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Research article

The relationship between the number of neurons and behavioral performance in Swiss mice

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ABSTRACT

Neuronal number varies by several orders of magnitude across species, and has been proposed to predict cognitive capability across species. Remarkably, numbers of neurons vary across individual mice by a factor of 2 or more. We directly addressed the question of whether there is a relationship between performance in behavioral tests and the number of neurons in functionally relevant structures in the mouse brain. Naïve Swiss mice went through a battery of behavioral tasks designed to measure cognitive, motor and olfactory skills. We estimated the number of neurons in different brain regions (cerebral cortex, hippocampus, olfactory bulb, cerebellum and remaining areas) and crossed the two datasets to test the *a priori* hypothesis of correlation between cognitive abilities and numbers of neurons. Surprisingly, performance in the behavioral tasks did not correlate strongly with number of neurons in any of the brain regions studied. Our results show that whereas neuronal number is a predictor of cognitive skills across species, it is not a good predictor of cognitive, sensory or motor ability across individuals within a species, which suggests that other factors are more relevant for explaining cognitive differences between individuals of the same species.

1. Introduction

Brain size varies by more than 100,000-fold across species [1], and it has long been expected that this variation is related to the animal's cognitive skills. For example, a measure of behavioral innovation derived from a systematic collection of field notes of previously unreported behaviors shows a positive correlation with forebrain size across bird species [2]. Deaner and colleagues [3] found that within the primate order, absolute brain size is a good predictor of a global cognition index extracted from meta-analyses. In a large, multi-group, coordinated effort to study many different species, MacLean and colleagues [4] showed that absolute brain size is the best neuroanatomical predictor of performance in a task of self-control.

Brain size may also be an indicator of cognitive ability across individuals within the same species. Anderson (1993) reported that in rats, cognitive performance correlates with brain size [5,6]. Witelson and colleagues found that brain volume explains ~34 % of the variance in verbal ability in women [7].

Brain size was considered a proxy for the number of brain neurons both across and within species, based on assumptions about universal scaling rules of neuronal density and uniform surface densities of neurons within and across cortical areas [8,9]. More neurons would bring larger information processing capacity, learning and flexibility [10,11]. Recent evidence suggests that brain mass and number of neurons do not scale in the same way across species, and that within a species, they are not correlated at all [12,13]. Thus, it has been proposed that numbers of neurons in the cerebral cortex - and not a brain size proxy - are the main neuroanatomical determinants of cognitive abilities across species [10,12,14,15]. The question remains as to whether the relationship holds true *within* a species.

Similarly aged individuals of a non-isogenic mouse strain exhibit variation of 1.33-fold in brain size and 1.63-fold in number of neurons [13]. Until recently [16], it was impractical to estimate numbers of neurons for large numbers of individuals, and few studies directly

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addressed the relationship between an animal's cognitive performance and its neuron number. The few studies that did studied induced changes, such as maternal treatment with growth hormone [17,18] and induced tetraploidy in salamander embryos [19,20]. Such studies showed that manipulating the number of neurons affects cognitive performance, but did not address the relationship in healthy, normally developed animals.

We sought to address this question directly: is there a relationship between naïve (untrained) performance in behavioral tests and numbers of neurons in functionally relevant brain structures? To this end, swiss mice underwent behavioral tasks designed to measure cognitive, motor and olfactory ability and then we counted the number of neurons in their brain regions. Specifically, we tested the following *a priori* hypotheses: performance in an olfactory test and in the rotarod test correlate with number of neurons in the olfactory bulb and cerebellum, respectively; performance in a navigation learning task correlates with number of neurons in the hippocampus and cerebral cortex; performances in tasks that require perceptual and executive functions correlate with number of neurons in the cerebral cortex and hippocampus.

2. Methods

2.1. Behavioral testing

All experiment procedures used in this study were approved by the Animal Care and Use Committee at Federal University of Rio de Janeiro (protocol number 01200.001568/2013-87).

Male mice were aged 2 months at the beginning of behavioral testing, and were naïve (inexperienced) in any type of behavioral tasks. All mice were housed in groups of 2–5 per cage. We performed the tests in the following order: olfactory test, rotarod, Morris water maze, puzzle-box and operant conditioning. To control for the effects of human manipulation and physical exercise, the control group underwent the physical part of each test, without the cognitive challenge, as explained below for each task. Mice were euthanized at the end of the behavioral testing when they were 4 months old.

