While the plots for SD and wild rats tend to overlap, the plots for LE rats are shifted to the left, indicating that they have smaller brain masses than the other two strains.

CHAPTER FOUR: GENERAL DISCUSSION

Overall, we found that strains vary in brain size, the proportional size of some brain regions (especially cerebellum (CB), olfactory bulbs (OBs) and rest of brain (ROB)), the cellular composition of some brain regions (especially CB and OB), as well as in CB foliation and Purkinje soma size. We did not, however, find consistent differences between wild and laboratory strains as we predicted. Taken together, these results indicate that domestication affects both brain composition and morphology, but such changes are strain and brain region specific. Indeed, we found that the differences between domestic laboratory strains may be as great as those between domestic and wild rats.

The current study provides some evidence for neuroanatomical differences between Sprague-Dawley (SD) rats, Long-Evans (LE) rats and wild rat strains. It should be noted, however, that we cannot disentangle the effects of strain from the potential effects of differential housing conditions. We found that LE rats were the most different to both the other domestic strain examined, SD rats, and from wild rats. However, the LE rats were reared in cages with less than half the floor space provided to the SD and wild rats obtained from the Mossakowski Medical Research Center at the Polish Academy of Sciences (Warsaw, Poland). This housing problem was compounded by the fact that LE rats had the largest bodies of all three strains, and therefore, they had even less space per unit body mass than SD and wild rats. If increased floor space constitutes a form of environmental enrichment, then it is possible that the strain differences we observed may be a result of housing effects (Greenough & Benefiel, 2004) instead of selective breeding. In the current study, it is impossible to determine whether the brains of rats differed as a result of housing or strain, but if environmental enrichment was having an effect, we might expect to see LE rats differ from the other two strains across more

measurements, especially the composition of the cortex (CX) (Diamond et al., 1964). In addition to differential housing conditions, captive breeding may also be having an effect on brain size (O'Regan & Kitchener, 2005). The wild rats used in this study were from the F3 generation in a captive environment, so it is possible that these rats that were reared in a captive environment may already differ from their free-living counterparts. To better assess the relative effects of captive breeding and/or housing on the brain, it would be advantageous for future studies to compare first generation in captivity wild rats to age-matched free-living wild rats. Furthermore, it will be important for future studies to fully characterize the neural effects of cage sizes.

Although these critical comparisons are yet to be done, there are several reasons why it is unlikely that our data cannot be fully explained as an artifact of housing. First, previous experiments indicate that standard housing differences across European and North American laboratories do not produce any differences in the play behaviour of Wistar rats (Himmler, Modlinska, et al., 2014). Second, equivalent results have been reported for SD rats; that is, whether reared in a standard European or North American cage, SD rats display the same behavioural play profile (Himmler, Lewis, et al., 2014). Together this suggests that strain differences in behaviour are not produced by small difference in cage size. Third, although the play behaviour of SD and wild rats is quantitatively similar in that they both engage in a higher frequency of evasive defense tactics compared to LE rats, the manner in which SD and wild rats evade their play partners is qualitatively different. Namely, while SD rats evade playful attacks by moving their head and neck (while the rest of their body remains stationary), wild rats evade with whole body movements, such as running, jumping or swerving (Himmler et al., 2013). Moreover, even though LE rats engaged in a lower frequency of evasion, most of their evasive actions involve whole body movements, thus, the evasive actions of LE rats are qualitatively

most similar to wild rats. In this way, irrespective of cage dimensions, SD rats do not experience as much acrobatic movement as either wild or LE rats. Given that wild and SD rats were housed identically, but their experiences were nonetheless different, the greater similarity in CB morphology shared by these strains cannot be explained by housing alone. Therefore, our finding that wild and SD rats have the most similar brain morphology is inconsistent with the interpretation that housing conditions produce different motor experiences which, in turn, affect the brain. For the reasons given above, the confounding effects of cage size are unlikely to explain how the brains of wild and SD rats are most similar.

It is also important to consider the limitations of the isotropic fractionators (IF) technique when drawing conclusions about our results. Although the IF method is much faster than traditional stereological methods and provides accurate cell counts (Bahney & von Bartheld, 2014; Ngwenya et al., 2017), it is limited in several ways. First, homogenization requires the gross dissection of brain regions, and any inconsistencies in these dissections can introduce error into cell counts. Second, this error may be amplified when dissecting smaller brain regions, so analyzing specific neuroanatomical regions using isotropic fractionation is more challenging and may be less reliable. Thus, one limitation of the present study is that we were unable to analyze smaller regions of interest where strain differences may become apparent if they were measured individually, especially in the sub-divisions of CX and ROB. Third, the IF technique can currently only classify nuclei as either neuronal or non-neuronal (Herculano-Houzel & Lent, 2005). Therefore, a potential area for future research would be to quantify differences in specific cell types across strains. For example, LE rats had more non-neuronal cells in the CB than wild rats, so determining whether this represents an increase in oligodendrocytes, astrocytes, ependymal cells, microglia, or endothelial cells may be important to understanding strain

differences in cellular composition. A fourth limitation of isotropic fractionation is that the homogenization process destroys everything expect cell nuclei, so it is impossible to study cell morphology or the structural arrangement of cells within tissue (Napper, 2018). Thus, valuable information regarding the functional relationships between cells within a network cannot be determined from this method.

