Charles University
Faculty of Science

Study programme: Zoology

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Evolution of brain complexity and processing and cognitive capacity in selected vertebrates

Evoluce komplexity a procesní a kognitivní kapacity mozku u vybraných obratlovců

Doctoral thesis

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Prague, 2022

Prohlašuji, že jsem závěrečnou práci zpracovala samostatně a že jsem uvedla všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla předložena k získání jiného nebo stejného akademického titulu.

V Praze, dne 24. 11. 2022

Kristina Kverková

"What source was there back then, save for our overelaborate nervous circuitry, for the evils we were seeing or hearing about simply everywhere? My answer: There was no other source. This was a very innocent planet, except for those great big brains."

– **Kurt Vonnegut, Galápagos**

Acknowledgements

Submitting a doctoral thesis is one of those moments that inspire people to express their undying gratitude for their parents, friends, extended family, neighbours, and the United Nations. Not being immune to this impulse, I'm going to indulge in some thanksgiving and reflection of my own. First and foremost I want to thank my advisor, who taught me not to worry and love the academia. I'm still nowhere near his level of carefree optimism but definitely less neurotic than when I started. Thanks to him I can now click the submit button five minutes before the deadline completely unfazed. And I feel like an impostor maybe only half of the time. When I first approached him, stating my interest in "brains and mole-rats", he suggested the perfect topic and I've been hooked ever since. I very much appreciate the generous amount of freedom, support and trust that he gave my throughout my studies.

I'm also grateful to all the other awesome people at the Faculty of Science and the Department of Zoology. With such inspiring classes and discussions and friendly atmosphere, my experience has been nothing short of amazing. And I have to thank the secretaries for being so kind to me, even though I still can't get any paperwork right on the first try. Special thanks go to my past and present labmates, who made working here such a blast. Every day has been fun, grinding in the lab, travelling to conferences, late night perfusions, parties, and board game sessions… and I hope we built lasting friendships. I'm of course grateful to the funding agencies that kept a roof over my head and to the many collaborators and people who provided us with animals.

On a more personal note, I wish to thank my wonderful husband, who unwittingly inspired me to let go of my comfortable career and plunge into the whirlwind world of science, and then supported me wholeheartedly. Since then I realized there is no better way to combine my many interests. Some of my favorite activities as a child involved growing different kinds of mold on orange peels, training house flies, and performing brain surgery on teddy bears. So I owe a lot to my parents, who not only put up with my experiments, but were always willing to discuss anything and kept a steady supply of books coming. My relationship with statistics goes back to rolling dice 1000 times to see if I can get more sixes by wishing really hard (sadly, I can't). Now, I actually get paid to dissect brains and analyse real data! This is a dream come true and I consider myself very lucky to be able to continue chipping away at the mysteries of science. Thanks everyone, I couldn't have done this without you!

Abstract

Brain processing capacity has traditionally been inferred from data on brain size. However, recent studies have shown that similarly sized brains of distantly related species can differ markedly in the number and distribution of neurons, their basic computational units. Therefore, a finer-grained approach is needed to reveal the evolutionary paths to increased cognitive capacity. This quantitative approach to the evolution of brain processing capacity at the cellular level is relatively new, since quick and reliable estimation of the number of neurons in whole brains or large brain regions has only become possible in the past 15 years or so with the introduction of the isotropic fractionator. This method of determining brain cellular composition is applicable to a wide range of questions. We can assess intraspecific variation, both at the individual and population level, examine the effect of sex and age, and the study selection at the intraspecific level. At the other side of the spectrum, we can study large macroevolutionary trends or try to isolate the effect of specific selective pressures by comparing more closely related and ecologically similar species. In this thesis, I explored variation in brain size and brain cellular composition across vertebrates at both intraspecies and interspecies level.

In Chapter 1, we showed that different populations of the Madagascar ground gecko can vary substantially in the number of brain neurons. There were no sex differences in brain size, number of neurons, or neuron density, even though the species is moderately sexually dimorphic. We also provided evidence that postnatal neurogenesis in geckos does not only replace lost neurons but adds new ones and that this is especially pronounced in the adult telencephalon.

In Chapter 2, we assessed the effect of artificial selection for large relative brain size in guppies on the numbers of neurons. We discovered that it leads to a corresponding increase in the number of neurons. Female guppies in the small-brained and large-brained groups did not differ in neuron density, so the larger brains translated linearly to an increase in neurons. This might explain a host of enhanced cognitive abilities previously described in the large-brained guppies.

In Chapter 3, we tested the social brain hypothesis by directly looking at neuron numbers for the first time. Using Bayesian phylogenetic generalized linear mixed models, we found no association between sociality and any measure of brain size or proxy of brain capacity, showing that sociality in and of itself does not necessarily lead to larger brains and intelligence. It seems that metabolic constraints and possibly increased hypoxia tolerance outweigh any potential benefits of higher brain processing capacity in this specific case and that the nature of social complexity (organisational vs. relational) might be an important factor.

Finally, in Chapter 4, we reconstructed the evolution of brain neuron numbers in amniotes. We analysed a dataset comprising brain sizes of almost 4000 species of amniotes and neuron numbers in three major brain parts of 251 species. We found that non-avian reptiles have rather low absolute numbers of neurons. Besides their low encephalization (brain size relative to body size), they also feature lower neuron densities, resulting in substantially fewer neurons per body mass compared to endotherms. This holds despite the fact that, across amniotes, neuron densities go down with increasing brain size. Using reversible jump MCMC, we were able to identify significant changes in neuron-brain scaling along amniote phylogeny, without any *a priori* hypotheses. We found that birds and mammals have independently increased not only brain size, but also neuron density, converging on a similar scaling relationship, while there were no significant shifts within non-avian reptiles over the span of 325 million years. This again highlights the importance of energetic constraints in brain evolution. Moreover, this difference between endotherms and ectotherms is most pronounced in the cerebellum, not in the telencephalon. Other two major increases in relative brain size and neuron density occurred in anthropoid primates and core landbirds (Telluraves), again resulting in similar scaling.

Abstrakt

Procesní kapacita mozku bývá tradičně odhadována na základě velikosti mozku. Z nedávných studií však vyplývá, že u vzdáleně příbuzných druhů s podobnou velikostí mozku se může podstatně lišit počet neuronů, tedy základních výpočetních jednotek, i jejich distribuce do různých částí mozku. Abychom tedy dokázali odhalit, jakým způsobem se v evoluci kognitivní kapacita mění, potřebujeme pracovat s daty na jemnější škále. Takový kvantitativní přístup k evoluci procesní kapacity mozku na buněčné úrovni je relativně nový. Možnost rychle získat spolehlivé odhady počty neuronů v celém mozku nebo jeho větších částech je totiž k dispozici až v posledních zhruba 15 letech díky rozšíření metody izotropní frakcionace. Ta otevírá dveře k řešení celé řady otázek. S její pomocí můžeme zjišťovat vnitrodruhovou variabilitu na úrovni jedinců i populací, podívat se na efekt pohlaví a věku, nebo studovat selekci na vnitrodruhové úrovni. Z druhé strany spektra pak můžeme sledovat velké makroevoluční trendy nebo se zaměřit na porovnání blízce příbuzných a ekologicky podobných druhů a pokusit se tak studovat vliv jednotlivých selekčních tlaků.

Ve své disertaci se zabývám variabilitou velikosti mozku a jeho buněčného složení mozku napříč obratlovci, a to jak na vnitrodruhové, tak na mezidruhové úrovni. V kapitole 1 jsme ukázali, že různé populace gekona madagaskarského se mohou významně lišit počtem neuronů v mozku. Zároveň jsme nenašli žádné rozdíly mezi pohlavími ve velikosti mozku, počtu neuronů ani hustotě neuronů, přestože se jedná o druh s mírným pohlavním dimorfismem. Prokázali jsme také, že postnatální neurogeneze u gekononů neslouží jen k nahrazení ztracených neuronů, ale že neuronů v průběhu života přibývá, což je obzvláště patrné v koncovém mozku dospělců.

V kapitole 2 jsme zkoumali, jak se umělá selekce na větší relativní velikost mozku u živorodek *Poecilia reticulata* odrazí v počtech neuronů. Zjistili jsme, že tato selekce má za následek odpovídající narůst v počtu neuronů. Samice živorodek ze skupiny s malými a velkými mozky se mezi sebou nelišily v neuronální hustotě, takže zvětšení mozku se přímo lineárně promítlo do vyššího počtu neuronů.

Vysvětluje to lepší výkon v celé řadě kognitivních úloh, který byl u těchto živorodek selektovaných na relativně větší mozky popsán.

V kapitole 3 jsme provedli první test hypotézy sociálního mozku s přímým porovnáním počtu neuronů. Bayesiánské lineární smíšené modely s fylogenetickou korekcí neprokázaly žádné spojení mezi socialitou a velikostí nebo procesní kapacitou mozku. Ukázali jsme tak, že socialita sama o sobě k evoluci velkých mozků a inteligence nestačí. Metabolická omezení a potenciálně vyšší tolerance k hypoxii mohou v tomto konkrétním případě jít proti potenciálním přínosům větší kapacity mozku. Dalším důležitým faktorem může být samotná povaha sociální komplexity u rypošů (organizační, nikoli relační).

V kapitole 4 jsme rekonstruovali evoluci počtu neuronů u amniot. Analyzovali jsme rozsáhlý dataset velikostí mozku, který čítal téměř 4000 druhů amniot, a počty neuronů ve třech velkých částech mozku u 251 druhů. Ukázalo se, že neptačí plazi mají poměrně malé absolutní počty neuronů. Kromě nízké encefalizace (velikosti mozku relativně k velikosti těla) mají také nižší hustoty neuronů, takže v porovnání s endotermními skupinami mají ve výsledku podstatně méně neuronů na stejnou hmotnost těla, a to přesto, že u amniot obecně hustoty neuronů s rostoucí velikostí mozku klesají. Pomocí MCMC s reverzibilními skoky jsme detekovali významné změny ve škálování počtu neronů s velikostí mozku a těla ve fylogenezi amniot, aniž bychom museli specifikovat *a priori* hypotézy ohledně toho, ve kterých skupinách k nim došlo. Zjistili jsme, že ptáci i savci nezávisle zvětšili nejen mozky, ale také hustoty neuronů, přičemž obě skupiny konvergentně dospěly k podobnému škálování. Naproti tomu uvnitř neptačích plazů žádné výrazné změny během 325 milionů let evoluce neproběhly. Znovu to poukazuje na důležitou roli, kterou v evoluci mozku hrají energetická omezení. Tento rozdíl mezi endotermními a ektotermními amnioty je navíc nejvýraznější v mozečku, nikoli v koncovém mozku. K dalším dvěma zvětšením relativní velikosti mozku a hustoty neuronů došlo u antropoidních primátů a ptačí skupiny Telluraves, přičemž výsledné škálování u těchto dvou skupin je opět podobné.

Contents

List of publications included in the thesis

1) **Kverková, K., Polonyiová, A., Kubička, L., Němec, P.** , 2020. Individual and age-related variation of cellular brain composition in a squamate reptile. *Biol. Lett.* 16, 20200280. https://doi.org/10.1098/rsbl.2020.0280

Contribution of Kristina Kverková: conceptualization, formal analysis, investigation, project administration, visualization, writing – original draft preparation, writing – review & editing

Mgr. Pavel Němec, Ph.D

2) **Marhounová, L., Kotrschal, A., Kverková, K., Kolm, N., Němec, P.**, 2019. Artificial selection on brain size leads to matching changes in overall number of neurons. *Evolution* 73, 2003–2012. https://doi.org/10.1111/evo.13805

Contribution of Kristina Kverková: formal analysis, investigation, visualization, writing – review & editing

Mgr. Pavel Němec, Ph.D

3) **Kverková, K., Bělíková, T., Olkowicz, S., Pavelková, Z., O'Riain, J.M., Šumbera, R., Burda, H., Bennett, N.C., Němec, P.** , 2018. Sociality does not drive the evolution of large brains in eusocial African mole-rats. *Sci. Rep.* 8, 9203. https://doi.org/10.1038/s41598-018-26062-8

Contribution of Kristina Kverková: formal analysis, investigation, visualization, writing – original draft preparation, writing – review & editing

Mgr. Pavel Němec, Ph.D

4) **Kverková, K., Marhounová, L., Polonyiová, A., Kocourek, M., Zhang, Y., Olkowicz, S., Straková, B., Pavelková, Z., Vodička, R., Frynta, D., Němec, P.** , 2022. The evolution of brain neuron numbers in amniotes. *Proc. Natl. Acad. Sci. U.S.A.* 119, e2121624119. https://doi.org/10.1073/pnas.2121624119.

Contribution of Kristina Kverková: conceptualization, formal analysis, investigation, project administration, visualization, writing – original draft preparation, writing – review & editing

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INTRODUCTION

The brain is the most multifunctional organ, involved in virtually everything we do. That makes it both an extremely attractive and extremely tricky subject of study. We are on a quest to understand its working and how we, humans, got to have such disproportionately large brains. Consequently, comparative neuroscience has to deal with the fact that brain complexity cannot be reduced to any one dimension; in fact, it would be foolish to assume that it can. That said, if we want to make any progress in understanding brain evolution, settling down on a convenient proxy for brain "capacity" or "complexity" is necessary. Brain mass or endocranial volume (often used interchangeably) has been the favorite proxy in the field over several decades. The obvious advantage of this approach is that volume can be easily measured (and converted to mass by multiplying by brain tissue density (Stephan, 1960), and only an intact skull is required, no need to obtain the actual brain. This allows people to make use of vast museum collections and, importantly, include fossil species. As a result, nowadays, datasets of mammalian and avian brain sizes count thousands of species (Ksepka et al., 2020; Smaers et al., 2021; Tsuboi et al., 2018). However, while convenient, this notion of "size" might be a little too oversimplified. It completely ignores the relative sizes of major brain parts and carries the implicit assumption (actually explicitly stated by Jerrison, 1985) that brains of the same mass or volume are composed of the same number of neurons.

As it turns out, this assumption does not, in fact, hold. Although it has long been clear from histological images and stereological counts that species and brain parts can differ substantially in neuronal density, this fact went largely ignored in studies of brain evolution. Given that the information processing capacity of the brain depends on the number of neurons, neuron packing density, interneuronal distance, and axonal conduction velocity (Dicke and Roth, 2016; Herculano-Houzel, 2017) as well as other physiological factors, it seems highly desirable to account for these variables. Admittedly, estimating these numbers would have been extremely time-consuming and not practical at a large scale, until recently.

The situation somewhat changed with the advent of the isotropic fractionator – a relatively fast and accurate method of counting cells in whole brains or brain parts (Herculano-Houzel and Lent, 2005). Studies using this method challenged some long-held ideas, such as the number of neurons in the human brain or its glia-to-neuron ratio (Herculano-Houzel, 2012). They also confirmed that brains of the same size are not necessarily composed of the same number of neurons; specifically, primates feature much higher neuron densities than rodents, but they have similar numbers of glial cells per brain mass (Herculano-Houzel et al., 2007, 2006). The "neuronal scaling rules" (the allometric relationship between brain structure mass and number of neurons) have subsequently been established in several high-level mammalian clades: Afrotheria (Neves et al., 2014), Artiodactyla (Kazu et al., 2014), Carnivora (Jardim-Messeder et al., 2017), Chiroptera (Herculano-Houzel et al., 2020), Eulipotyphla (Sarko et al., 2009), Glires (Gabi et al., 2010), Marsupialia (Santos et al., 2017), and Primates (Herculano-Houzel et al., 2007). These studies revealed lineage-specific differences in neuronal densities and neuron allocation to different brain parts within mammals.

Later, a surprising result came to light: birds seem to have much higher brain neuron densities than mammals in general, especially in the pallium, making their absolute neuron numbers comparable to those of much larger primates (Olkowicz et al., 2016). This resolved a long-standing conundrum of how birds with rather modestly sized brains can perform some impressive cognitive feats. Clearly, taking a more in-depth look at the actual brain composition can yield solutions to problems that have previously been hard to figure out. However, it also opens up a range of new questions. The bird species included in the study were predominantly corvids and parrots, known for their intelligent behaviour and high encephalization. So is this brain composition common to all birds or is it specific to this clade? And is it the case that birds increased their neuron densities, possibly due to the constraints of flight that preclude having a large head, or are mammals the odd ones out among amniotes and actually decreased brain neuron densities? To provide some

answers, it is necessary to broaden the sampling of bird groups and to get data on all sauropsids, including non-avian reptiles, as these are essential for making inferences about evolutionary changes in amniotes. This is what I set out to do in the main part of my thesis.

Another controversial aspect in the field of comparative neuroscience and cognition is whether "cognitive capacity" is better explained by absolute or relative brain size (or neuron numbers, for that matter), with evidence supporting both sides of the argument (e.g. Benson-Amram et al., 2016; Deaner et al., 2007; Horschler et al., 2019; Kotrschal et al., 2013; MacLean et al., 2014; Sol et al., 2007). On the one hand, absolute numbers seem to make more sense, but we would have to yield our primacy to other, much larger animals. Elephants and many cetaceans have larger brains than humans in absolute terms (Haug, 1987). On the other hand, the notion that brain size relative to body size governs cognitive capacity does not have much theoretical justification besides humans coming on top. It rests on the assumptions that a larger body requires proportionally more neurons to control it and that any spare capacity above that is used for "higher cognition", and both are somewhat contentious. Having a relatively larger brain may (or may not) be the result of selection "on brain size". (Of course, when we say selection on brain *size*, we actually mean selection on some behaviour mediated by brain *function* that also translates into an increase in brain size. This sort of shorthand is so common in the literature that we might sometimes forget.) In fact, the same relative brain size can arise from any combination of increase/decrease in brain/body size (Smaers et al., 2012). Looking at both brain size and neuron numbers across large datasets might reveal some useful patterns, which is why I wanted to examine the relationship between relative neuron densities and relative brain size.

The assumption that to maintain the same level of cognitive power, larger bodies need more neurons (recently explicitly formulated as the cognitive equivalence hypothesis (Triki et al., 2022)) can be directly tested at the intraspecies level. Having robust data on individual variation and ontogenetic changes in brain size and

neuron numbers is essential for testing this hypothesis and determining adequate sample sizes for comparative studies. However, these data are rare and, on the neuronal level, they were only available for mammals, specifically mice (Fu et al., 2013; Herculano-Houzel et al., 2015b). Nothing was known about the individual variation in animals that exhibit extended postnatal neurogenesis, potentially translating into a higher variation in neuron numbers in adulthood. We addressed this question by looking at guppies and geckos. Additionally, there was no literature about the effect of age on neuron numbers in non-mammalian species. It is possible that neuron numbers decline much more slowly with advancing age in species with prolonged neurogenesis; they might also stay stable or even increase. Lastly, sexual differences in size or neuron numbers are well-documented in specific brain areas (Guillamón and Segovia, 1997; Panzica and Melcangi, 2016; Reid and Juraska, 1992), but are less straightforward when it comes to the whole brain (Joel and Fausto-Sterling, 2016). Using the gecko *Paroedura picta* as a model organism, we addressed the extent of individual variation both within and between populations, sex differences, and the effect of age on brain size and neuron numbers and densities in the whole brain and the telencephalon.