2.1.1. Olfactory test

We used a modified version of the hidden peanut butter finding test [21]. On the first day, mice were food-deprived and given one peanut each to prevent bait shyness. On the next day, we exposed them to an arena of 40 × 40 cm filled with bedding, and a buried peanut in it. In three trials starting at different positions, we allowed mice 15 min to find the peanut. The latency to find the peanut in each of the three trials was recorded and summed, giving the total latency used as a score, so smaller scores indicate better performance. We exposed control animals to the same procedure but without the buried peanut in the arena.

2.1.2. Rotarod

To assess balance and motor learning, we measured the latency to fall from a mouse rotarod (Insight Equipamentos, Brazil). On the first day, we exposed mice to a rotating rod starting at 14 RPM and accelerating until 35 RPM for a maximum of 4 min for trial. Mice underwent 5 trials and we recorded the latency to fall in three trials, after discarding the highest and lowest latencies. On the next day, mice underwent the same experiment for three trials, and the final score was the sum of the latency to fall over 6 trials in the first and second days. Control mice were exposed to the rotarod at a constant 14 RPM for a similar duration.

2.1.3. Morris water maze

The maze (circular tub, 110 cm diameter) was filled with water at 22 °C made opaque by the addition of milk powder. All testing occurred under dim lighting. After thirteen training trials across three days to find a hidden platform, mice were left in the maze for 90 s without the platform. The goal quadrant was the one in which the platform was located, and the opposite quadrant was the one not adjacent to the goal quadrant. The score was calculated by subtracting the amount of time a mouse spent on the goal quadrant from the time it spent in the opposite quadrant.

2.1.4. Puzzle-box

The test was adapted from [22]. Briefly, the arena consisted of a wooden white box divided by a removable barrier into two compartments: a brightly-lit start zone (58 cm long, 28 cm wide) and a smaller covered goal zone with bedding and food (15 cm long, 28 cm wide). Lightly food-deprived mice always started in the brightly-lit zone, and were given a 5 min opportunity to go to the goal zone three times per day, undergoing a total of nine trials (T1–T9) over 3 consecutive days. Over the nine trials, they were challenged with obstructions of increasing difficulty placed at the underpass. The sum of the latencies to reach the goal zone in each trial was used as a readout of the puzzle-box test, so smaller scores indicate better performance.

2.1.5. Operant conditioning

We adapted an auditory operant conditioning task from [23]. For 32 days, testing consisted of different phases that involved the following cognitive capacities: auditory tone discrimination, attention, memory, problem solving and cognitive flexibility. All testing sessions consisted of 1 h per day, except the second phase, which consisted of two sessions of 30 min per day. To motivate mice to participate, they were lightly food-deprived in order to maintain around 90 % of the baseline weight. In the first phase of testing, they got a food reward for making a “go” response. The reward consisted of a 20 mg food pellet (BioServe, Frenchtown, NJ). After making more than 40 responses for two sequential days, the mice went to the second phase, in which they had to make a go response only during a 3 s window after the presentation of a 600 ms tone. Mice graduated after making a correct go response at least 70 % of the time again for two sequential days, which was the same criteria for the following phases. On the next phase, there were 3 different sounds and mice had to discriminate the correct one. In the fourth phase, mice had to find a correct tone in a new set of 3 different sounds of 200 ms each. Finally, the fifth phase consisted of a 1-back, in which mice had to perform a go response when a 200 ms tone A was played following another specific 200 ms tone B. All tones were presented at 70 dB SPL. Testing was performed in an acoustically transparent operant training chamber contained within a sound-attenuated chamber. The performance was calculated multiplying the number of days spent on each phase by its number and summing the results (if an animal stayed 16 days in phase 1 and 16 days in phase 2, the score would be (16*1) + (16*2) = 48). Therefore, mice that reached higher phases faster over the 32 days of testing got higher scores.

2.2. Perfusion and dissection

Animals were killed at 4 months of age by an overdose of ketamine and xylazin (300 mg/kg and 30 mg/kg, respectively) through intraperitonial injection and perfused with 4% phosphate-buffered paraformaldehyde (PFA) Brains were removed, post-fixated and then dissected into cerebral cortex, hippocampus, cerebellum, olfactory bulb and remaining areas (composed of all remaining regions of the brain, here referred as RoB for “rest of brain”). The cerebral cortex was further divided into anterior and posterior regions. Details of the perfusion and dissection are available in the Supplementary Material.

2.3. Isotropic fractionator

After post-fixation, brains were processed to obtain estimates on their number of neuronal and non-neuronal cells, using the isotropic fractionator [16]. This method has been shown by two independent groups to give estimates comparable to stereological estimates [24–26] and to have high reliability [27].