Nonetheless, comparing cell populations across strains is critical for understanding the quantitative networks that comprise the brains of our animal models (Napper, 2018). Likewise, quantitatively comparing the brains of wild and laboratory rats provides an avenue by which to investigate how selective breeding and the domestic environment affect our animal models. For instance, we found evidence for strain differences in the anatomy of specific brain regions that may parallel functional differences in strain-specific behaviour. It is also interesting to note that differences in the mass of brain regions were not necessarily associated with parallel changes in cell numbers. In addition to cell numbers, the sizes of brain regions may reflect the size of cells, dendritic branching patterns, neuropil (i.e., microvascular, glial cell processes, axons and dendrites) (Spocter et al., 2012), water content, or other unknown factors. Our results highlight the importance of cell counts in quantitative neuroanatomical studies, because inferring functional differences based on either cell density or the size of brain regions may lead to erroneous conclusions (Napper, 2018).

A better understanding of the functional implications of our results could be provided by quantifying the number, size and morphology of specific cell types. In the OB, for instance, it will be critical to determine whether the anatomical differences we reported here are associated with strain-specific olfactory abilities. In the olfactory epithelium, olfactory receptors send odorant information into glomeruli in the OB, and these signals are transmitted to mitral cells,

which integrate this information and send it to other brain regions for higher-level processing (Slotnick, 2001). Thus, differences in olfactory sensitivities across strains may be better understood by studying glomeruli as well as mitral cells (which are the principal output neuron of the OB), neither of which can be measured using isotropic fractionation (Herculano-Houzel & Lent, 2005). It will also be important for future studies to characterize strain differences in the anatomy of the vomeronasal organ and its sensory receptors, as these receptors project to the accessory OB, which in turn, projects to the amygdala, hypothalamus and other brain regions. Similarly, the accessory OB might be an important region to examine with respect to strain differences because in addition to processing odorant information, accessory OB neurons convey pheromone signals (detected by the vomeronasal organ) which are relevant to species-specific social and reproductive behaviour (Trinh & Storm, 2003).

Our finding that LE and SD laboratory rats differ in OB anatomy may have major implications for the use of laboratory rats as animal models. Although little is known about strain differences in olfactory abilities, there is some evidence to indicate that strains may differ in olfactory sensitivities. For example, laboratory rat strains vary in their level of responsiveness to specific odors, including the artificial predator odor 2,4,5-trimethylthiazoline (Rosen et al., 2006) as well as the so-called “alarm substance” which is produced by conspecifics (Abel, 1992). Because the olfactory system projects to the prefrontal cortex, limbic system and hypothalamus, differences in olfactory sensitivities across strains could affect various aspects of cognition, emotionality as well as the regulation of basic physiological processes, respectively (Slotnick, 2001). Thus, the results of experimental studies that include a component of olfaction may be dependent on the rat strain that is selected.

It will also be important for future comparative studies to better characterize the precise variation in CB neuroanatomy between strains. For example, we found that LE rats had less CB foliation than other strains, but little is known about strain differences in the size or cellular composition of specific folia. Individual CB folia receive unique cortical inputs and are responsible for specific integrative functions (Welker, 1990), thus, examining strain differences in folia morphology may be informative to understanding strain differences in CB processing. LE rats also had smaller Purkinje somas compared to the other strains, so it may also be relevant for future studies to examine strain differences in Purkinje cell morphology, such as dendritic branching and synaptic density. Studying Purkinje cells is particularly relevant to understanding strain differences in CB functionality because they are the ultimate destination of all information entering the cerebellar cortex as well as the sole output neuron of the cerebellar cortex (D'Angelo & De Zeeuw, 2009). Thus, strain differences in Purkinje cell morphology may have major implications for any research concerning either cerebellar pathology or motor behaviours. Another way of assessing strain differences in CB complexity and connectivity would be to measure the volume of the white matter layer. In the CB, the white matter consists of efferent Purkinje cell fibers, as well as afferent climbing, and mossy fibers, which originate from the inferior olive and pontine nuclei (as well as other regions), respectively. Strain differences in white matter volumes could be assessed using histology, as well as imaging techniques such as Diffusion Tensor Imaging (DTI) and Magnetic Resonance Imaging (MRI). Because the white matter of the CB facilitates communication with the cerebral cortex and brainstem, examining white matter morphology through these techniques may grant insight into the differences in functional connectivity across strains.

Laboratory rats are among the most common animal models used to study brain function, pathology and behaviour (Nestler & Hyman, 2010). In neuroscience, LE and SD rats serve as popular models in which to study cognition and neuropsychological dysfunction, respectively (e.g., Gallagher & Nicolle, 1993; Kim et al., 2017; Turner & Burne, 2014). Given the critical role of LE and SD rats in neurological research, variation in neuroanatomy across strains may have implications for the interpretation of findings as well as the generalizability of research. One implication is that using only one strain to study a neurological phenomenon may lead to strain-specific results that are not generalizable to other strains, let alone translatable to other animals, including humans. In addition to strain differences, the unreliability of results may be further compounded by the fact that the laboratory environment itself can cause animals of the same-strain to perform differently in standardized behavioural tests (Wahlsten, 1972). In other words, researchers should be cognisant of the fact that the brain and behaviour of our animal models is influenced by their genetics, environment, as well as gene-environment interactions. To improve reliability, studies should ideally be run across multiple strains. Moreover, even when strains appear to be identical, it is important to verify such assumptions.

We hope that the current study helps bring attention to the similarities and differences among strains to improve future neuroscience research. Again, we believe that studies should more often be replicated in other strains to ensure that findings are not idiosyncratic to the strain, or the laboratory environment. In general, using a wider range of model organisms will better allow us to disentangle principals of brain and behaviour as they can be applied more broadly. To improve upon our research, it would be beneficial to investigate neuroanatomical differences across a wider range of laboratory strains as well as to examine potential sex differences.

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