Apart from the total number of brain neurons, we might be also interested in numbers of neurons in specific brain areas. These will yield a more fine-grained picture of the brain. Advances in whole-brain clearing have made it possible to collect data on specific cell type counts in different brain areas in mammalian species (Costantini et al., 2021; Murakami et al., 2018; Zhang et al., 2017), while the isotropic fractionator is useful for quantifying neurons in larger brain compartments. It turns out that high-level mammalian clades and even species within a single order can differ substantially in neuron distribution. As a case in point, while the brain of the African elephant contains 3 times more neurons than the human brain, the vast majority of these is located in the cerebellum and only about 2% in the cerebral cortex (Herculano-Houzel, 2009; Herculano-Houzel et al., 2014). Humans have almost 20% of all neurons in the cerebral cortex, so the numbers are flipped and they outnumber the elephant by a factor of 3. The elephant's

enlarged cerebellum is likely connected to processing sensory-motor information from its unique appendage, the highly flexible and sensitive trunk. But in fact, all mammals are characterized by having the majority of their neurons in the cerebellum, although the preponderance is not quite as extreme as in the elephant (Herculano-Houzel et al., 2015a). So it came as a bit of a surprise that in parrots and corvids, most neurons are located in the telencephalon instead, while this is not the case for the emu, domestic pigeon and junglefowl (Olkowicz et al., 2016). Only a handful of outgroup species were included in that study, prompting the question of whether there are distinct patterns of brain neuron distribution in different bird groups and whether they are related to cognitive abilities.

The association between neuron numbers and densities and actual cognitive abilities is still controversial, and animal "intelligence" is notoriously difficult to study, especially across species. Devising tests that accurately reflect cognitive capacity and are not biased by lack of motivation and interspecies differences in perception, preferences and locomotion or manipulation abilities has proven tricky, but there are comprehensive studies that include a wide range of species (e.g. Bryer et al., 2022; MacLean et al., 2014). Studies comparing individuals of the same species are much easier to perform. The guppy *Poecilia reticulata* has long been used as a model organism for studying the effect of artificial selection for relative brain size on various behavioural measures. Larger-brained guppies tend to outperform smaller-brained ones, but it was not clear if and how the selection affects brain cellular composition. This is another question that we were able to address using the isotropic fractionator.

CHAPTER 1

Individual and age-related variation of cellular brain composition in a squamate reptile

BIOLOGY LETTERS

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Research

Cite this article: Kverková K, Polonyiová A, Kubička L, Němec P. 2020 Individual and age-related variation of cellular brain composition in a squamate reptile. *Biol. Lett.* **16**: 20200280. http://dx.doi.org/10.1098/rsbl.2020.0280

Received: 24 April 2020
Accepted: 7 September 2020

Subject Areas:
evolution

Keywords:
number of neurons, number of glial cells, brain size, intraspecific variation, cellular scaling rules, evolution

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Electronic supplementary material is available online at https://doi.org/10.6084/m9.figshare.c.5120436.

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Neurobiology

Individual and age-related variation of cellular brain composition in a squamate reptile

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Within-species variation in the number of neurons, other brain cells and their allocation to different brain parts is poorly studied. Here, we assess these numbers in a squamate reptile, the Madagascar ground gecko (*Paroedura picta*). We examined adults from two captive populations and three age groups within one population. Even though reptiles exhibit extensive adult neurogenesis, intrapopulation variation in the number of neurons is similar to that in mice. However, the two populations differed significantly in most measures, highlighting the fact that using only one population can underestimate within-species variation. There is a substantial increase in the number of neurons and decrease in neuronal density in adult geckos relative to hatchlings and an increase in the number of neurons in the telencephalon in fully grown adults relative to sexually mature young adults. This finding implies that adult neurogenesis does not only replace worn out but also adds new telencephalic neurons in reptiles during adulthood. This markedly contrasts with the situation in mammals, where the number of cortical neurons declines with age.

1. Introduction

Even though numerous studies have pointed out the importance of brain neuron number, density and distribution as a proxy for brain processing capacity [1–4], individual variation in brain composition is virtually unknown. Data are available only for the laboratory mouse *Mus musculus* [5] and the guppy, *Poecilia reticulata* [6]. Both studies uncovered substantial individual variation in neuronal density, but despite that, guppies with bigger brains have more neurons on average, which is not true in mice. Unlike birds and most mammals (and the guppy), reptiles generally continue to grow for longer periods of time, even after attaining sexual maturity. Limited information is available as to what happens to the brain at the cellular level. Studies in the Nile crocodile (*Crocodylus niloticus*) have shown that the brain continues to grow, albeit with significant negative allometry [7] and the number of neurons grows with an even shallower slope, approaching an asymptote long before reaching maturity [8]. A postnatal increase in the number of neurons in several cortical areas has also been reported in the lizard *Podarcis hispanica* [9].

However, the existence of substantial neurogenesis throughout life [10–14] (reviewed in [15]) might potentially result in a higher variation in neuronal numbers in adult animals, complicating quantitative comparative studies. To assess this, we tested individual variation in the brain (divided into cerebral hemispheres and rest of the brain) mass and number of neurons and non-neuronal cells in a model reptile. As a suitable species, we used the Madagascar ground gecko (*Paroedura picta*), a fast-growing reptile, attaining sexual maturity at around 3 months of age and fully grown between 1 and 2 years [16].

We hypothesized that reptiles may have higher individual variation in the number of neurons compared with mammals owing to their extensive adult neurogenesis.

When evaluating within-species variation, we should account for the fact that there are, in essence, two components of this variation, one being the inter-individual differences within a population, and the other being differences between populations potentially under different selective pressures and adapted to different environments [17,18]. Many studies assessing individual variation within a species are limited to one captive population. On the one hand, this can mitigate problems with mixing animals of unknown history, subjected to different environmental conditions. On the other hand, it might also lead to underestimating the actual variability of the trait. To overcome this issue, we included two captive populations of recent wild ancestry (F1, and in a few cases F2 generation) and assessed both interpopulation and intrapopulation variation. Age and sex are other important factors contributing to within-species differences. Sexual dimorphism in relative brain size is present in some squamate species but not others [19] and sex differences in the volume of specific brain parts (e.g. [20–23]) are well documented in reptiles. Owing to prolonged neurogenesis, the numbers of brain neurons in reptiles might potentially decrease with age at a slower pace than in mammals, or even increase. To be able to assess the effect of sex and age, we used animals of both sexes in close to equal proportion and included three different age groups from one population, encompassing hatchlings, young adults and fully grown adults.

2. Material and methods

(a) Animals

For this study, we used the Madagascar ground gecko (*Paroedura picta*), a well-studied reptile, e.g. [24–27]. We compared two sources of variation—intrapopulation and interpopulation—for that purpose, we obtained 10 adult animals (5 males, 5 females) from a private breeder (population A) and 10 animals (5 males, 5 females) from the breeding facilities of the Faculty of Science of Charles University, Prague (population B). All the individuals were fully grown adults between 1 and 2 years of age, close to maximum body size (mean snout–vent length (SVL): 84 mm, 93% of the maximum reported SVL [28]). We included a group of fourteen 14-day-old hatchlings (5 males, 9 females) and ten 6-month-old sexually mature but not fully grown animals (herein referred to as 'young adults') (5 males, 5 females) from the breeding facilities of the Faculty of Science of Charles University (population B) to compare the variation in different age groups from the same population.

(b) Brain processing

The animals were euthanized by anaesthetic overdose and perfused with 4% paraformaldehyde. Brains were removed, post-fixed and divided into the telencephalon (cerebral hemispheres, excluding olfactory bulbs) and the rest of the brain. To explore differences between brain regions, in a subset of individuals (adults from population A) we analysed the distribution of neurons in six brain divisions, namely in the cerebral hemispheres, olfactory bulbs, diencephalon, optic tectum, cerebellum and brainstem (electronic supplementary material, figure S1). In these brain components the total numbers of cells, neurons and non-neuronal cells were estimated using the isotropic fractionator [29], a fast and accurate technique that provides results comparable to unbiased stereology [30–32]. The protein NeuN was used as a nuclear marker of neurons [33]. Further details are provided in the electronic supplementary material.

(c) Statistical analysis

All the statistical tests were performed in the base package of R 3.6.2. [34]. To ensure normality, the continuous variables were $\log_{10}$-transformed. The relationships between cell numbers or density and brain region or body mass were analysed using linear models. The differences between group means were tested with one-way ANOVA and Tukey's *post-hoc* test and the variances were compared with Bartlett's test. Although the assumption of homogeneity of variances was violated in a few cases, similar results were obtained when using non-parametric tests.

3. Results

(a) The Madagascar ground gecko brain in cell numbers

The 20 fully grown animals varied 2.26-fold in body mass (10.54–23.8 g), 1.41-fold in brain mass (73.6–103.6 mg) and 1.94-fold in number of brain neurons (2.9–5.6 millions) and 2.28-fold in number of other cells (2.8–6.3 millions) (electronic supplementary material, table S1). Unlike in mice [5], brain mass and body mass were correlated ($F_{1,18} = 12.76$, $p = 0.002$, $R^2 = 0.38$), the number of brain neurons and brain mass were correlated ($F_{1,18} = 14.21$, $p = 0.001$, $R^2 = 0.41$; figure 1*a*), but there was not a significant correlation between the number of neurons and body mass ($F_{1,18} = 3$, $p = 0.1$; figure 1*b*), whereas the number of other brain cells and body mass were weakly correlated ($F_{1,18} = 5.21$, $p = 0.035$, $R^2 = 0.18$).

In addition, we assessed brain cell distribution in six brain parts in adults of population A (electronic supplementary material, table S2). The number of neurons was significantly correlated with structure mass only in the cerebellum ($F_{1,8} = 5.517$, $p = 0.006$, $R^2 = 0.57$). In all structures, neuronal density decreased with increasing structure mass ($p < 0.001$ in all cases).

(b) Intrapopulation and interpopulation variation and sex differences

We also assessed brain variation within the two populations (electronic supplementary material, table S1; figure 1*c–f*). The intrapopulation variances in both groups are comparable, except for brain mass, which has a significantly higher variance in population A (Bartlett's $K^2 = 4.76$, $p = 0.03$). The population means were significantly different in all the parameters ($p < 0.03$ in all cases) except neuronal density ($F_{1,18} = 3.77$, $p = 0.068$), with population B having larger brains with more cells. There were no statistically significant differences in variance or mean between the sexes in any of the traits examined (in all cases $p > 0.3$, table S3; figure 1*c–f*).

(c) Differences in age groups within population B

The 6-month-old geckos had significantly smaller brains than the fully grown geckos (Tukey HSD: $p < 0.0001$), but they did not significantly differ in the number of whole-brain neurons (Tukey HSD: $p = 0.78$) and non-neurons (Tukey HSD: $p = 0.94$). The 14-day-old geckos had significantly smaller brains and fewer neurons and other brain cells than either of the adult groups (Tukey HSD: $p < 0.001$ in all cases) (electronic supplementary material, table S4; figure 2*a*,*b*).

The 6-month-old geckos had a significantly smaller telencephalon than the fully grown geckos (Tukey HSD: $p < 0.001$) and fewer telencephalic neurons (Tukey HSD: $p < 0.001$), but

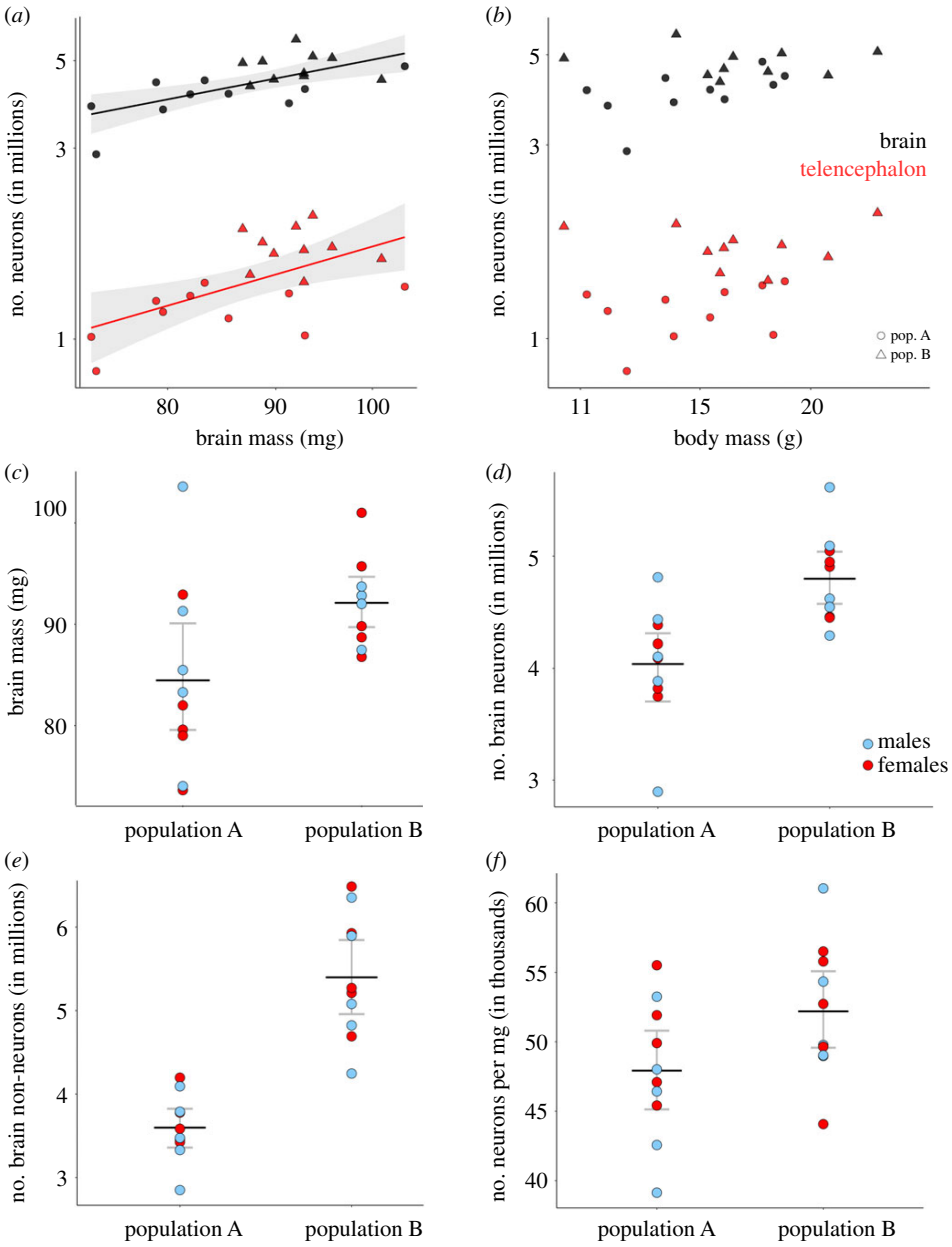

Figure 1. Intraspecific neuronal scaling and comparison between two captive populations for the Madagascar ground gecko. (*a*,*b*) Number of brain neurons (black symbols) and number of neurons in the cerebral hemispheres (red symbols) plotted as function of brain mass (*a*) and body mass (*b*). Shapes of points denote the two populations of fully grown animals. (*c*–*f*) Brain mass (*c*), number of brain neurons (*d*), number of non-neuronal cells (*e*) and neuronal densities (*f*) are compared between fully grown adults of the two populations. The horizontal bars denote the means, and 95% confidence intervals are shown for the population mean. Values for males are in blue and values for females in red.

there was no difference in non-neurons ($p = 0.52$). The 14-day-old geckos had again significantly smaller telencephalon and fewer neurons and other cells than either of the adult groups (Tukey's HSD: $p < 0.001$ in all cases) (electronic supplementary material, table S4; figure 2*c*,*d*).

Variance in brain mass in the 14-day-old group was significantly smaller than that of both adult groups (across all groups, Bartlett's $K^2 = 8.01$, $p = 0.018$), while the variance in brain neurons and non-neurons was homogenous across age groups. Variance in the number of telencephalic neurons in the 14-day-old group is significantly smaller than that of both adult groups (across all groups, Bartlett's $K^2 = 11.81$, $p = 0.003$). On the other hand, variance in neuron numbers in the rest of the brain is the same across all groups (Bartlett's $K^2 = 0.005$, $p = 0.998$).

4. Discussion

We assessed the individual variation in several brain traits both within and across two populations of Madagascar ground geckos and also evaluated sex differences and compared three different age groups within one population.

Across-population variation is larger than within-population, but not substantially so. Surprisingly, variation across adult geckos from both populations is comparable to within-population variation in 19 laboratory mice of the same strain, sex and age [5] in brain mass (1.41-fold versus 1.33-fold), number of neurons (1.94-fold versus 1.63-fold) and number of other cells (2.28-fold versus 2.98-fold); within-population variation in the geckos is even smaller than in mice in some traits (see electronic supplementary

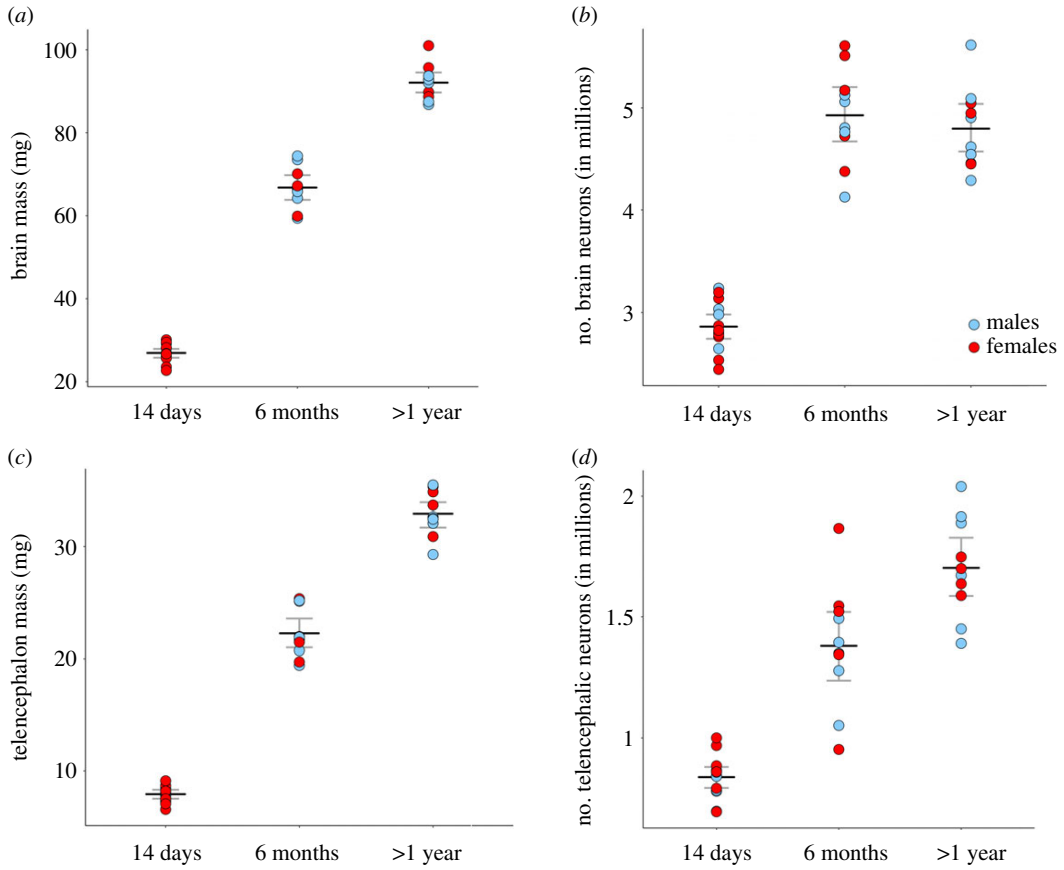

Figure 2. Comparison between three age cohorts. Brain mass (*a*), number of brain neurons (*b*), telencephalon mass (*c*) and number of telencephalic neurons (*d*) are compared across the three age groups of population B. The horizontal bars denote the mean and 95% confidence interval for the population mean. Values for males are in blue and values for females in red.

material, table S1). Combined with the data on the guppy and limited information on individual variation in mammals and birds [1,6,35] and our own unpublished data, it does not seem that there is generally a higher variability in brain traits in species with prolonged neurogenesis, although more species need to be investigated.