Briefly, structures were mechanically dissociated then stained with DAPI (4′-6-diamidino-2-phenylindole dihydrochloride, Invitrogen, USA) to identify the nuclei. The density of DAPI-stained nuclei in the suspension was estimated by counting in a fluorescence microscope using a Neubauer chamber. Similarly, neuronal nuclei were identified by immunocytochemistry for NeuN (Millipore mab377; RRID: AB_11204707; [28,29]. This allowed us to estimate the absolute number of cells and neurons in the tissue. The number of non-neuronal cells was determined by subtraction.

2.4. Statistics

All statistical analyses were performed in R 3.4.1 ([40]; RRID: SCR_001905). Performance in the behavioral tasks was ranked and the ranks were considered in the analyses. Pairwise correlations between the cellular composition of brain structures and the performances in the behavioral tasks were calculated using Spearman rank correlation, which allows the identification of non-linear associations as well. We chose an alpha level of 5% for statistical significance. For the power analysis, we followed [30,31]. Attained power is similar to the usual statistical power, but instead of considering a fixed alpha, it considers the obtained p-value – i.e. what is the chance of obtaining a p-value smaller than the one we got, for a given effect size, instead of the chance of obtaining a p-value smaller than alpha [30]. For the test of the comparison between control and trained groups, the power curve was calculated for a one-sided *t*-test with different sample sizes, using R.

3. Results

First, we investigated whether performance between tasks was correlated. We hypothesized a priori that the three tasks that depend the most on higher cognitive functions (puzzle-box, operant conditioning and Morris water maze) would have positively correlated performances. However, all correlations were non-significant, except for Hidden Peanut Butter Test x Puzzle Box (Supplementary Table 1). Therefore, in the analysis, we used the individual scores.

We then proceeded to test our *a priori* hypotheses regarding the number of neurons and performance in functionally-relevant brain regions. However, we found no significant correlations between the number of neurons in the olfactory bulb and the latency to find the peanut in the olfactory test, number of neurons in the cerebellum and latency to fall in the rotarod task, number of neurons in the hippocampus and performance in the Morris Water Maze, and number of neurons in the cerebral cortex and performance in the Morris Water Maze, number of neurons in the hippocampus and the cerebral cortex with performance in the puzzle-box and with performance in operant training (Supplementary Figs. 2–9). Taken together, these results indicate that the number of neurons is not a predictor of individual behavioral performance in mice (see Table 1 for all correlations, including the ones that were not planned). Similarly, for our *a priori* hypotheses, we did not find strong correlations between behavioral performance and neither the numbers of non-neuronal cells in the relevant structures above nor the total number of cells, neuronal densities and structure mass (Supplementary Tables 2–5).

To rule out the possibility that exposing mice to our battery of cognitive tests could alter the numbers of neurons, we compared the number of neurons of control and tested mice in the cortex and the hippocampus, because the number of neurons of these two brain regions are labile by environmental manipulations, such as physical exercise [42,43]. We did not find significant differences between control and tested animals in the number of neurons in the cortex and in the hippocampus (Supplementary Fig. 1).

We also investigated the possibility that our design did not find any statistically significant correlation because of lack of statistical power. The confidence interval for some correlations include values that could be relevant (e.g. number of neurons in the cerebellum and performance

Table 1
Correlations between behavioral performance in the tasks and number of neurons in different structures. Each cell in the table shows the Spearman correlation and 95 % confidence interval between behavioral performance and number of neurons for the various tasks and brain regions. No correlations but one, which was not an *a priori hypothesis* (Cerebral cortex, posterior x Rotarod) were significant at the 5% level. Sample size is 26-32, depending on the comparison. *Cerebral cortex, total* includes the hippocampus. *Whole brain* excludes the olfactory bulb.

Number of Neurons								
	Cerebellum	Cerebral Cortex, anterior	Cerebral Cortex, posterior	Cerebral cortex, total	Hippo-campus	Olfactory Bulb	Rest of Brain	Whole Brain
Hidden Peanut Butter Test	−0.27 [−0.57, 0.09]	−0.26 [−0.56, 0.1]	−0.23 [−0.53, 0.13]	−0.19 [−0.51, 0.17]	0.06 [−0.3, 0.4]	−0.13 [−0.46, 0.23]	−0.24 [−0.54, 0.12]	−0.3 [−0.59, 0.05]
Morris Water Maze	0.17 [−0.22, 0.52]	0.03 [−0.35, 0.41]	−0.32 [−0.62, 0.07]	−0.29 [−0.6, 0.1]	0.03 [−0.35, 0.4]	−0.03 [−0.4, 0.35]	0.03 [−0.36, 0.4]	−0.02 [−0.4, 0.36]
Operant Conditioning AUC	0.16 [−0.19, 0.48]	−0.05 [−0.39, 0.3]	−0.13 [−0.45, 0.23]	−0.21 [−0.52, 0.14]	−0.14 [−0.46, 0.21]	0.06 [−0.29, 0.4]	−0.19 [−0.5, 0.16]	−0.02 [−0.36, 0.33]
Puzzle Box	0.05 [−0.31, 0.39]	−0.2 [−0.52, 0.16]	−0.16 [−0.48, 0.2]	−0.17 [−0.49, 0.19]	−0.11 [−0.44, 0.25]	−0.13 [−0.46, 0.23]	0 [−0.35, 0.35]	−0.02 [−0.37, 0.33]
Rotarod	0.18 [−0.18, 0.49]	0.3 [−0.05, 0.58]	0.35 [0.01, 0.62]	0.33 [−0.01, 0.61]	−0.09 [−0.42, 0.26]	0.03 [−0.33, 0.37]	−0.16 [−0.48, 0.19]	0.2 [−0.15, 0.51]

Table 2
Summary of power analysis results. Following [31] and Mayo (2018, section 5.3), we calculated power, attained power for each of our *a priori* hypotheses, shown here for three different assumed "true" effect sizes (0.1, 0.3, 0.5). Power was calculated at a 5% significance level. Except for very small effects (0.1), the study is well-powered, in general.

Hypothesis	Expected signal, *a priori*	N	Spearman correlation with 95 % CI (our estimate)	P-value	Assumed "True" Effect Size	Power	Attained Power
Olfactory Bulb x Hidden Peanut Butter Test	negative correlation	30	−0.13 [−0.46, 0.23]	0,98	0,1	8,2 %	98,3 %
					0,3	37,1 %	99,5 %
					0,5	82,5 %	100,0 %
Cerebellum x Rotarod	positive correlation	32	0.18 [−0.18, 0.49]	0,22	0,1	8,5 %	28,9 %
					0,3	39,3 %	68,3 %
					0,5	85,0 %	96,3 %
Hippocampus x Morris Water Maze	positive correlation	26	0.03 [−0.35, 0.4]	0,97	0,1	7,7 %	97,4 %
					0,3	32,6 %	99,1 %
					0,5	76,3 %	99,9 %
Cerebral Cortex x Morris Water Maze	positive correlation	26	−0.29 [−0.6, 0.1]	0,06	0,1	7,7 %	9,1 %
					0,3	32,6 %	35,6 %
					0,5	76,3 %	78,8 %
Hippocampus x Puzzle Box	negative correlation	31	−0.11 [−0.44, 0.25]	0,87	0,1	8,3 %	88,8 %
					0,3	38,2 %	96,7%
					0,5	83,8 %	99,8 %
Cerebral Cortex x Puzzle Box	negative correlation	31	−0.17 [−0.49, 0.19]	0,32	0,1	8,3 %	39,0 %
					0,3	38,2 %	75,4 %
					0,5	83,8 %	97,5 %
Hippocampus x Operant Training	positive correlation	32	−0.14 [−0.46, 0.21]	0,07	0,1	8,5 %	11,2 %
					0,3	39,3 %	45,2 %
					0,5	85,0 %	88,3 %
Cerebral Cortex x Operant Training	positive correlation	32	−0.21 [−0.52, 0.14]	0,15	0,1	8,5 %	21,1 %
					0,3	39,3 %	60,2 %
					0,5	85,0%	94,1 %

on the rotarod task shows a correlation of 0.18, with a 95 % confidence interval going from -0.18 to 0.49). To address this possibility we did a post-hoc power analysis. Complete results are shown in Table 2. For a correlation of $\rho = 0.3$ - comparable to the one reported for human brain volume and IQ in [6] -, the attained power is typically above 60 %, sometimes over 90 %. In our study, however, we expected a correlation larger than that, for two main reasons: (1) as we have argued before, neuron number should be more strongly correlated with behavioral performance across species than brain volume and (2) greater variability of subjects in human studies adds noise to the estimate, compared to the present study with lab mice, raised in a controlled environment. For a larger correlation of 0.5, for our *a priori* hypotheses, the average power for the sample size in our study is over 90 % (at the 0.05 significance level), which is what we considered in our original sample size calculation, before the experiment. For the comparison between the control and tested mice, the study was well-powered (80 %) for an effect size (Cohen's d) of 0.66, and even more so (> 95 %) for the large effects reported in the literature for the effect of exercise (Cohen's d between 4 and 6, see Supplementary Table 13) [41,42]. While all of this assumes reliable measurements, this suggests that the design is well-powered for these medium to large effects.