Differences between the population means were significant in most traits, except for neuronal density. The population B had larger brains with more cells overall, but similar neuron to glia ratio and similar average cell size. Inter-population variation is thus mostly driven by the number of cells and probably genetically determined. It has to be noted that both populations were captive and were not currently under any particular artificial selection and we do not have information about the selective pressures shaping their ancestral populations. It is also possible that the differences are due to plasticity [15,36], as the housing conditions of the two populations were similar but not identical (see electronic supplementary material, Methods for details). Contrary to previous findings [15,36], smaller brains were observed in population A, which was kept in larger enclosures in small groups, and larger brains in population B, kept in smaller enclosures individually. One can speculate that, rather than acting as environmental enrichment, group housing in this species may have created social stress. In the wild, the sources of variation are likely more complicated.

In contrast with mice [5], geckos show a significant positive relationship between body mass and brain mass and between brain mass and the number of neurons, but not directly between the number of neurons and body mass, weakening the notion that larger bodies need more neurons to control them [37]. The neuronal density also goes down with increasing brain mass, resulting in a shallow slope of the relationship between brain mass and neuron counts.

We found no sex differences in any of the brain measures, even though sexual dimorphism in brain size has been reported in reptiles, e.g. [19,21–23]. Surprisingly, there was also no difference in body mass, although males in this species tend to be moderately larger [38]. This might be a bias of our particular sample. It is possible that in species with more pronounced sexual dimorphism, sex differences in brain neuronal density might arise, owing to brains in the larger sex 'keeping up' with the body in size but not necessarily adding more neurons, which are metabolically expensive (e.g. [39]) and likely not needed to control a larger body.

Within population B, we additionally compared three different age cohorts. Between hatchlings and fully grown adults, the change in brain size was 4.5-fold, in the number of neurons 2.3-fold, in the number of other cells 2.02-fold, in telencephalon mass 5.41-fold, in telencephalic neurons 2.94-fold and in other telencephalic cells 2.74-fold. These changes are consistent with those reported in the Nile crocodile [8] in that the brain size grows much more quickly than the number of neurons and glial cells. However, only sub-adult crocodiles were included in that study, whereas our sample included fully grown adults. Increase in the number of brain neurons during early postnatal life and adolescence has been reported also in rodents [40–42].

While there were large differences between the hatchlings and adults in every measure, young and fully grown adults did not differ in the total number of brain neurons and non-neurons. This is potentially important for comparative studies, since it implies that including adult but not fully grown animals may not significantly affect the results in terms of absolute numbers of brain cells. However, fully grown adults had significantly more neurons in the telencephalon than sexually mature young adults, implying that adult neurogenesis [10–12] does not only replace worn out but also adds new telencephalic neurons in reptiles during adulthood. This markedly contrasts with the situation in mammals, where the number of cortical neurons declines with age [40,41].

The variance in brain mass in the 14-day-old group was significantly smaller than in adults, which is not true for the variance in brain neurons and other cells, suggesting that the higher variation in brain mass in adults is largely due to differences in cell size, dendritic arbours and connections, resulting in more bulk in the 'rest of brain'. However, in the telencephalon, the variance in the number of neurons in hatchlings is significantly smaller than that of adults, implying differences in the rate of neurogenesis and/or neuronal death in the telencephalon are responsible for a substantial portion of the individual variation seen in adults. In any case, neuronal plasticity might play an important role in intrapopulation differences.

5. Conclusion

Our study provides the first data on inter-individual and interpopulation variation in the number of neurons in reptiles and suggests that despite the reptile brain growing in adulthood, within-species variation in neuronal numbers and densities is not substantially higher than in mammals. Furthermore, young adults do not have significantly lower numbers of neurons than fully grown adults, except for the telencephalon. Including adult but not fully grown animals thus should not significantly bias comparative studies based on numbers of neurons, although using very young individuals might skew neuronal densities and the number of neurons relative to body size. However, potential differences between populations might be a source of concern when directly comparing species, as examining just one population may mask small interspecific differences or create the appearance of a difference where there is none.

Ethics. The experiments presented in this work were approved by the Institutional Animal Care and Use Committee at Charles University in Prague and by the Ministry of Education, Youth and Sports of the Czech Republic (Permission No. MSMT-11721/2018-2).

Data accessibility. All data are available in the electronic supplementary material.

Authors' contributions. K.K. and A.P. conducted experiments and analysed data, K.K. and P.N. conceived of the project, L.K. provided experimental animals, and all authors wrote the manuscript. All authors approved the final version of the manuscript and agree to be held accountable for the content therein.

Competing interests. We declare we have no competing interests.

Funding. This study was supported by the Grant Agency of Charles University (810216) and Czech Science Foundation (18-15020S).

Acknowledgements. We thank Zuzana Starostová, Lukáš Kratochvíl and Jan Červenka for providing the animals, and Martin Kocourek for help with perfusions.

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CHAPTER 2

Artificial selection on brain size leads to matching changes in overall number of neurons

BRIEF COMMUNICATION

doi:10.1111/evo.13805

Artificial selection on brain size leads to matching changes in overall number of neurons

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Received April 12, 2019
Accepted June 19, 2019

Neurons are the basic computational units of the brain, but brain size is the predominant surrogate measure of brain functional capacity in comparative and cognitive neuroscience. This approach is based on the assumption that larger brains harbor higher numbers of neurons and their connections, and therefore have a higher information-processing capacity. However, recent studies have shown that brain mass may be less strongly correlated with neuron counts than previously thought. Till now, no experimental test has been conducted to examine the relationship between evolutionary changes in brain size and the number of brain neurons. Here, we provide such a test by comparing neuron number in artificial selection lines of female guppies (*Poecilia reticulata*) with >15% difference in relative brain mass and numerous previously demonstrated cognitive differences. Using the isotropic fractionator, we demonstrate that large-brained females have a higher overall number of neurons than small-brained females, but similar neuronal densities. Importantly, this difference holds also for the telencephalon, a key region for cognition. Our study provides the first direct experimental evidence that selection for brain mass leads to matching changes in number of neurons and shows that brain size evolution is intimately linked to the evolution of neuron number and cognition.

KEY WORDS: **Artificial selection, brain size, cognition, isotropic fractionator, number of neurons.**

The relationship between brain size and its functional capacity remains controversial. Several decades of comparative research on brain size variation in relation to body size have been based on the assumption that a larger brain also contains more neurons (Jerison 1973; Herculano-Houzel 2017; Tsuboi et al. 2018) and it has been argued that it is the difference in neuron number that underlies the commonly found association between measures of brain size and cognitive abilities (McDaniel 2005; Kotrschal et al. 2013; MacLean et al. 2014; Benson-Amram et al. 2016; Buechel et al. 2018; Horschler et al. 2019; Hwang et al. 2019).

The isotropic fractionator (Herculano-Houzel and Lent 2005), a recent methodological breakthrough to quantify neuron numbers quickly and accurately (Bahney and von Bartheld 2014; Miller et al. 2014; Ngwenya et al. 2017), has now made it possible to test this assumption by quantifying how neuron numbers scale with brain mass both within and across species. This method involves mechanical dissociation of fixed brain tissue into a homogenous suspension of free cell nuclei, which are then counted and immunocytochemically identified to estimate the proportion of nonneuronal (glial and endothelial) cells and neurons (Herculano-Houzel and Lent 2005). Data collected with this method have shown that similarly sized brains of vertebrates can differ in neuron number, neuronal densities, and allocation of neurons into different brain regions (Herculano-Houzel et al. 2015a;

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Olkowicz et al. 2016). For instance, a primate brain accommodates many more neurons than a rodent brain of similar size (Herculano-Houzel et al. 2007), and a parrot or songbird brain contains on average twice as many neurons as an equivalently sized primate brain (Olkowicz et al. 2016). These insights into differences in neuron numbers and densities offer a possible explanation as to why brain size sometimes does not predict cognitive ability, especially when comparison is made across distantly related species (Dicke and Roth 2016; Güntürkün and Bugnyar 2016). Moreover, neuronal density often shows a pattern of negative allometry with body and brain size across species (Herculano-Houzel et al. 2015a; Olkowicz et al. 2016). Hence, small-bodied species with smaller absolute brain size show higher neuronal densities than larger species with larger absolute brain size, although exceptions from this rule have been observed (Kverková et al. 2018). Whether this negative allometry pattern also exists within species or whether neuronal density is a species-specific characteristic is currently unknown (but see Herculano-Houzel et al. 2015b). When trying to understand the consequences of having a larger brain, it is therefore necessary to consider neuron number, and also ideally to test the effect of variation in brain size and neuron number on cognitive abilities.

The total number of neurons, elementary building blocks of the brain, is an important, though not the only determinant of brain information-processing capacity. Other factors at play include the number of neuronal connections, neuron packing density, interneuronal distance, and axonal conduction velocity (Dicke and Roth 2016). Thus, besides the total number of neurons and their connections, diversification of neuronal types and their properties (Markram et al. 2015; Tasic et al. 2018; Tosches et al. 2018; Zeisel et al. 2018), and diversification of molecular machineries subserving neuronal signaling (Grant 2016; Zhu et al. 2018), all contribute to the broad behavioral repertoires seen in various vertebrates. Because quantitative mapping of cell type and synaptic density distributions across the brain is challenging and difficult to interpret, and because consequences of cellular and molecular diversification for cognitive processes remain poorly understood, here we focus on the number of neurons, which currently is the most feasible, easy-to-measure proxy for cognitive abilities. Areas controlling higher cognitive functions involve mainly telencephalic associative regions, which, in turn, rely on telencephalic sensorimotor and also cerebellar processing (Barton 2012). Indeed, a growing body of comparative evidence suggests that the absolute number of neurons in the telencephalon is a particularly good correlate of cognitive abilities (Dicke and Roth 2016; Olkowicz et al. 2016; Herculano-Houzel 2017).

To investigate how evolutionary changes in brain size are related to changes in neuron number, we use artificial selection lines of guppies (*Poecilia reticulata*) that have been selected for relative brain size for five generations, resulting in >15 % differences in brain mass (Kotrschal et al. 2013). Importantly, several tests of various aspects of cognition in these selection lines have revealed substantial advantages of increased brain size in cognitive abilities, including numerical learning (Kotrschal et al. 2013), maze learning (Kotrschal et al. 2014), mate discrimination (Corral-López et al. 2017a; Bloch et al. 2018), predator avoidance (Kotrschal et al. 2015; van der Bijl et al. 2015), and reversal learning (Buechel et al. 2018). At the same time, perception aspects, such as visual acuity (Corral-López et al. 2017b), remained constant between the lines. Body size was not affected by artificial selection on relative brain size (Kotrschal et al. 2013, 2014). These selection lines thus offer the opportunity to test how evolutionary changes in brain size, demonstrably associated with changes in cognitive abilities, affect neuron number and neuron density independently of body size.

The vertebrate brain is divided into several regions with different functions and these regions can have strikingly different neuron numbers and densities (Herculano-Houzel et al. 2015a; Olkowicz et al. 2016). It is therefore important to also examine differences in neuron numbers between key brain regions to get the complete picture. Importantly, the guppy lines used here and selected for large and small brains did not differ in relative volumes of 11 major brain regions (Kotrschal et al. 2017a). Here, we quantify numbers of neurons and nonneuronal (glial and endothelial) cells in the whole brain and the telencephalon of large- and small-brained guppies using the isotropic fractionator. In a subset of individuals, we further quantify cell numbers in three other key brain regions. As these selection lines clearly differ in cognitive ability (see examples above), and we assume that more neurons provide higher computing power (Dicke and Roth 2016; Olkowicz et al. 2016; Herculano-Houzel 2017), we expect that absolute (and relative) number of neurons in the brain and telencephalon will be higher in the large-brained than in the small-brained selection lines.

Methods

BRAIN SIZE SELECTION LINES

We quantified neuron number in 53 adult female guppies from the brain size selection lines (Kotrschal et al. 2013; Supporting Information Dataset S1). A total of 26 females originated from large-brained selection lines, and 27 females from small-brained selection lines. The selection regime consisted of three up-selected and three down-selected lines (see Kotrschal et al. 2013 for full description of the selection experiment). The individuals in this assay came from the fifth generation of selection and were adult virgin females. We focused on females in the study because most of the previous cognitive assays have been done on females.

TISSUE PREPARATION

The fish were euthanized by an overdose of benzocaine and kept in 4% paraformaldehyde solution during transport from

Table 1. Relative distribution of mass and cells in female guppy brain.

Structure	Mass (mg)	Number of neurons	Neuronal density (N/mg)	Nonneuronal cells	Nonneuronal cells density (N/mg)	Glia/neurons ratio
Whole brain ($n = 42$)	4.8 $\pm$ 0.617	4.3×10^6 $\pm$ 4.97×10^5	9.05×10^5 $\pm$ 8.9×10^4	2.2×10^6 $\pm$ 3.65×10^5	4.7×10^5 $\pm$ 7.6×10^4	0.52 $\pm$ 0.092
Telencephalon ($n = 49$)	0.83 $\pm$ 0.115	6.33×10^5 $\pm$ 8.6×10^4	7.48×10^5 $\pm$ 8.3×10^4	3.93×10^5 $\pm$ 6.86×10^4	4.79×10^5 $\pm$ 7.1×10^4	0.65 $\pm$ 0.113
Tectum ($n = 17$)	1.36 $\pm$ 0.189	1.1×10^6 $\pm$ 1.67×10^5	8.79×10^5 $\pm$ 1.4×10^5	6.31×10^5 $\pm$ 1.4×10^5	4.76×10^5 $\pm$ 1.08×10^5	0.55 $\pm$ 0.147
Cerebellum ($n = 16$)	0.48 $\pm$ 0.098	1.7×10^6 $\pm$ 3.2×10^5	3.8×10^6 $\pm$ 6.89×10^5	4.3×10^6 $\pm$ 1.27×10^5	1×10^6 $\pm$ 3.799×10^5	0.27 $\pm$ 0.095
Diencephalon and brainstem ($n = 17$)	2.11 $\pm$ 0.291	7.45×10^5 $\pm$ 1.25×10^5	3.56×10^5 $\pm$ 5.1×10^4	6.33×10^5 $\pm$ 1.54×10^5	2.9×10^5 $\pm$ 5.83×10^4	0.83 $\pm$ 0.224

Stockholm University to Charles University in Prague, where body was weighted to the nearest 0.1 mg using a Kern ALJ (Kern & Sohn GmbH, Balingen-Frommern, Germany) 120-4 balance and standard body length (from the tip of the snout to the end of the caudal peduncle) was measured to the nearest 0.01 mm using an electronic digital calliper IP67. Immediately afterward, the brains were removed using an Olympus SZX (Olympus Corporation, Tokyo, Japan) 16 stereomicroscope and weighed to the nearest 0.001 mg using a Mettler Toledo (Mettler Toledo, Columbus, Ohio) MX5 microbalance. We divided the brains into two parts, the telencephalon and the "rest of the brain" comprising the diencephalon, tectum, cerebellum, and brainstem. To quantify neuron number and neuronal density in three additional brain regions, a subsample of 20 brains (11 and nine from the large- and small-brained lines, respectively) were dissected into telencephalon, tectum (comprising the tectum opticum, torus semicircularis, and torus longitudinalis), cerebellum, and a division consisting of the brainstem and diencephalon. Cerebral hemispheres including the olfactory bulbs were detached from the rest of the brain by a transverse cut separating the telencephalon from the rostral pole of the tectum and diencephalon. The remaining brain was divided into left and right halves by a midsagittal cut. Subsequently, the cerebellum was excised from the surface of the brainstem together with the valvula cerebelli extending into the ventricle of the tectum. The tectum was then cut off from the remaining division, which consisted of the brainstem and the diencephalon. The latter more detailed dissections do not allow for statistical brain size selection line comparisons due to (1) small sample sizes and (2) potentially higher measurement errors as dissecting and homogenizing such small quantities is extremely challenging. Nevertheless, they provide an opportunity to coarsely characterize the guppy brain in numbers, at least for our study population. The final sample sizes for the different dissection protocols are given in Table 1. Immediately after dissection, all the brain divisions were weighed to the nearest 0.001 mg, and then kept in antifreeze solution (30% glycerol, 30% ethylene glycol, 40% phosphate buffer) at –25°C for later processing.

ISOTROPIC FRACTIONATOR METHODOLOGY

We estimated the total number of cells, neurons, and nonneuronal cells using the isotropic fractionator (Herculano-Houzel and Lent 2005). Each dissected brain division was homogenized in 40 mM sodium citrate with 1% Triton X-100 using Tenbroeck tissue grinders (0.5 mL, Ningbo Ja-Hely Technology Co., Ltd., China). When turned into an isotropic suspension of isolated cell nuclei, the homogenate was transferred to an Eppendorf tube and the walls of the grinder were rinsed with dissociation solution to transfer all the cells to the tube. Then we measured the exact volume of the homogenate using an Eppendorf Xplorer (Eppendorf, Hamburg, Germany) 5–1000 µL electronic pipette and added a fluorescent DNA marker DAPI (4,6-Diamidino-2-Phenylindole, Dihydrochloride) (5% of the total volume) to stain all nuclei. The total number of nuclei in suspension, and therefore the total number of cells in the original tissue, was estimated by determining the density of nuclei in small fractions drawn from the homogenate. At least six 10 µL aliquots were sampled and the number of cells was counted in a Neubauer-improved counting chamber (BDH, Dagenham, Essex, UK) using an Olympus BX51 fluorescent microscope; additional aliquots (four to six) were counted when any coefficient of variation (CV) exceeded 0.05 (CV was always $\leq$ 0.10). After determining the total number of cells, the proportion of neurons was determined by immunocytochemical detection of neuronal nuclear marker NeuN (Mullen et al. 1992). We used rabbit polyclonal antibody anti-NeuN (ABN78, dilution 1:800; Merck). The binding sites of the primary antibody were revealed by Alexa Fluor 594-conjugated goat anti-rabbit IgG (dilution 1:800; ThermoFisher Scientific). An electronic hematologic counter (Alchem Grupa, Torun, Poland) was used to count simultaneously DAPI-labeled and NeuN-immunopositive nuclei in the Neubauer chamber. A minimum of 500 nuclei were counted per

sample to estimate percentage of double-labeled neuronal nuclei. The number of nonneuronal cells was derived by subtracting the number of neurons from the total number of cells.

DATA ANALYSIS

All statistical analyses were conducted in the R software environment version 3.5.1 (R Core Team 2018). As all dependent variables were normally distributed, we used linear mixed models (LMMs) implemented in the packages "lme4" (Bates et al. 2015) and "lmerTest" (Kuznetsova et al. 2017), with the trait of interest as the dependent variable, brain size selection treatment as a fixed effect (and, in case of relative measures, body/brain mass as a covariate), and replicate nested in brain size selection treatment as a random effect. We then selected the best model by stepwise backward elimination of nonsignificant effects, starting from the full model with an interaction between the covariate and selection treatment. In all cases, the interaction term was nonsignificant and removed from the model. Satterthwaite's approximation was used to estimate the effective degrees of freedom. Results are presented using the best-fitting model parameters.

The Kendall's τ rank correlation coefficient was used to assess the association between brain tissue mass and the number of neurons and nonneuronal cells at the individual level using the base R package (R Core Team 2018).

ETHICS

The experiment was performed in accordance with ethical applications approved by the Stockholm Ethical Board (Dnr: C50/12, N173/13, and 223/15).

Results

THE GUPPY BRAIN IN CELL NUMBERS

In this study, 53 females weighed between 247 and 580 mg, their brain mass ranged between 3.83 and 6.35 mg, and their brains contained between 3.35 and 5.73 million neurons (Table 1, Supporting Information Dataset S1). Whole brain neuron density ranged between 7.05×10^5 and 1.76×10^6 N/mg and was negatively associated with both body mass (LMM, $t_{1,40} = 18.39$, $R^2 = 0.188$, $P = 0.002$) and brain mass (LMM, $t_{1,40} = 14.97$, $R^2 = 0.302$, $P = <0.001$). We found that neuron density varied greatly among the principal brain divisions examined. The highest average neuron density was detected in the cerebellum ($\sim 3.82 \times 10^6$ N/mg), and the lowest in the division comprising the diencephalon and brainstem ($\sim 3.56 \times 10^5$ N/mg). Consequently, different brain divisions harbored different amounts of neurons. The telencephalon constituted 17% of the brain mass and contained 15% of all brain neurons, the tectum constituted 28% of the brain mass and contained 26% of brain neurons, the brainstem and diencephalon together made up 44% of the brain mass but contained only 17% of brain neurons, and the densest brain region, the cerebellum, contained 40% of brain neurons despite representing only 10% of the total brain mass.

Nonneuronal (glial and endothelial) cells constituted a minor cellular fraction in all brain divisions (Table 1, Fig. S1A and S1B). Among individual females, the proportion of nonneuronal cells to neurons in the brain ranged between 27% and 44%. Hence, the maximum glia/neuron ratio (if all nonneuronal cells were glial cells) for the whole brain ranged between 0.36 and 0.77. Density of nonneuronal cells varied across brain regions, although to lesser extent than the density of neurons, and was loosely correlated with neuron density. We found the highest nonneuronal density in the cerebellum ($\sim 1.03 \times 10^6$ N/mg; Table 1), and the lowest in the division comprising the diencephalon and brainstem ($\sim 2.91 \times 10^5$ N/mg; Table 1).

COMPARISON BETWEEN SELECTION LINES

We found that females from large- and small-brained selection lines differed in both relative brain mass (LMM, $t_{3,50} = 5.281$, $P < 10^{-5}$; Fig. 1A) and absolute brain mass (LMM, $t_{2,51} = 4.804$, $P < 10^{-4}$; Fig. 1B), with females of large-brained lines having approximately 15.4% larger brains compared to those of the small-brained lines. Large-brained females were also slightly heavier than small-brained females (LMM, $t_{2,51} = 2.09$; $P = 0.042$; Fig. 1C).

The relationship between brain mass and number of neurons could be described by similar linear functions in the large- and small-brained selection lines (see Supporting Information Results and Table S1). Thus, the number of neurons relative to overall brain mass did not differ between the selection lines (LMM, $t_{3,39} = 1.360$, $P = 0.182$), meaning that large- and small-brained lines showed similar neuronal densities (LMM, $P = 0.292$). However, due to their larger brains, the large-brained lines had a higher total number of neurons than those of the small-brained lines (LMM, $t_{2,40} = 3.573$, $P < 0.001$; Fig. 2B). This amounted to an 11.9% difference in neuron number between selection lines. To control for body size, we also examined residuals from a neuron number versus body mass regression to determine the "neuronal index" (Herculano-Houzel 2007) and found a significantly higher neuronal index in large- compared to small-brained individuals (LMM, $t_{2,40} = 2.906$, $P < 0.006$; Fig. 2D).

Telencephalon mass correlated tightly with brain mass (LMM, $t_{2,51} = 12.833$, $P < 10^{-15}$, $R^2 = 0.76$; Fig. 1D) but the telencephalon mass fraction did not differ between the lines (LMM, $P = 0.521$; Fig. 1E). Likewise, the number of telencephalic neurons correlated with telencephalon mass (LMM, $t_{2,47} = 6.537$, $P < 10^{-7}$, $R^2 = 0.47$; Fig. 3A), but neuron density in the telencephalon did not differ between the lines (LMM, $P = 0.203$; Fig. 3B). In absolute terms, the telencephalon of the large-brained lines was heavier (LMM, $t_{2,51} = 4.756$, $P < 10^{-4}$; Fig. 1F) and harbored more neurons than the telencephalon of the

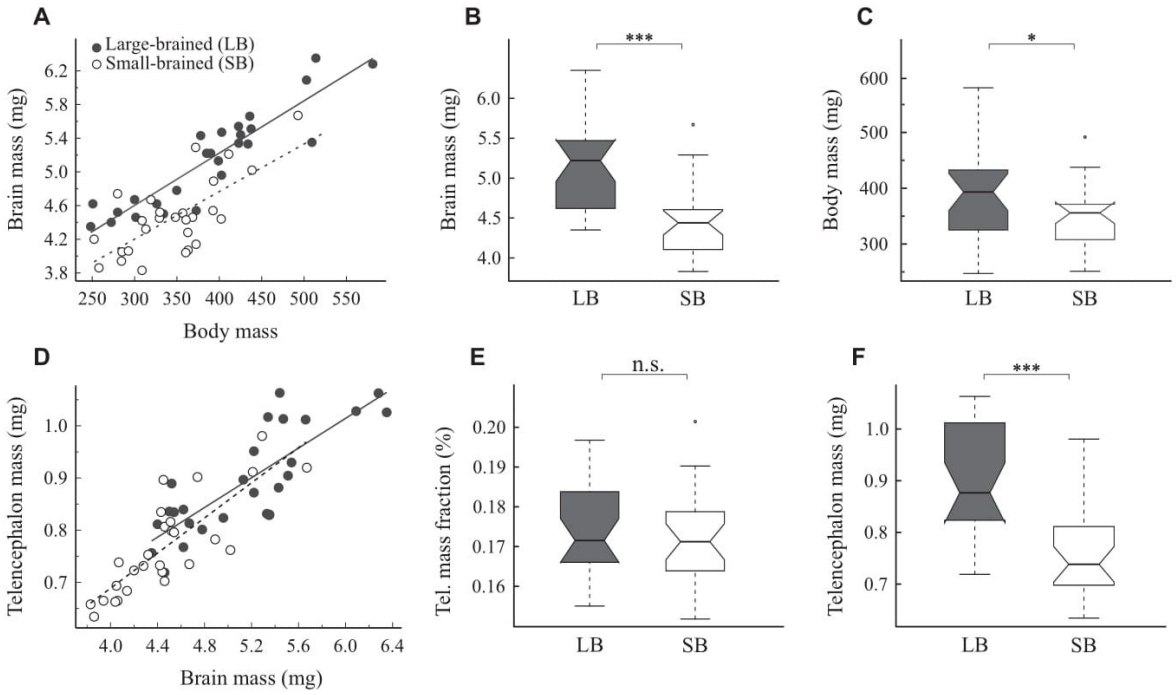

Figure 1. Brain and telencephalon size compared between small- and large-brained selection lines. (A) Brain-body scaling in female guppies. Note that allometric lines for small- and large-brained guppies have significantly different intercepts, clearly indicating difference (grade shift) in relative brain mass (for statistics, see SI Results). Absolute brain mass (B) and body mass (C) compared between selection lines. (D) Telencephalon mass plotted as a function of brain mass. Note that relative mass of the telencephalon does not differ between the selection lines (for statistics, see Supporting Information Results). Telencephalon mass fraction (E) and absolute telencephalon mass (F) compared between selection lines. Each point in the scatterplots represents the values for one individual, the lines represent the ordinary least squares regressions for small-brained (the dashed lines) and large-brained (the solid lines) female guppies. Box plots denote median, 95% confidence intervals of median, first and third quartiles, total range, and outliers. The statistical significance level in box plots is indicated as follows: $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; n.s., nonsignificant). LB, large-brained line; SB, small-brained line.

small-brained lines (LMM, $t_{2,47} = 3.670$, $P < 0.001$; Fig. 3B). Although the sample size for additional brain regions was too small to allow formal statistical comparisons of selection lines, we found qualitatively similar differences between the selection lines for all other brain regions, except the brain stem (Table S2).

The scaling of brain mass and number of nonneuronal cells did not differ between the selection lines (see Supporting Information Results, Fig. S1C). Likewise, no difference was observed in glia/neuron ratio (LMM, $P \geq 0.683$ in all cases). But fish from the large-brained lines tended to have higher absolute numbers of nonneuronal cells compared to fish from the small-brained lines, although the difference was only significant for the telencephalon (LMM, telencephalon: $t_{2,47} = 2.297$, $P = 0.026$; whole brain: $t_{2,40} = 1.773$, $P = 0.084$; Fig. S1D).

INDIVIDUAL DIFFERENCES IN CELLULAR DENSITIES

Apart from variation in brain mass, the mass of the examined brain regions and number of neurons and nonneuronal cells (see above), we also observed considerable individual differences in densities of neurons and nonneuronal cells (Fig. 4 and Fig. S2). Thus, although we detected clear differences between the brain size selection lines, individuals with the largest brains did not necessarily have the most neurons and/or nonneuronal cells. Nevertheless, the rank correlation between brain tissue mass and number of neurons was significant both in the whole brain (Kendall's $\tau = 0.46$, $P < 0.001$; Fig. 4A) and in the telencephalon (Kendall's $\tau = 0.49$, $P < 0.001$; Fig. 4B). The same pattern was observed for the association between brain tissue mass and number of nonneuronal cells (whole brain: Kendall's $\tau = 0.29$, $P = 0.007$, Fig. S2A; the telencephalon: Kendall's $\tau = 0.39$, $P < 0.001$, Fig. S2B). The observed individual differences in neuronal densities were much larger than expected measurement error (see Methods section), therefore it is unlikely that they represent mere technical artifacts.

Discussion

Our results show that selection for larger and smaller brains also generates a matching increase in number of neurons. Moreover,

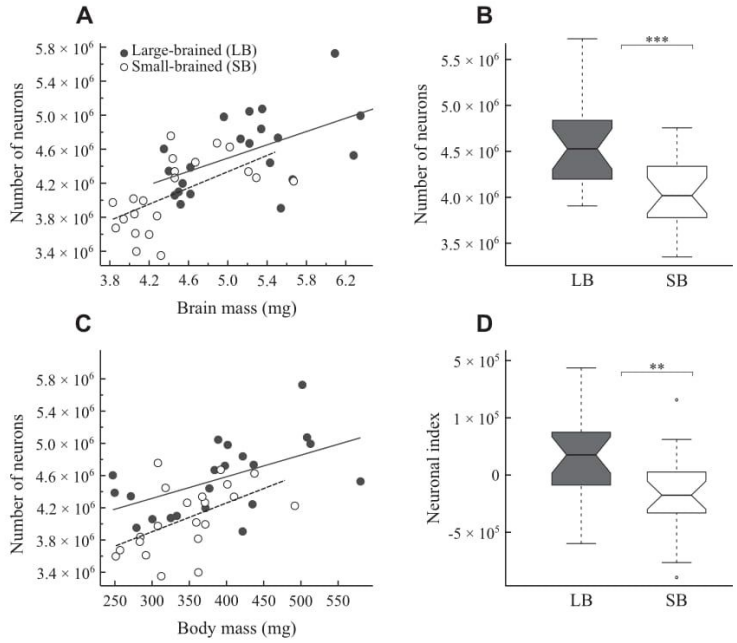

Figure 2. Neuronal scaling and neuron numbers compared between small- and large-brained selection lines. (A) Number of neurons plotted as a function of brain mass. Note that the relationship between brain mass and the number of brain neurons does not differ between the selection lines (for statistics, see Supporting Information Results). (B) The total number of brain neurons compared between selection lines. (C) Number of neurons plotted as a function of body mass. Note that guppies of large-brained line have significantly more neurons for a given body mass (for statistics, see Supporting Information Results). (D) Neuronal index (i.e., residuals from the neurons-body regression line for all female guppies) compared between selection lines (see Figure 1 for explanation).

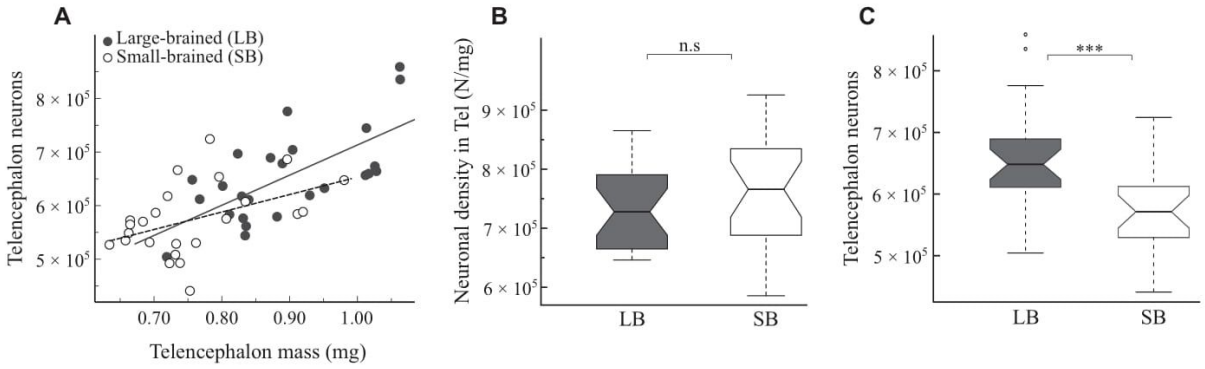

Figure 3. Neuronal scaling, densities, and numbers in the telencephalon. (A) Number of telencephalic neurons plotted as a function of telencephalon mass. Note that the relationship between telencephalon mass and number of telencephalic neurons does not differ between the selection lines (for statistics, see Supporting Information Results). (B and C) Neuronal densities in the telencephalon (B) and absolute number of telencephalic neurons (C) compared between selection lines (see Figure 1 for explanation).

the number of neurons increases linearly with increasing brain mass in the selection lines. The implications of this result are manifold. First, it suggests that brain mass can be an appropriate predictor of neuron number, at least at the within species level. It is important to acknowledge that the correlation between brain mass and neuron number was not very strong due to pronounced variation in neuronal densities. Yet, brain mass accounted for 47% of the observed variation in the number of neurons. This finding, however, should be generalized with caution as a much weaker correlation between brain mass and number of neurons has been observed in laboratory mice (Herculano-Houzel et al. 2015b) and captive-bred Madagascar ground geckos *Paroedura picta* (our

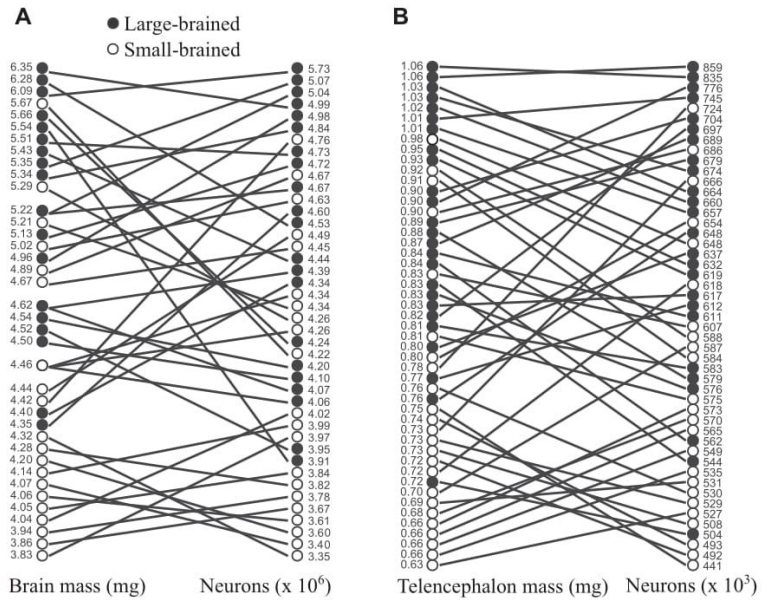

Figure 4. **Individual differences in brain and telencephalon size, neuron numbers, and densities. Relationship between brain mass (A), telencephalon mass (B), and neuron counts. These variables are ranked in descending order from the largest to the smallest and individual values are given on the sides of the graphs. Solid lines connect values measured in the same individual. Crossed lines indicate individual differences in neuronal densities.**

own unpubl. data). This difference might be attributable to the relaxed selective pressure in captive-bred populations. It is well known that animals bred in captivity often have smaller brains and behave differently than their wild counterparts (e.g., Price 1999; Kruska 2007; Guay and Iwaniuk 2008; Burns et al. 2009; LaDage et al. 2016; Jensen 2017). The mice and geckos were likely kept under low cognitive pressure, whereas guppies in this study were subjected to strong artificial selection on brain size. We hypothesize that the association between brain mass and neuron number might be stronger in wild populations. Natural selection in the wild acts on behavior, not on brain size, as the artificial selection performed here. Thus, individual variation in neuronal density in wild populations inhabiting the same selective environment might be decreased due to directional selection and therefore lower than that observed in captive populations. On the other hand, changes in the environment can trigger substantial changes in brain region size (for review, see Kotrschal et al. 2017b; Kotrschal et al. 2012; Gonda et al. 2013; Fong et al. 2019) and potentially also region-specific changes in neuronal numbers and densities. Local control and variation in cell proliferation or survival may facilitate mosaic brain evolution in wild populations, when favored by selection (Montgomery et al. 2016). Assessment of neuronal density variation in natural populations is required to test these hypotheses.