4. Discussion

Although it is established that neuronal death is accompanied by worse behavioral performance across many tasks [32,33], to our knowledge, our study is the first that attempts to investigate whether the number of neurons in normally-developed mice is associated with individual variation in behavioral performance. Our results suggest that naturally occurring variation in neuron number is not associated with variation in performance at the level of inexperienced individual animals within a species.

Similarly to environmental enrichment or exercise [42,43], exposing mice to our cognitive battery could have changed neuronal numbers in ways that masked initial individual differences associated with behavioral performance. However, it is unlikely that this played a role in our results, because our battery of behavioral tests did not influence neuronal number in the cortex nor in the hippocampus compared with untrained mice (Supplementary Fig. 1).

While we focused on neurons, it is known that glial cells also influence behavior. Han and colleagues [34] investigated the effect of astrocytes on cognition, using a chimeric mouse model with grafted human astrocytes. They report that the chimeric mice perform better than control mice in an object location memory test and fear conditioning tasks. However, we did not find strong correlations for any of our *a priori* hypotheses between individual behavioral performance and numbers of non-neuronal cells, nor total cells, neuronal density and mass (Supplementary Tables 2–5).

Precise measures are essential to detect small effects. Regarding the behavioral measures, it is known that typically uncontrolled variables can have effects on animal behavior [35]. The isotropic fractionator is a precise enough for detecting within-species differences, for instance, between isogenic (C57BL/6) and non-isogenic (Swiss) mice, and its intrinsic error from the counting procedure and immunostaining amounts only to ~15 % [27]. Power analysis showed our design had enough power to detect large effect sizes, based on what has been described on the literature (Table 2). Therefore, we argue that whatever correlation might exist between the quantities analyzed here is likely to be as small or smaller than what one could reasonably expect to be found in humans (such as in McDaniel, 2005), explaining less than 10 % of the variance in cognitive performance in these tasks. Whereas the number of neurons is a strong predictor of cognitive performance across species [10,12,14,15], our study suggests that this is not the case within a species. It may be simply that the magnitude of the differences within a species is not large enough to have a consistent, detectable impact on cognitive abilities, even if neurons are the limiting resource, evolutionarily speaking, for increasing the information processing capacity of the brain, due to the high optimization of most brain components [36].

A small correlation between behavioral performance and neuron number at the individual level invites a number of other possible explanations. First, we might not have tested the abilities for which having more neurons is beneficial. One recent study reviewed the literature in search of measures of cognitive performance of three animals - pigeons, corvids, and apes, as a function of increasing neuron number -

on different tasks [37]. They found that the maximum level of performance is the same for all three animals in some of the tasks (e.g. short-term memory and abstract numerical competence). Remarkably, while pigeons do reach the same level of performance as primates and corvids on some tasks, they are slower to learn and have difficulty generalizing and transferring the associations to new contexts [37]. This suggests that the number of neurons might matter for the speed of learning, storage or generalization, but not to the final level of performance. Although the measures extracted from the cognitive tasks used in this study do take into account the learning rate, they also depend on the final level of performance.

One possibility is that having more neurons makes a larger difference for sensorimotor abilities, but not higher-order ones: more neurons could result in more precision in stimulus representation. Having more neurons available would decrease interference and overlap between stored patterns, reducing confusion in retrieval. Accordingly, a training-induced increase in the number of auditory neurons that respond preferentially to a given sound frequency correlates positively with a rat's ability to identify said sound frequency [38,39,41]. However, we could not determine the number of neurons in sensorimotor cortices because their dissection is not reliable.

Future studies should aim to identify better predictors of variation in behavioral performance across individuals of the same species, such as genetic differences, numbers of synapses, numbers of specific glial cell subtypes, degree of myelination or dendritic arborization. In light of our results, we expect that, at the individual level, these variables may better predict the experience-dependent plasticity that underlies learning according to the individual history of interaction with the environment.

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Author statement

KN, GDG, SHH and RP designed research; KN, GDG, YAT, SC, AP and KM performed research; KN and GDG analyzed data; KN, GDG, SHH and RP wrote the paper.

CRediT authorship contribution statement

Kleber Neves: Conceptualization, Funding acquisition, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Gerson Duarte Guercio:** Conceptualization, Methodology, Funding acquisition, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. **Yuri Anjos-Travassos:** Investigation, Methodology. **Stella Costa:** Investigation. **Ananda Perozzo:** Investigation. **Karine Montezuma:** Investigation. **Suzana Herculano-Houzel:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing. **Rogério Panizzutti:** Conceptualization, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors report no conflict of interest.

Appendix A. Supplementary data

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