Second, the remarkable finding that a 12% difference in neuron numbers arose within just five generations of artificial selection for brain size have important implications. It suggests that selection on individuals with more neurons or larger brains within a population can be an important microevolutionary mechanism underlying the evolution of brain size and information processing capacity, at least at the population and species level. This result also shows that such evolutionary changes can be very fast. It is notable in this context that two or three generations of guppies per year occur in the wild (Houde 1997; Magurran 2005).

Third, the evidence that the large-brained guppies also have higher cognitive abilities (Kotrschal et al. 2013, 2014, 2015; van der Bijl et al. 2015; Corral-López et al. 2017a; Bloch et al. 2018; Buechel et al. 2018) supports the idea that the number of neurons, either absolute or in relation to body size, is an important factor contributing to cognitive abilities. We propose that it is indeed the higher number of neurons in the larger brains in these selection lines that have yielded their cognitive advantages, especially because the differences in neuron number between large- and small-brained females were consistent across whole brain and the telencephalon. It is worth noting that the large-brained fish in this study were slightly larger than the small-brained fish. This is the first time this has been encountered in more than 20 comparisons of body mass that have been done on subsamples of these selection lines, and the most likely explanation is therefore that we randomly picked differently sized individuals from the brain size selection lines. Importantly, the differences in neuron number between the large- and small-brained lines were substantial and robust also when the analyses controlled for body mass.

Fourth, and finally, the observed neuronal scaling rules, that is, the relationship between brain mass or brain region mass and number of neurons (Herculano-Houzel et al. 2006), were very similar in the large- and small-brained lines. Despite the observed decreasing neuronal densities with increasing brain mass across all our animals and a slightly smaller body mass in the small-brained lines, there were no significant differences in neuronal density between the brain size lines. Hence, it is not cellular composition but rather brain size that sets the large-brained lines apart from the small-brained lines. This supports what we have previously shown for brain region volumes (Kotrschal et al. 2017a), namely that the brains of the large-brained lines are scaled-up versions of the brains of the small-brained lines. This is similar to what has been shown when comparing the large human brain to smaller nonhuman primate brains (Herculano-Houzel 2009), and the large corvid brains to smaller non-corvid songbird brains (Olkowicz et al. 2016). Hence, it seems that these mentioned differences in neuron numbers are generated mainly by the relative (and absolute) size of the brain.

Apart from the comparisons between the guppy brain size selection lines, we provide the first quantification of neuron number in the guppy brain. Although our quantification is done on selected laboratory populations, this makes it the second fish species with a known number of neurons and the first one with a known neuron count and known densities in specific brain regions. An extremely miniaturized cyprinid fish *Danionella translucida* from Myanmar has the smallest known adult vertebrate brain possessing only 650 thousand neurons (Schulze et al. 2018), which is almost eight times less than reported here for the guppy. By contrast, the zebrafish *Danio rerio* seems to have a higher number of brain neurons, because its brain contains on average 36% more cells (Hinsch and Zupanc 2007). All these small fish have tiny brains and extremely high neuronal densities. For instance, whole brain neuron densities reported here for the guppy are approximately twofold and approximately 4.4-fold higher than the highest whole brain neuronal densities reported in birds and mammals, respectively (Sarko et al. 2009; Olkowicz et al. 2016). These observations confirm a trend found in other taxa (Herculano-Houzel et al. 2015a; Olkowicz et al. 2016), namely that smaller bodied species with correspondingly smaller brains have higher neuron density than larger species with larger brains. We suggest that this is one mechanism to compensate for the small absolute brain size in small-bodied vertebrates that have to solve relatively complex ecological and social problems in their natural environments. As already mentioned, neuron numbers in most brain regions examined matched the relative size of the region with the exception of the cerebellum, which showed an almost fourfold higher neuron number than expected for its size. This is a similar pattern as found in birds and mammals (Herculano-Houzel 2010; Herculano-Houzel et al. 2015a; Olkowicz et al. 2016). Just like in other vertebrates (for review, see Barton 2012; Baumann et al. 2014; Sokolov et al. 2017), the cerebellum in teleost fishes is important for many functions such as motor coordination and movement but also cognitive processes (Kotrschal et al. 1998; Butler and Hodos 2005; Rodríguez et al. 2005; Braithwaite 2006; Kolm et al. 2009; Gómez et al. 2010; Warren and Sawtell 2016). The high absolute numbers of cerebellar neurons thus indicate that this region is important in computationally demanding tasks also in the guppy. The neuron richness of the cerebellum highlights the need to estimate neuron numbers and not just region size to fully appreciate brain region functional capacity (for further discussion, see Herculano-Houzel 2010; Barton 2012).

The density of nonneuronal cells was also high in the population of guppies studied here. Depending on the brain region and taxon, they are two to four times higher than the highest nonneuronal cell densities reported in birds and mammals (Sarko et al. 2009; Herculano-Houzel et al. 2015a; Olkowicz et al. 2016; Dos Santos et al. 2017; Kocourek et al. unpubl. data). It remains unclear whether the high nonneuronal cell densities in the guppy represent the corollary of miniaturization or a feature that is shared by all teleost fishes. Interestingly, the degree of variation in nonneuronal cell density across different brain regions in our fish is comparable to that of birds and mammals but generally much less pronounced than variation in densities of neurons (Herculano-Houzel et al. 2015a; Olkowicz et al. 2016). Although these findings indicate that nonneuronal scaling rules are much more conserved than neuronal scaling rules, they weaken the notion that nonneuronal densities are largely independent of brain size, brain region, and taxon investigated (Herculano-Houzel et al. 2014; Olkowicz et al. 2016).

To conclude, we demonstrate that selection for brain size in the guppy has generated matching changes in the number of neurons, and these differences are similar across the whole brain and the telencephalon, a key region for cognition. We also show that neuronal density scales negatively with brain size at the intraspecific level, replicating previous findings across species in other taxa. Importantly, together with earlier studies assessing behavior in large- and small-brained guppies (see above), this study provides the first direct demonstration of a close association between brain size, neuron numbers, and cognitive abilities at the intraspecific level. Thus, our findings provide experimental support for the idea that neuron numbers adequately predict cognitive abilities.

AUTHOR CONTRIBUTIONS

PN and NK designed the research and drafted the manuscript; AK and NK performed the selection experiment and provided experimental animals; LM collected experimental data; KK, LM, and AK analyzed the data; and all authors wrote the paper.

ACKNOWLEDGMENTS

We thank Severine Buechel, Anna Rennie, and Annika Boussard for help with fish husbandry. This study was supported by the Grant Agency

of Charles University (1502317 to LM), Czech Science Foundation (18-15020S to PN), the Swedish Research Council (2012-03624 and 2016-03435 to NK and 2017-04957 to AK), the Austrian Science Fund (J3304-B24 to AK) and the Knut and Alice Wallenberg Foundation (1022013.0072 to NK).

DATA ARCHIVING

The entire dataset used in our analyses can be found in the Supporting Material of this manuscript.

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Associate Editor: A. M. Rice
Handling Editor: Mohamed A. F. Noor

Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1. Scaling rules for female guppy brains from brain size selection lines. Power laws were calculated from the individual values listed in dataset S1.
Table S2. Relative distribution of mass and cells in female guppy brains from brain size selection lines. G/N ratio, glia to neuron ratio.
Figure S1. Glia/neuron ratios, nonneuronal cell scaling, and numbers compared between small- and large-brained selection lines.
Figure S2. Individual differences in brain and telencephalon size, nonneuronal cell numbers, and densities.
Guppies dataset.

CHAPTER 3

Sociality does not drive the evolution of large brains in eusocial African mole-rats

SCIENTIFIC REPORTS

Sociality does not drive the evolution of large brains in eusocial African mole-rats

Received: 26 May 2017
Accepted: 2 May 2018
Published online: 15 June 2018

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The social brain hypothesis (SBH) posits that the demands imposed on individuals by living in cohesive social groups exert a selection pressure favouring the evolution of large brains and complex cognitive abilities. Using volumetry and the isotropic fractionator to determine the size of and numbers of neurons in specific brain regions, here we test this hypothesis in African mole-rats (Bathyergidae). These subterranean rodents exhibit a broad spectrum of social complexity, ranging from strictly solitary through to eusocial cooperative breeders, but feature similar ecologies and life history traits. We found no positive association between sociality and neuroanatomical correlates of information-processing capacity. Solitary species are larger, tend to have greater absolute brain size and have more neurons in the forebrain than social species. The neocortex ratio and neuronal counts correlate negatively with social group size. These results are clearly inconsistent with the SBH and show that the challenges coupled with sociality in this group of rodents do not require brain enlargement or fundamental reorganization. These findings suggest that group living or pair bonding *per se* does not select strongly for brain enlargement unless coupled with Machiavellian interactions affecting individual fitness.

The social brain hypothesis (SBH) contends that the demands imposed on individuals by living in cohesive social groups exert a selection pressure favouring the evolution of large brains and complex cognitive abilities[1]. It was originally proposed to explain the exceptional cognitive abilities in primates, but it has since been extended to a wider range of vertebrate taxa, including cetaceans, carnivores, bats, insectivores, ungulates, various birds and cichlids (for a review see[2,3]). While the SBH has gained great traction in evolutionary anthropology, what the underlying mechanisms are, or how broadly it applies to other animals remains an area of active research. Recent studies incorporating phylogenetic corrections and more stringent measures have failed to provide strong support[4–7] and even new analyses in primates, incorporating a substantially larger number of species and phylogenetic uncertainty, challenge its validity[8,9]. An exception is a recent study reporting larger brain size in cetaceans living in mid-sized groups[10]. The hypothesis has only recently been tested in rodents for the first time and the results revealed that, in ground squirrels, sociality is not associated with larger relative brain size, but that social species tend to have larger bodies and correspondingly absolutely larger brains[6], suggesting that a possible link between body size and sociality may be mediating the effect on brain size.

Over the past decades, different factors have been proposed as the main driving force of cognitive enhancement mediated by sociality in birds and mammals generally, and primates in particular (reviewed in[2,11]). The original idea emphasized competition and tactical deception (as reflected in the name "Machiavellian intelligence")[12], but the mechanism was later reformulated by Dunbar and Shultz[13,14] as the need to maintain group cohesion through individual recognition and affiliative interactions to diffuse conflict. According to this latter view, cognitively demanding social behaviours are believed to take the form of behavioural coordination and pair bond formation in non-primates, but might become generalized to all group members in primates (reviewed in[2]). Mating system thus represents another domain of sociality that is pertinent to brain evolution. Indeed, association between monogamy and larger relative brain size has been reported in ungulates, carnivores, and birds[13,15].

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Cooperative breeding itself is another factor that has been suggested as potentially facilitating large brain evolution[15–17] (but see[18,19]).

Despite recent progress in comparative methods that take phylogenetic relatedness into account, broad comparative studies, while allowing for greater statistical power, remain inherently prone to spurious findings due to large variations in ecology and life history traits, the unrecognized influence of hidden variables, heterogeneity in evolutionary trajectories and selection pressures, and data inconsistencies across datasets[3,9,20,21]. One way to limit the effects of biological heterogeneity and statistical interference is to study brain evolution within closely related but behaviourally diverse clades[21]. Here, we use this approach and test the SBH in African mole-rats (Rodentia: Bathyergidae). This group is ideal to provide insights into some of the unanswered questions without introducing confounding factors associated with differences in general biology and ecology that have been implicated in brain size evolution. Major factors besides sociality include substrate use, habitat complexity, diet and foraging mode, activity pattern, home range, developmental mode and maternal investment (for a review, see[20]). Mole rats are uniform in most of these traits. They are all strictly subterranean, burrowing and feeding on underground parts of plants[22–26], but cover the whole social spectrum, from strictly solitary to the remarkably social cooperative breeders, warranting the term "eusocial"[27,28]. They all give birth to altricial young and from the limited information available, it seems there are no systematic differences in maternal investment (gestation length, litter size, lactation length) connected to sociality[29]. The naked mole-rat is somewhat exceptional, though, in having substantially larger litters than the other species[30]. Solitary species, however, seem to be seasonal breeders[31–33], in contrast to mostly aseasonally breeding social species[34–36]. Sociality also goes hand in hand with larger burrow systems and thus increased "home range", but reliable data for all species are not available and there is substantial intraspecific variation[37,38].

Solitary mole-rats are highly territorial and aggressive towards conspecifics. Their affiliative social interactions are confined to short periods of time during the breeding season and maternal care for juveniles, which disperse shortly after weaning[31–33]. Social species live in stable, multigenerational families in which only few individuals (often just a single bonded pair) reproduce and most of their offspring stay permanently within the family as non-reproductive helpers. Typically, members of this cohesive group cooperate through digging and maintaining the burrows, foraging for food and bringing it to communal storage, engaging in colony defence against intruders and predators, and taking care of the pups – grooming, huddling, returning them to the nest chamber when they wander off and providing them with cecotrophs[22,39–43]. In the genus *Cryptomys* the groups tend to be smaller and much less stable, especially in the mesic parts of the range[44]. Moreover, social mole-rats, in contrast to solitary ones, seem to be monogamous[45–48], which is another purported driver of cognitive abilities in non-primate mammals[13]. There is also evidence of individual recognition[43,49] and elaborate vocalization and social interactions in the social species[30,50–52] so these are not just simple aggregations. Mole-rat sociality is based on long-term (lifelong in eusocial species) pair bonds and stable social relationships among all members of an extended family[27,28,53]. Due to limited opportunity for dispersal and new burrow formation, there seems to be little flux in the composition of the social group, especially in eusocial species, colonies of which are characterized by extensive overlap of adult generations and permanent (lifelong) philopatry[27]. Importantly, manipulative or Machiavellian behaviour is likely selected against in mole-rat colonies with monopolized reproduction because it would harm an individual's inclusive fitness.

While social environment is a complex system, where various components come into play, some patterns in the data could provide insight into their relative importance. The general prediction is that monogamous social species of mole-rats should have bigger brains than solitary species. If social bonding, individual recognition, maintaining group cohesion and cooperation exert the major selection pressure[13,54], then the eusocial species with extremely high reproductive skew towards a single breeding pair might be expected to show the largest brains and cognitive potential, since they live in the largest and most cohesive groups, with a decreasing trend towards the solitary end of the social spectrum. If, however, the competitive aspect of sociality is more important, eusocial species should not face a pressure to increase brain size, since outcompeting other colony members would not improve an individual's fitness. Mole-rats that are still social, but not with such an extreme reproductive skew (genus *Cryptomys*)[44,55], could perhaps be expected to show greater cognitive capacities and larger brains, since they could potentially benefit by becoming dominant and taking over or starting their own colony, or realise their direct fitness by extra-colonial paternity[55]. However, as noted above, it is highly unlikely that complex Machiavellian interactions are present in mole-rats. No difference in brain size between the groups would thus indirectly point to these competitive interactions being the most important factor.

The social organization of eusocial mole-rats resembles that of eusocial insect societies in several aspects, such as monopolization of reproduction[27,28] and division of labour among non-reproductive group members[39,56–58] (but see[59,60]). Alternative hypotheses for social brain evolution have been recently developed for (eu)social insects[61] and African mole-rats have been suggested as a possible vertebrate group where they may apply. The distributed cognition hypothesis (DCH) seems to be particularly pertinent, as its predictions are opposite to those of the SBH. It assumes that in multi-generational colony groups characterized by high reproductive skew and therefore subjected to strong colony-level selection, members can rely on social communication to supplement individual cognition. The hypothesis therefore predicts relaxed selection for individual cognitive abilities and reduced brain investment in such (eu)social species[61]. If cooperative information sharing among individual mole-rats outweighs within-colony conflicts, solitary species should have the largest brains, with a decreasing trend toward the eusocial end of the social spectrum, where the potential for "distributed cognition" is highest.

Most comparative studies dealing with the SBH published to date have focused on relative brain mass or volumes of specific brain regions (particularly the neocortex) and the results were largely based on the analysis of previously published data[5,13,54,62–65]. In this study, we test predictions of the SBH and the DCH, using new, unprecedentedly comprehensive data on brains of 11 species representing all six existing genera of mole-rats. In light of recent studies on cognition[66,67] and neuronal scaling rules[68,69], it becomes clear that regarding cognitive abilities as

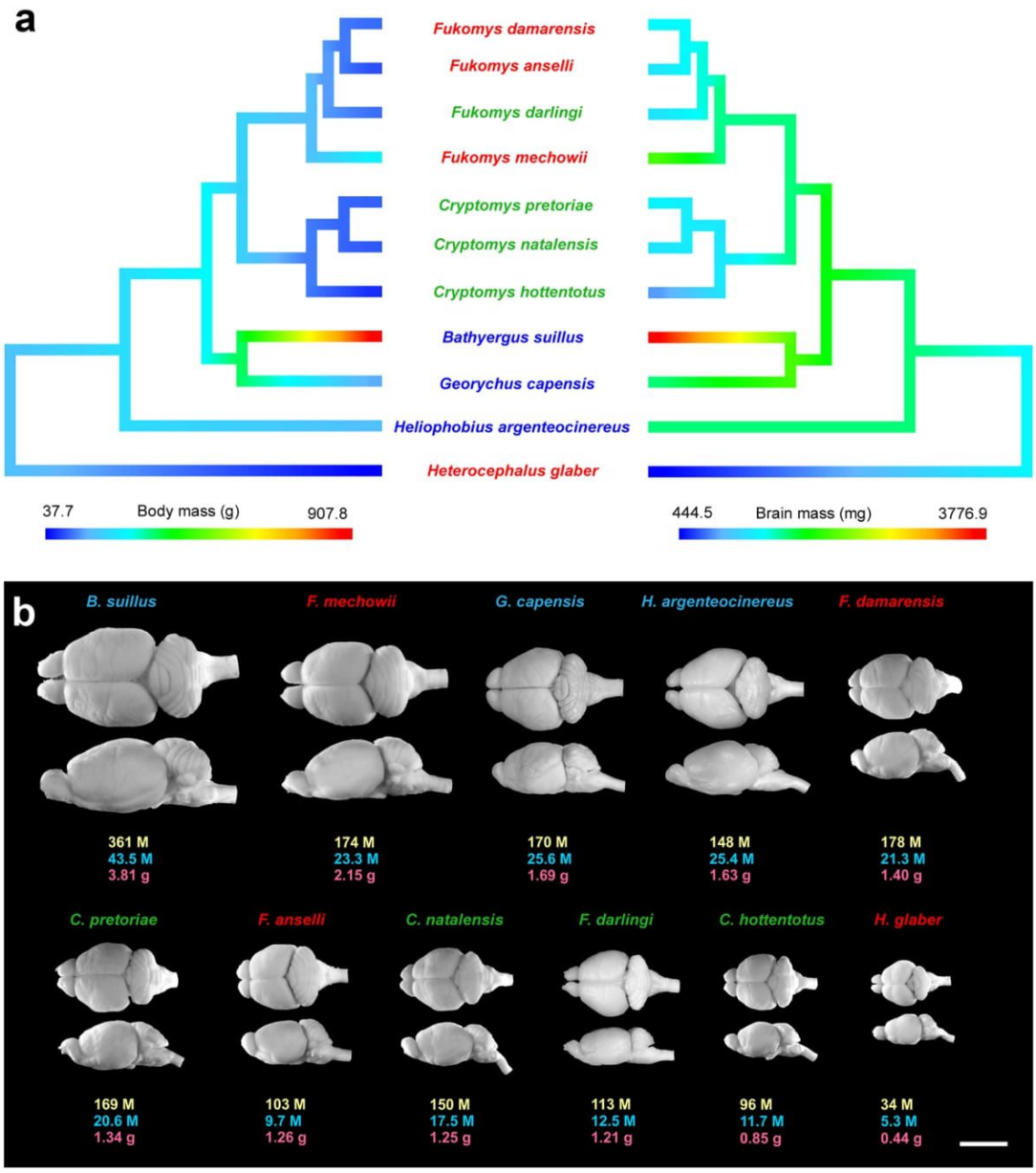

Figure 1. Body size, brain size and number of neurons for the mole-rat species examined. (**a**) The phylogeny of the 11 African mole-rat species included in the analyses with body mass (the left tree) and brain mass (the right tree) mapped as a continuous trait with the ancestral states reconstructed using the phytools package in R. The topology of the tree follows a published report[113]. (**b**) Dorsal and lateral views of representative brains are accompanied by information concerning total numbers of brain neurons (yellow), numbers of pallial neurons (blue) and brain mass (red). M, million. Scale bar, 10 mm. Species names are colour-coded by sociality: red – eusocial, green – social, blue – solitary.

a function of relative brain size is a gross oversimplification, and might be even misleading[70]. There are at least two factors at play – brain size and neuronal density[69,71]. Thus, at neuroanatomical level, more cognitive power can be achieved by increasing brain size or size of specific brain regions, or by increasing the neuronal density without that necessarily manifesting as a substantial increase in volume. Investigating a broad range of brain size measures enables us to pinpoint which brain parts, if any, are under selection, or if the whole brain responds in concert.

Results

Absolute and relative brain size. While it might be possible that subterranean microphthalmic mammals are somehow aberrant in the way their brains are built, we show that this is not a concern in the choice of mole-rats as our model group. With the exception of the naked-mole rat, bathyergids do not significantly differ from

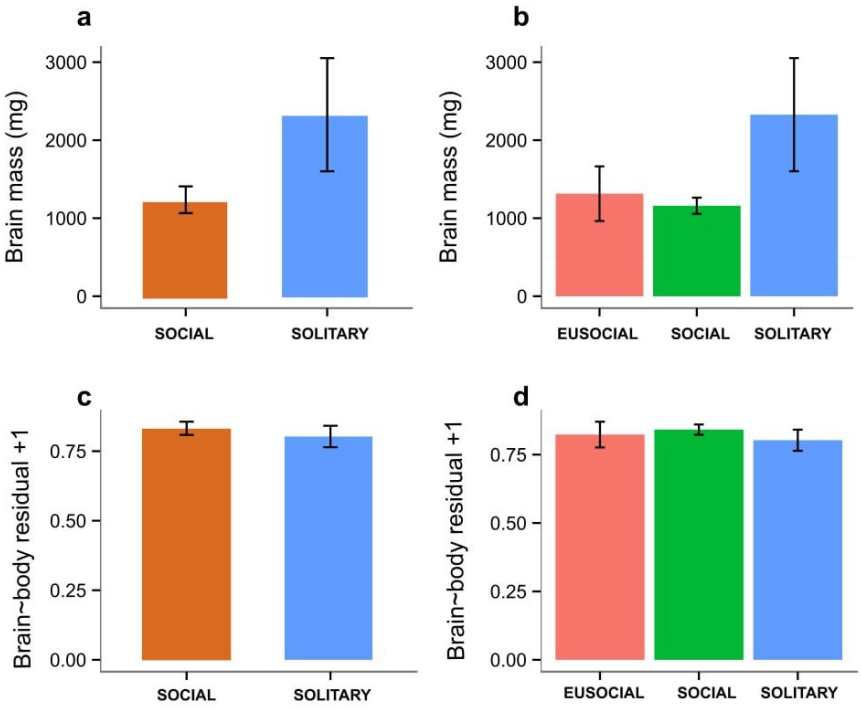

Figure 2. Absolute and relative brain size by sociality. Bar plots illustrating the differences in absolute (**a**,**b**) and relative brain size (**c**,**d**) between social and solitary (left column graphs) and eusocial, social and solitary species of African mole-rats (right column graphs). Note that solitary mole-rats tend to have absolutely, but not relatively larger brains than social ones. Relative brain size is expressed as a residual from the brain-on-body regression line for Rodentia, with 1 added to get positive numbers. Data are represented as mean ± SEM.

other rodents in either their allometric brain-body relationship or previously published neuronal scaling rules (Fig. S1). Notably, the naked mole-rat not only has a smaller brain than expected for a rodent of its body size, but also a lower number of neurons than predicted for its brain size.

The studied species range in average body mass from 38 g to 908 g and in average brain mass from 0.44 g to 3.81 g (Fig. 1, Table S1). Solitary species have significantly larger body mass than social species (posterior mean = 1.1089, CI = [0.1481, 2.2049], pMCMC = 0.0321, lambda mean = 0.75; for other comparisons, see Table S2). Likewise, absolute brain mass tends to be higher in solitary species, although the difference is not significant (posterior mean = 0.6486, CI = [−0.0018, 1.4556], pMCMC = 0.0741, lambda mean = 0.84; for other comparisons, see Table S2) (Fig. 2a,b).

Relative brain size, a measure previously shown to be associated with sociality[2,13,72] (expressed as a residual from the regression line for rodents) shows no connection to the social system in mole-rats (Fig. 2c,d; for statistics, see Table S2).

Volumetric analyses. To assess whether there is any evidence of mosaic evolution (disproportional enlargement of specific brain parts, see e.g.[73]) in response to selective pressures associated with sociality, we measured the volumes of 14 brain regions and determined the scaling rules for those structures with brain size (Tables S1 and S3). All measured volumes correlate significantly and very tightly with whole brain volume (Fig. 3). In fact, brain volume accounts for over 90% of variance in all structure volumes measured, except for the amygdala ($R^2 = 0.86$) (Table S3). We then compared relative volumes of these brain structures between sociality grades. Not surprisingly, given the high proportion of variance explained by brain size, relative volumes of all the structures are independent of sociality (Table S4). Mole-rats are thus no exception to the broad rule that conserved scaling rules explain an overwhelming proportion of variance in brain region volumes, as has been clearly shown in a much larger sample of mammals[74].

The neocortex ratio [C_R: neocortex volume/(brain volume − neocortex volume)] has been traditionally used as a proxy for intelligence in tests of the SBH. We found that in mole-rats, there are no significant differences between the social categories, but there is a potential trend towards higher C_R in solitary species (Fig. 4, Table S2). C_R also decreases significantly with maximum group size (PGLS: −0.0278, p = 0.0294; Fig. 5a) and mean group size (PGLS: −0.0358, p = 0.0218; Fig. 5d), but the relationship is not significant after removing the naked mole-rat from the analysis (maximum group size: −0.0297, p = 0.0721; mean group size: −0.0337, p = 0.1405).

Number of neurons. Neuronal numbers in the whole brain and specific brain regions are presented in Fig. 1 and Table S5, results of the statistical analyses in Table S6. Mole-rats generally conform to the neuronal scaling rules previously established for rodents[75] (Fig. S1b,c). Solitary species tend to have higher absolute numbers

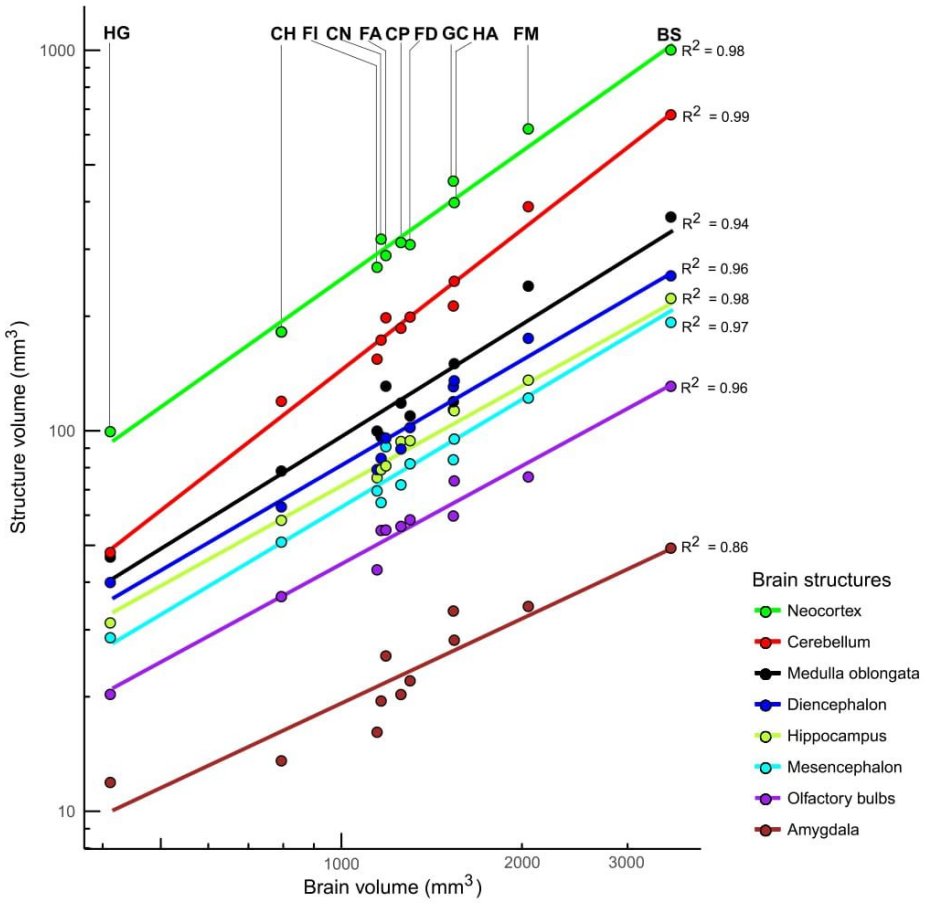

Figure 3. Scaling of selected brain structures with brain volume. Log-transformed structure volumes are plotted against log-transformed total brain volumes. The diencephalon volume was calculated as the sum of the thalamic and hypothalamic volumes, the mesencephalon volume as the sum of the tectal and tegmental volumes. Fitted lines and coefficients of determination are taken from the OLS regressions of species averages. Note that all structures scale very predictably with total brain volume. BS, *Bathyergus suillus*; CH, *Cryptomys hottentotus*; CN, *Cryptomys natalensis*; CP, *Cryptomys pretoriae*; FA, *Fukomys anselli*; FD, *Fukomys damarensis*; *FI*, *Fukomys darlingi*; FM, *Fukomys mechowii*; GC, *Georychus capensis*; HA, *Heliophobius argenteocinereus*; HG, *Heterocephalus glaber*.

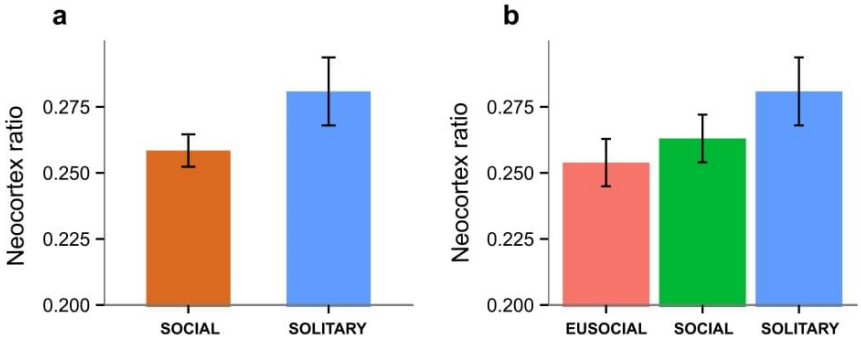

Figure 4. Neocortex ratio by sociality. Bar plots illustrating the differences in neocortex ratio (the ratio of neocortex volume to the rest of the brain volume) between (**a**) social and solitary, and (**b**) eusocial, social and solitary species of African mole-rats. Data are represented as mean ± SEM. Note that solitary species tend to have higher neocortex ratios than social ones.

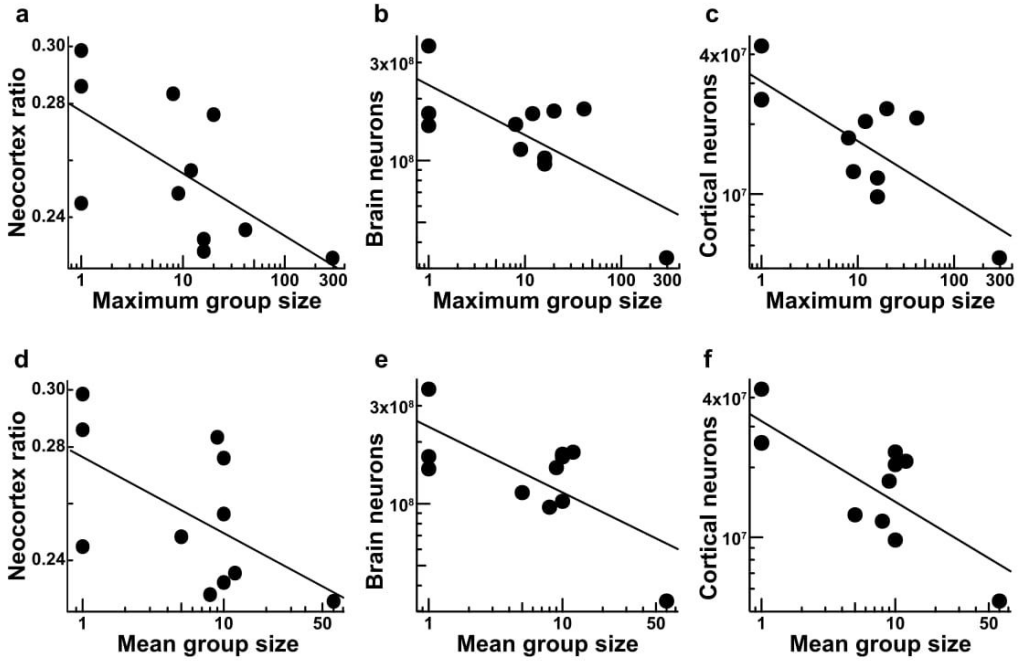

Figure 5. The relationship of selected neuronal correlates of cognitive capacity and social group size. Scatter plots showing negative correlation between neocortex ratio (**a**,**d**), number of brain neurons (**b**,**e**), number of cortical neurons (**c**,**f**) and maximum (**a**–**c**) and mean group size (**d**–**f**). The fitted lines represent the phylogenetic least squares regressions.

of neurons compared to social species (Table S6; Fig. 6a,b). Importantly, this difference is most pronounced and statistically significant in the number of cortical neurons (posterior mean = 0.7928, CI = [0.0694, 1.5191], pMCMC = 0.0396, lambda mean = 0.48) and neurons in the subcortical forebrain (posterior mean = 0.6884, CI = [0.0306, 1.3882], pMCMC = 0.0480, lambda mean = 0.44), i.e., solitary species have significantly more neurons in the forebrain (posterior mean = 0.7603, CI = [0.0405,1.4421], pMCMC = 0.0332, lambda mean = 0.48) (Fig. 6c,d).

Consistent with these results, the number of brain neurons decreases with both maximum group size (PGLS: −0.2167, p = 0.0322; Fig. 5b) and mean group size (PGLS: −0.2804, p = 0.0492; Fig. 5e), although this relationship is not significant after removing the naked mole-rat from the analysis (maximum group size: −0.1423, p = 0.1048; mean group size: −0.1643, p = 0.133). Number of cortical neurons also decreases with maximum group size (PGLS: −0.2724, p = 0.0019; Fig. 5c) and mean group size (PGLS: −0.3680, p = 0.0021; Fig. 5f), and, notably, this relationship remains significant even when analysed without the naked mole-rat (maximum group size: −0.2905, p = 0.0272; mean group size: −0.2342, p = 0.018).

Numbers of neurons contained in the brain regions examined correlate significantly and very tightly with their mass (Table S3) and, because the size of these regions scales highly predictably with brain size (Fig. 7b), also with brain mass (Fig. 7a). Numbers of neurons relative to the brain mass do not differ between the social grades in the whole brain or any of the five brain parts (Table S6).

We also examined residuals from the neurons-body regression line for rodents, essentially the neuronal index proposed by Herculano-Houzel[76] as an adequate proxy for cognitive abilities, and the ratio of cortical neurons to the neurons in brain stem, another index of cognitive power, analogous to the neocortex ratio (Fig. 6e–h). No significant differences were found between the solitary and social groups for either the neuronal index (posterior mean = −0.2467, CI = [−1.7914, 1.2497], pMCMC = 0.72, lambda mean = 0.07; for other comparisons, see Table S2) or cortical neurons ratio (posterior mean = 0.4113, CI = [−0.0363, 0.8235], pMCMC = 0.0585, lambda mean = 0.36; for other comparisons, see Table S2), although there is a trend for higher cortical neurons ratio in solitary species (Fig. 6g,h).

Discussion

The analyses performed in this study do not indicate a positive association between the neuroanatomical correlates of brain information processing capacity and sociality in African mole-rats. Despite examining measures ranging from overall brain size to neuronal numbers, we found no differences between the social grades in any of the relative measures, whether previously reported (relative brain size, neocortex ratio)[13,62], or tested for the first time (neuronal index, cortical neurons ratio). The few significant differences we revealed relate to absolute measures and were in favour of solitary mole-rats. Most importantly, solitary species have more neurons in the forebrain than social ones. Because the forebrain subserves higher cognitive functions and because the number of forebrain neurons is one of the major determinants of brain computational capacity[69,71,77], the high number

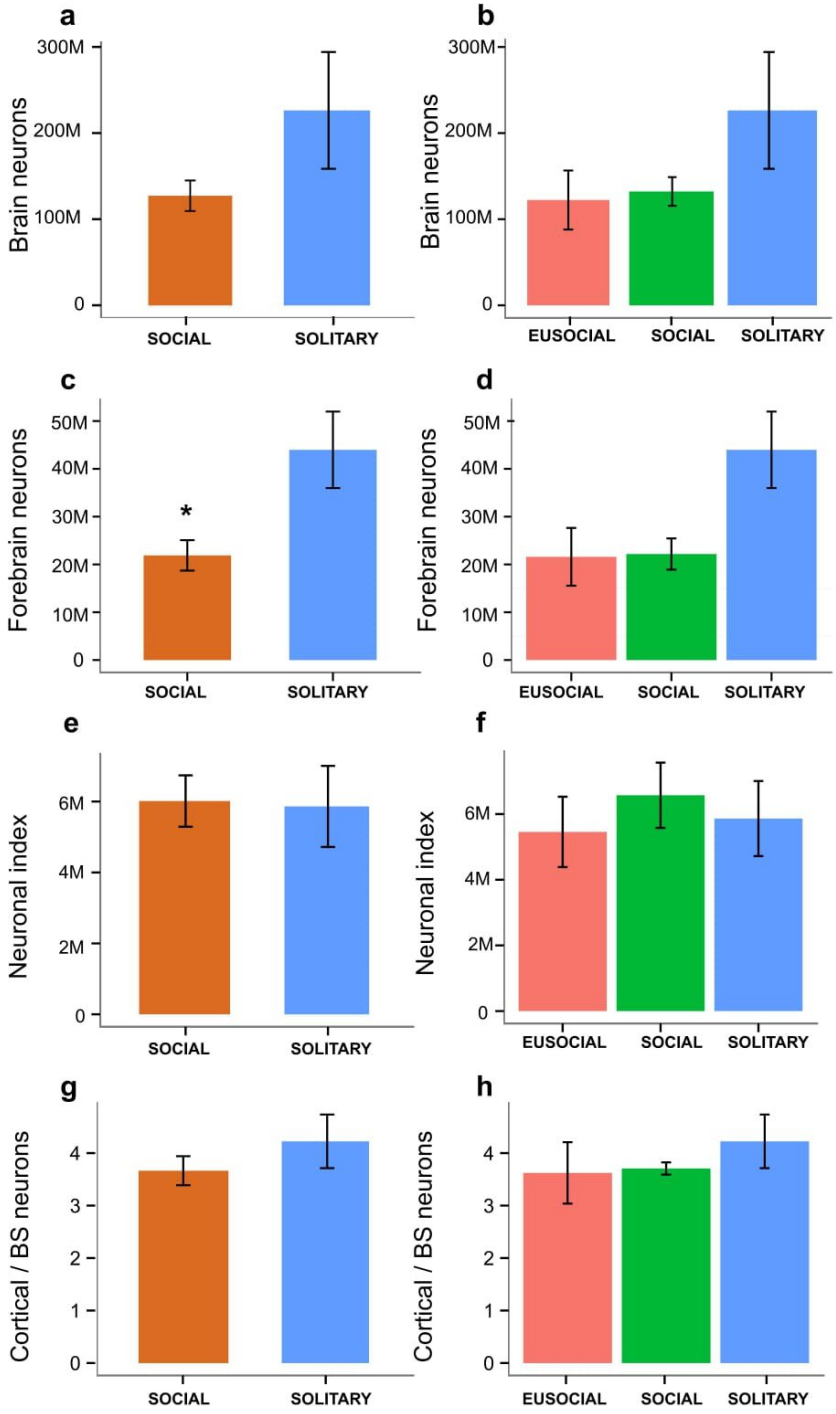

Figure 6. Neuronal approximations of cognitive capacity by sociality. Bar plots illustrating the differences in the average number of brain neurons (**a**,**b**), the average number of forebrain neurons (**c**,**d**), neuronal index (**e**,**f**) and the ratio of cortical neurons to brain stem neurons (**g**,**h**) between social and solitary (left column graphs) and between eusocial, social and solitary species of African mole-rats (right column graphs). Note that solitary mole-rats have significantly more forebrain neurons and tend to have more brain neurons and higher cortical neurons ratios than social ones. The neuronal index is expressed as a residual from the neurons-on-body mass regression line for Rodentia, adjusted by adding the largest negative value to get positive numbers. Data are represented as mean ± SEM; asterisk marks a significant difference (95% confidence interval does not include 0).

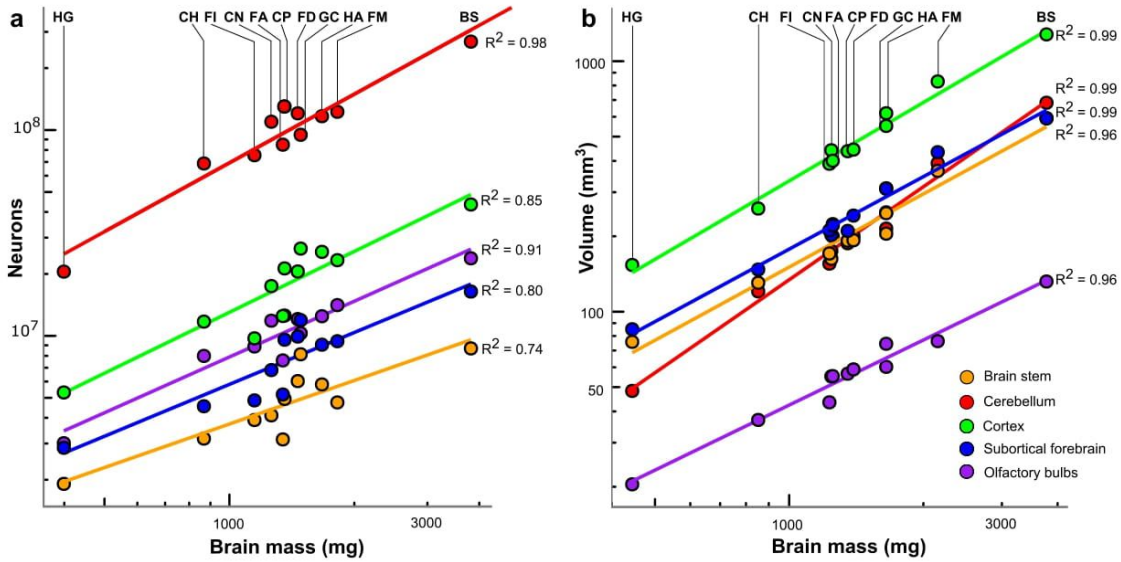

Figure 7. Scaling of neuronal numbers and volumes of major brain divisions with brain mass. (**a**) Number of neurons contained in the brain divisions plotted as a function of brain mass. (**b**) Division volumes plotted as a function of brain mass, for comparison. Data points correspond to species averages. Coefficients of determination are reported for the OLS regressions. See caption to Fig. 3 for abbreviations.

of forebrain neurons likely endows solitary species with improved cognitive abilities and increased behavioural flexibility. General cognitive abilities aside, it could be hypothesized that social mole-rats would have relatively larger brain areas related to individual recognition and/or emotional processing, such as olfactory areas or the amygdala[78,79]. This is not the case, however. Brain structure scaling is very conservative in mole-rats and we found no evidence of mosaic evolution. These results show that social living that entails maintaining group cohesion, individual recognition, behavioural coordination, monogamous pair bonding and cooperative breeding does not drive the evolution of large brains harbouring large numbers of neurons in African mole-rats. Importantly, our failure to find support for the SBH is not due to lack of statistical power. If that were the case, there would be no significant results and the trends would be in the opposite direction.

Although the debate about the importance of relative vs. absolute brain size for cognition is still ongoing and recent evidence for both is available[66,80], our results do not support the SBH in any case. Since we included both absolute and relative measures of whole brains and several brain regions, the results are not tied to any particular assumptions about the neural substrate for cognitive capacity. Drawing an analogy with insect eusociality (see Introduction), it is tempting to interpret the lower number of forebrain neurons in social mole-rats as evidence supporting the DCH. The very fact that the naked mole rat, the species that forms the largest colonies of up to 295 members[24] and in which non-breeding individuals of both sexes are physiologically suppressed from reproduction[81,82], has the smallest brain and the lowest number of neurons (both in absolute and relative terms; Tables S1 and S5, Fig. S1) is in line with the hypothesis. However, in contrast to DCH predictions[61], a reduced brain size and lower numbers of neurons were not observed in the other eusocial species, in which reproductive skew is maintained solely by incest avoidance[43] or by combination of incest avoidance and a suppression of female reproductive physiology[83]. While it is well possible that physiological reproductive suppression of non-breeders is necessary to achieve the level of group selection needed to relax the selection for individual cognitive abilities, alternative explanations cannot be excluded. For instance, the small, hairless and semi-poikilotermic[84] naked mole-rat may face more severe metabolic constraints than its larger hairy relatives. All other differences between social and solitary species reported in this study seem to be attributable to differences in body size. Taken together, the results obtained in this study are inconsistent with the SBH and do not provide a sound support for the DCH, they highlight the importance of viewing body size not just as a confounding factor to be corrected for, but as intrinsically connected with and driving brain size and computational capacity. Technically, body size is tightly coupled to absolute brain size and that, in turn, with the total number of neurons. There is substantial evidence and growing consensus that the total number of neurons and their densities are decisive for brain computational power[67,69,71,77]. Moreover, it has been posited that increased numbers of neurons lead to increased brain complexity, as neurons are the brain's "computational units" and more neuronal assemblies can be created, a notion supported by recent experimental evidence in mice[85].

The special case of mole-rats might also provide an insight into a more general problem with the SBH. Considering that, across vertebrates, the single best determinant of brain size is body size[86], we might have to deal with a confounding factor responsible for driving both sociality and larger bodies. Because the evolution of group-living is generally believed to have evolved as a response to predation[3,87], which can select for greater body size[88,89], and a growing body of evidence suggests that predation also directly selects for larger brains, it has been suggested by van der Bijl and Kolm (2016) that predation may confound the SBH by causing spurious

correlation between sociality and brain size[3]. The subterranean niche confers relative protection from predators and predation is not a driver of social evolution in mole-rats (see below). Therefore, we argue that low predation pressure in subterranean burrows may partly explain the lack of positive relationship between the correlates of brain processing capacity and sociality in African mole-rats.

These findings add to the series of recent papers that have reported no link between relative brain size and sociality in mammals[5,6,8,9,80] (but see[10]) and fish[4,90]. However, they are in stark contrast to previous studies in primates, cetaceans, carnivores and insectivores[62–65,91] that have found a positive relationship between C_R and social group size. In mole-rats, the trend goes in the opposite direction: solitary species tend to have larger C_R and C_R tends to correlate negatively with group size. This makes sense in light of the findings of Schillaci[92], who reports that C_R in primates correlates highly positively with body size and is not a significant predictor of group size, after controlling for body size. In other words, C_R is in fact indicative of absolute brain size, and that is what drives the correlation in primates. Interestingly, a recent test of the SBH in another rodent group (ground squirrels of the tribe Marmotini)[6], revealed that there is no link between relative brain size and sociality, but that social species tend to be larger and hence have absolutely larger brains. This relationship between body mass and sociality (and, correspondingly, the neocortex ratio) is opposite in mole-rats, and thus contrary to the SBH. Once again, these results point to a tight coupling between body size and absolute brain size. The latter seems to be generally linked with the brain's intrinsic complexity: the proportional and absolute size of the neocortex, the number of cortical areas and the total number of cortical neurons increase with absolute brain size (for reviews, see[93,94]).

The results presented here in no way challenge the existence of more subtle neurobiological differences between solitary and social mole-rats. Indeed, differences in neuropeptide receptor distributions and densities and in adult hippocampal neurogenesis were reported[95–97], though only limited data on a handful of species are currently available. Likewise, our findings cannot rule out that sociality does select for larger brains in mole-rats, as all we can observe is the end result of all selective pressures and constraints put together. Some hidden factors might be confounding the results, since not enough reliable data is available on all aspects of life-history in mole-rats. However, from the information available, there does not seem to be a systematic difference in maternal investment (gestation length, litter size, weaning age) between social and solitary species[29]. Solitary species, however, are seasonal breeders, in contrast to mostly aseasonally breeding social species[31–36]. To our knowledge, this has not been previously linked to differences in brain size, but it is another difference that cannot be separated from sociality and deserves further investigation.

Furthermore, it is possible that solitary mole-rats are subject to selection for larger size, or that social mole-rats face some constraints on body and/or brain size that the solitary ones are free from. Factors contributing to mole-rat sociality, or lack thereof, are still not well understood, although the aridity food distribution hypothesis is currently the prevalent explanation[53] (for alternative explanations, see[27]). Social mole-rats, generally living in harsher environments with fewer resources, may be prevented from attaining larger body (and brain) size due to the need to reduce energetic demands. Brains are metabolically expensive[98] and, simultaneously, excavating the burrow systems, especially in hard soils, carries an enormous energetic cost[99]. Lowering the metabolic demands might therefore be of utmost importance. Smaller body size and communal foraging means improving the chances of subsisting on scarce and dispersed food sources. The fact that this reduction in body size is not accompanied by an increase of relative brain size (which would result purely from decelerated brain mass reduction compared to body mass reduction) suggests that sociality does not exert enough selective pressure on brain size to outweigh these metabolic constraints. This is not to say that sociality does not act on cognitive abilities, but its importance may be more limited than generally assumed by the SBH.

To conclude, the absence of any evidence for selection acting on larger brain size or higher neuronal numbers in eusocial mole-rats, the pinnacle of cooperative breeding in vertebrates, weakens the notion that behavioural coordination or stable bonding is cognitively demanding and drives the evolution of cognitive capacity across vertebrates[13]. The fact that the challenges coupled with sociality do not entail brain enlargement or fundamental reorganization in this group resonate with an alternative view that dyadic and polyadic social interactions might not require flexible cognitive solutions in real-time, but could be solved by simpler evolved rules-of-thumb[100]. To our knowledge, there is no evidence that mole-rats engage in any Machiavellian interactions. But even if they were involved in sophisticated strategies like formation of coalitions or tactical deception, such behaviours would not increase individual fitness in species with monopolized reproduction; hence Machiavellian interactions should not effectively select for larger brains and improved cognitive abilities in eusocial mole-rats. Taken together, mole-rat sociality involves most putative drivers of cognitive abilities except for Machiavellian interactions. Therefore, our findings suggest, albeit indirectly, that Machiavellian interactions rather than social bonding and cooperation underlie the previously found link between social complexity and brain size.

Future stringent tests assessing the validity and generality of the SBH should encompass both (i) broad-scale comparative analyses incorporating various measures of social complexity as well as ecological and life-history variables including potentially confounding factors (such as appropriate proxies of predation pressure) and (ii) studies of variation in brain composition among closely related species that have similar ecologies and life-history traits but exhibit different levels of sociality. It will be equally important to direct further efforts to move from using readily measured traits such as brain size to more reliable proxies for cognitive abilities such as neuronal numbers and sizes of brain regions involved in specific behaviours. Integration of these approaches will provide deeper insights into the causal relationship between brain processing capacity and sociality.

Methods

Animals. African mole-rats (Bathyergidae) are endemic to sub-Saharan Africa. They form a monophyletic group within the rodent clade Ctenohystrica. Recently, it was suggested that the naked mole-rat *Heterocephalus glaber* be moved into its own family Heterocephalidae based on the time of divergence and distinctive

Species	Origin of Animals				Experimental Use		
	Colony			Wild-caught	Volumetry	Isotropic fractionator	Brain-body relationship
	Duisburg-Essen	České Budějovice	Cape Town				
Heterocephalus glaber	7	3	3		5	3	13
Bathyergus suillus				11	5	3	11
Georychus capensis				8	4	3	8
Heliophobius argent.		8			4	3	8
Fukomys mechowi	8	3			3	3	11
Fukomys anselli	9				3	3	9
Fukomys damarensis				11	4	3	11
Fukomys darlingi		3		4	4	3	7
Cryptomys hottentotus				8	4	3	8
Cryptomys pretoriae				10	5	3	10
Cryptomys natalensis				13	5	3	13
Total	24	17	3	65	46	33	106

Table 1. Origin and use of the experimental animals.

morphological and genetic traits[101]. Since this taxonomical revision does not change the phylogenetic relationships in any way, all the species are treated here as belonging to the monophyletic family Bathyergidae.

Eleven species of African mole-rats were examined: the Cape dune mole-rat *Bathyergus suillus* (BS), silvery mole-rat *Heliophobius argenteocinereus* (HA), Cape mole-rat *Georychus capensis* (GC), common mole-rat *Cryptomys hottentotus* (CH), Natal mole-rat *Cryptomys natalensis* (CN), highveld mole-rat *Cryptomys pretoriae* (CP), Ansell's mole-rat *Fukomys anselli* (FA), Mashona mole-rat *Fukomys darlingi* (FI), Damaraland mole-rat *Fukomys damarensis* (FD), giant mole-rat *Fukomys mechowii* (FM) and naked mole-rat *Heterocephalus glaber* (HG). All animals were adults and in the case of cooperatively breeding species non-reproductive individuals. Reproductive animals are usually the largest in the colony and can even substantially increase their body size after gaining reproductive status[102–105]. On the other hand, it is highly unlikely that reproductive status has any significant effect on absolute brain size or composition, because all reproductive animals are recruited from helpers well after reaching maturity. As reproductive individuals were not available in sufficient numbers and for all species, they were excluded from the analysis because including them could introduce a potential bias in relative brain size. Both sexes were close to equally represented (females: 52, males: 49, unknown: 4), with at least one male and one female of each species for each analysis. Animals were obtained either from colonies in the University of Duisburg-Essen, the University of South Bohemia (České Budějovice) and the University of Cape Town or wild-caught and housed at the University of Pretoria and University of Cape Town. Details on origin and use of experimental animals are provided in Table 1.

Animals were killed by halothane overdose and perfused transcardially with heparinized phosphate-buffered saline, followed by 4% phosphate-buffered paraformaldehyde (PFA). Brains were dissected and weighed immediately after perfusion, post-fixed overnight in the same fixative, and stored in 0.5% PFA or in anti-freeze solution at −20 °C until further processing.

All experimental procedures were conducted in accordance with the Guidelines for Animal Care and Treatment of the European Union, and were approved by the animal care and ethics representatives of the Faculty of Science of Charles University in Prague, University of Duisburg-Essen and University of Pretoria (AUCC 030110-002, AUCC 040702-015 and AUCC 000418-006). Captive animals originated from breeding colonies, the maintenance of which was approved by the Veterinary Office of the City of Essen, Germany (AZ: 32-2-1180-71/328) and by Ministry of Agriculture of the Czech Republic (22395/2014-MZE 17214); wild animals were collected under permit from the relevant Nature Conservation authorities of Gauteng, Western Cape and Northern Cape Provinces, South Africa. All efforts were made to minimize animal numbers and suffering.

Sociality. Given the lack of a generally accepted measure of social complexity and problems associated with even simple measures such as group size[106], we decided to adopt a simple approach and treat sociality either as a binary variable (solitary: BS, GC, HA; social: all others), or a categorical variable with tree levels (solitary: BS, GC, HA; social: CH, CN, CP, FI; eusocial: FA, FD, FM, HG). While crude, it is not subject to intraspecific variation and research effort bias and the categories also roughly correspond to group size[24]. The categories were delimited based on reproductive skew (the number of overlapping generations). Although it remains controversial whether solitary or social life-style is ancestral for African mole-rats[27], eusociality has evolved at least two times, once in the naked mole-rat and once within the genus *Fukomys* (Fig. 1a). Social group size (see electronic supplementary material, Table S7) was also used in a subset of analyses for the sake of comparison with earlier studies.

Relative brain size. A total of 106 animals were used to investigate the brain-body scaling in African mole-rats. The interspecific allometry of brain mass was determined by ordinary least square (OLS) linear regression of brain mass on body mass. Brain-body allometry at the order level (Rodentia) was used to calculate residuals for mole-rats. This relationship was based on a separate dataset of brain and body masses for rodent species

(n = 414) collated from the literature (for references, see Dataset S1). The regression line is thus kept independent of the data and provides an unbiased reference.

Volumetric analysis. Forty-five brains were used to perform the volumetric analysis. Brains were embedded in gelatine blocks fixed in sucrose-paraformaldehyde solution (30% sucrose, 4% PFA) and sectioned on a cryostat in the coronal plane at a thickness of 60 µm. Every second section was mounted on a slide and stained with cresyl violet. Total brain volume and the volume of 14 distinct regions of the brain (olfactory bulbs, olfactory cortices, neocortex, entorhinal cortex, hippocampus, amygdala, striatum, septum, thalamus, hypothalamus, midbrain tectum, midbrain tegmentum, cerebellum and medulla oblongata) were determined. Contours of the brain and the measured regions were drawn from the sections using a camera lucida. These drawings were then digitized using a Wacom tablet and the areas measured using the Scion Image software. The total area of the drawn structures was multiplied by the section thickness and sampling ratio to obtain the structure volume. Final volumes were then corrected for shrinkage. The extent to which a brain shrinks during histological processing is different in each brain. To obtain comparable values, each structural volume was multiplied by a correction factor (C_{ind}) calculated for each brain as follows: C_{ind} = volume of the perfused brain/sum of serial section volumes. The volume of the perfused brain was calculated by dividing the brain mass by the fixed brain tissue density (1.036 g/cm^3)[107]. Note that brain mass does not change significantly within the first hours of fixation[107]. Because all brains used in this study were weighed immediately after perfusion, i.e., after very short fixation, these measurements correspond to mass/volume of fresh brain.

Isotropic fractionator. Three brains per each species (33 in total) were used for quantification of total numbers of cells, neurons and nonneuronal cells using the isotropic fractionator method[108]. Brains were postfixed in 4% PFA for at least two weeks. After fixation, brains were dissected into the following five compartments: olfactory bulbs, cerebral cortex (including the underlying white matter and comprising the neocortex, hippocampus, olfactory cortices such as piriform and entorhinal cortex, and pallial amygdala), subcortical forebrain (comprising the diencephalon, caudate putamen, nucleus accumbens, globus pallidus, ventral pallidum, olfactory tubercle and septum), cerebellum, and brain stem (comprising the mesencephalon and medulla oblongata). Each dissected brain division was homogenized in 40 mM sodium citrate with 1% Triton X-100 using Tenbroeck tissue grinders (Wheaton, Millville, NY, USA). When turned into an isotropic suspension of isolated cell nuclei, homogenates were stained with the fluorescent DNA marker DAPI, adjusted to a defined volume, and kept homogenous by agitation. The total number of nuclei in suspension, and therefore the total number of cells in the original tissue, was estimated by determining the density of nuclei in small fractions drawn from the homogenate. At least four 10 µl aliquots were sampled and counted using a Neubauer improved counting chamber (BDH, Dagenham, Essex, UK) with an Olympus BX51 microscope equipped with epifluorescence and appropriate filter settings; additional aliquots were assessed when needed to reach the coefficient of variation among counts $\leq$0.15. Once the total cell number was known, the proportion of neurons was determined by immunocytochemical detection of the neuronal nuclear marker NeuN[109]. This neuron-specific protein was detected by an anti-NeuN rabbit polyclonal antibody (Merck Millipore, dilution 1:800). The binding sites of the primary antibody were revealed by a secondary anti-rabbit antibody conjugated with Alexa Fluor 594 (Life Technologies, Carlsbad, CA, USA; dilution 1:400). An electronic hematologic counter (Alchem Grupa, Torun, Poland) was used to count simultaneously DAPI-labelled and NeuN-immunopositive nuclei in the Neubauer chamber. A minimum of 500 nuclei was counted to estimate the percentage of double-labelled neuronal nuclei. Numbers of nonneuronal cells were derived by subtraction.

Data analysis. All data analyses were performed in R Studio with R 3.3.2.[110]. Prior to statistical analyses data were log-transformed. For estimating the differences between social and non-social species (sociality as a fixed effect), we used Bayesian generalised linear mixed models with Markov chain Monte Carlo (MCMC) estimation in the package MCMCglmm[111], with phylogenetic correction and multiple measurements per species taken into account as random effects. The lambda parameter was estimated for each MCMC model. This parameter potentially varies between 0, indicating that the trait evolution is independent of phylogeny, and 1, indicating that the traits are evolving according to Brownian motion on the given phylogeny, while intermediate values correspond to an effect of phylogeny weaker than under the Brownian model[112]. Mole-rat phylogeny was constructed from a published report[113]. Each model was run for 5 million iterations, with a burnin of 5000, and a thinning interval of 1000, that means approximately 5000 estimations were sampled. Convergence was confirmed by visual inspection of trace plots. Estimates of the differences between the levels of sociality were calculated from a posterior distribution created by subtracting the estimates for each level obtained during each MCMC iteration. Parameter estimates were considered statistically significant when 95% credible intervals (CI) did not include 0.

All linear regression coefficients, used to describe allometric scaling relationships, were determined by the ordinary least squares (OLS) method from species averages. For analyses of the relationship between selected brain measures and social group size, phylogenetic least squares (PGLS) method implemented in the R package nlme[114] was used with Pagel's lambda model for scaling the phylogenetic variance-covariance matrix. Statistical significance was evaluated at α level of 0.05.

Relative sizes and indexes of cognitive power were calculated as follows: relative brain size as a residual from the brain-body mass OLS regression for 414 species of rodents, excluding mole-rats; relative volumes of brain regions as residuals from the OLS regression of the brain region volume on the whole brain volume; relative numbers of neurons as residuals from the neurons-brain mass OLS regression for mole-rats; the neuronal index as residuals from the neurons-body mass OLS regression for rodents[76], excluding mole-rats; the cortical neurons ratio as the ratio of the number of cortical neurons to the number of brain stem neurons.

Data availability. All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Acknowledgements

This study was supported by the Czech Science Foundation (14–2758 S, to P.N.), Grant Agency of Charles University (325515, to K.K.) and the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/30.0022, to S.O.). We thank Kate Arnold, Thomas Bugnyar and W. Tecumseh Fitch for reading of the manuscript and discussions, Heiko Frahm, Marcela Lucová and Ivana Rašpličková for their assistance with experiments. P.N. thanks Jennifer U.M. Jarvis for her hospitality during his stay in Cape Town.

Author Contributions

P.N., K.K., H.B. and N.C.B. designed the research; H.B., R.S., M.J.O. and N.C.B. provided experimental animals; P.N., K.K. T.B., S.O. and Z.P. collected the data; K.K. analysed the data and all authors wrote the paper.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-018-26062-8.

Competing Interests: The authors declare no competing interests.

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CHAPTER 4

The evolution of brain neuron numbers in amniotes

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The evolution of brain neuron numbers in amniotes

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Edited by Gene Robinson, University of Illinois at Urbana–Champaign Institute for Genomic Biology, Urbana, IL; received December 9, 2021; accepted January 28, 2022

Reconstructing the evolution of brain information-processing capacity is paramount for understanding the rise of complex cognition. Comparative studies of brain evolution typically use brain size as a proxy. However, to get a less biased picture of the evolutionary paths leading to high cognitive power, we need to compare brains not by mass but by numbers of neurons, which are their basic computational units. This study reconstructs the evolution of brains across amniotes by directly analyzing neuron numbers by using the largest dataset of its kind and including essential data on reptiles. We show that reptiles have not only small brains relative to body size but also low neuronal densities, resulting in average neuron numbers over 20 times lower than those in birds and mammals of similar body size. Amniote brain evolution is characterized by the following four major shifts in neuron–brain scaling. The most dramatic increases in brain neurons occurred independently with the appearance of birds and mammals, resulting in convergent neuron scaling in the two endotherm lineages. The other two major increases in the number of neurons happened in core land birds and anthropoid primates, which are two groups known for their cognitive prowess. Interestingly, relative brain size is associated with relative neuronal cell density in reptiles, birds, and primates but not in other mammals. This has important implications for studies using relative brain size as a proxy when looking for evolutionary drivers of animal cognition.

intelligence | cognition | evolution | brain size | number of neurons

Significance

The evolution of brain processing capacity has traditionally been inferred from data on brain size. However, similarly sized brains of distantly related species can differ in the number and distribution of neurons, their basic computational units. Therefore, a finer-grained approach is needed to reveal the evolutionary paths to increased cognitive capacity. Using a new, comprehensive dataset, we analyzed brain cellular composition across amniotes. Compared to reptiles, mammals and birds have dramatically increased neuron numbers in the telencephalon and cerebellum, which are brain parts associated with higher cognition. Astoundingly, a phylogenetic analysis suggests that as few as four major changes in neuron–brain scaling in over 300 million years of evolution pave the way to intelligence in endothermic land vertebrates.

The evolution of cognitive capacity or "intelligence" and its underlying neural substrate has been of long-standing interest to biologists. Great strides have been made in understanding the evolution of brain size in vertebrates, with studies analyzing data on thousands of species (1–3). Since larger animals have larger brains but are not necessarily smarter, most studies of cognitive evolution use relative brain size (corrected for body size), which is thought to reflect extra neurons beyond those needed for controlling the body (4). We now have a good idea where major changes in brain–body scaling happened within birds (2) and mammals (3), and it is also clear that both mammals and birds have relatively larger brains than nonavian sauropsids (hereafter referred to as reptiles), although this has been rarely formally quantified because data on reptilian brain sizes are scarce (5).

However, we still lack a clear picture of the evolution of actual brain processing capacity. This is because the same increase in relative brain size can be reached by different evolutionary paths, not always involving actual brain enlargement, and might often result from selection on body size (3). Moreover, similarly sized brains of distantly related species can harbor substantially different numbers of neurons overall and in major brain parts (6, 7). These two caveats invalidate the very idea that we can estimate extra neurons and glean information about cognitive capacity from absolute or relative brain size alone.

This capacity is better determined by the number of neurons in the brain or specific brain parts (although their relative importance is still debated), their connections, interneuronal distance, and axonal conduction velocity (8, 9). Unlike brain size, though, these measures are not readily available for a sufficient number of species to be of practical use. Nevertheless, thanks to methodological advances (10), neuronal scaling rules (the allometric relationship between brain mass and neuron numbers) have now been determined for eight high-level mammalian clades (6, 11–13) as well as for a limited sampling of birds (14, 15).

To get the big picture of amniote brain evolution, we have to include data on nonavian reptiles. The deepest split in amniote evolution occurred between the synapsid lineage, leading to mammals, and the sauropsid lineage, including reptiles and birds. We cannot tell if similarities between birds and mammals are due to shared ancestry or convergent evolution without considering reptiles. Yet, the dearth of quantitative data

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Author contributions: K.K. and P.N. designed research; R.V. provided animals; D.F. provided animals helped to devise methodology; K.K., L.M., A.P., M.K., Y.Z., S.O., B.S., Z.P., and P.N. performed research; K.K. analyzed data; and K.K., L.M., A.P., M.K., Y.Z., S.O., B.S., Z.P., R.V., D.F., and P.N. wrote the paper.

The authors declare no competing interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at http://www.pnas.org/lookup/suppl/doi:10.1073/pnas.2121624119/-/DCSupplemental.

Published March 7, 2022.

on reptile brains is striking—brain mass is available for 183 species (5, 16), compared to thousands for birds and mammals, and neuron numbers are known for a mere 4 reptile species (17–19).

Taken together, to understand the evolution of brain processing capacity in amniotes, we need to include nonavian reptiles, consider changes in both brain–body and neuron–brain scaling, and examine the allocation of neurons to different brain parts. In this study, we provide these much needed data and reconstruct the big picture of brain evolution in amniotes in terms of neuron numbers.

Results

Using the isotropic fractionator (10), we quantified brain cellular composition in 107 species of squamate reptiles and turtles, covering all major lineages and a wide range of body sizes, and in an additional 37 species of birds. We then combined this with previously published data on birds, mammals, and reptiles, resulting in the largest dataset of vertebrate neuron numbers to date, comprising 251 species. Additionally, we compiled data on brain and body sizes in almost 4,000 species of amniotes, including 312 species of reptiles. Mapping these quantitative traits on a time-calibrated phylogenetic tree reveals that birds and mammals have convergently increased both absolute and relative brain size (Fig. 1) and neuron numbers (Fig. 2*A*), resulting in a disproportionate expansion of brain processing capacity. While there is substantial overlap among the distributions of absolute brain sizes in all three groups, relative brain sizes are almost entirely distinct in reptiles (Fig. 1), with birds and mammals having on average about sixfold and eightfold larger brains, respectively, than expected for a reptile with the same body mass (*SI Appendix*, Table S1). Importantly, this increase in brain size goes hand in hand with an increase in neuron density (number of neurons per brain structure mass), even though, across amniotes, neuron densities go down as brains get larger (*SI Appendix*, Fig. S1). The difference in nonneuronal (glial) cells is much less pronounced, although reptiles still show lower numbers (*SI Appendix*, Fig. S2*B*). As a result, reptiles have dramatically lower neuron numbers for a given body size. On average, birds and mammals harbor about 21- and 20-fold more neurons in their brains, respectively, than would be expected for equivalently sized reptiles (*SI Appendix*, Fig. S2 and Table S1). As an illustrative example, the squamate reptile with the most neurons in our dataset, the Asian water monitor, with a body mass of 3.9 kg, has 78 million brain neurons, which is comparable to the 168-g Golden hamster (84 million) or the 44-g King quail (80 million). The 90-kg Nile crocodile has only one-half as many neurons (83 million) as the 4.5-g goldcrest (164 million), to mention an extreme example.

These differences are not homogenous across brain regions. Not only can brains of the same size differ in the number of neurons but also the total number of neurons can be allocated to different brain parts. Here, we divided the brain into three parts, namely, the telencephalon, cerebellum, and the "rest of brain," comprising the diencephalon, mesencephalon, and medulla oblongata. While the telencephalon has traditionally taken center stage as the "seat of higher cognition," it is the cerebellum that accounts for most of this striking increase in neuron numbers. Birds and mammals have on average about 17- and 9-fold more neurons, respectively, in the telencephalon than expected for reptiles of equivalent body mass, but about 45- and 69-fold more neurons in the cerebellum. In the rest of brain, however, this amounts to about a ninefold and fourfold increase, which is less than the increase in relative brain size in mammals (*SI Appendix*, Table S1). Consequently, the allocation of brain neurons to the three major brain parts is distinct in reptiles, mammals, basally diverging birds, and core land birds (Fig. 2*B*). The ratio between telencephalic and cerebellar neurons varies among reptilian and avian groups but remains similar across mammals, implying the previously reported coordinated scaling of neurons in these structures (20) is specific to mammals.

To further explore these changes in neuron scaling rules across amniotes, we fitted Bayesian reversible-jump bivariate multiregime Ornstein–Uhlenbeck models (21), which allow for the automatic detection of significant shifts in allometry (slope and intercept) on a phylogeny without the need to specify the shift locations a priori. These analyses identified several major macroevolutionary shifts in neuron scaling within amniotes (Fig. 3*A* and *SI Appendix*, Figs. S3–S6). Consistently, for the whole brain and major brain parts, the shifts were uncovered at the branches leading to mammals and birds, with the exception of the rest of brain, where the shift was located on the branch leading to placental mammals, assigning marsupials to the ancestral condition. Additional shifts happened in core land birds (comprising hawks and eagles, owls, falcons, songbirds, and parrots in our dataset) and anthropoid primates (monkeys and apes). The relatively low number of transitions to different optima in over 300 million years of evolution implies strong constraints are in place.

To confirm these shifts and to determine whether they result in distinct or convergent allometric regimes, we tested the differences in the slope and intercept of the putative grades in a phylogenetic least squares (PGLS) framework (*SI Appendix*, Table S2). The best fit model for whole-brain neuron scaling comprises three groups, namely, reptiles, anthropoid primates and core land birds, and other birds and mammals, with similar convergences in the scaling of individual brain parts (Fig. 3 *B*–*E*). Although emphasis has previously been placed on the differences, here, we show a remarkably similar pattern of evolution of neuronal scaling in birds and mammals, despite their different brain organization. However, the sampling is still far from complete, so additional scaling shifts might be uncovered in the future. No major shifts in brain neuron scaling were identified within nonavian reptiles, despite their long evolutionary history. Similar changes were uncovered for the scaling of neurons with body mass (*SI Appendix*, Fig. S7 and Table S3), where an additional decrease in the number of cerebellar neurons was found in snakes. This is due partly to their elongated bodies skewing the brain–body relationship and partly due to the reduction of the cerebellum, which is common to limbless squamates and associated with the pattern of locomotion (22). The resulting changes in the number of neurons for body mass follow different paths in different brain parts (Fig. 4 and *SI Appendix*, Fig. S8).

The above findings about the differential distribution of neurons to major brain parts in mammals are slightly complicated by the fact that the telencephalon was dissected differently in the mammalian studies; the number of telencephalic neurons, therefore, excludes the striatum in mammals, which is included with the rest of brain instead. The number of telencephalic neurons also excludes the olfactory bulbs (OBs) in 26 species of mammals because they were not available for analysis. The results are unlikely to be significantly affected by this difference in brain division or the missing OBs, as the striatum accounts for a small fraction of telencephalic neurons and OBs account for a small proportion of brain neurons in these mammalian

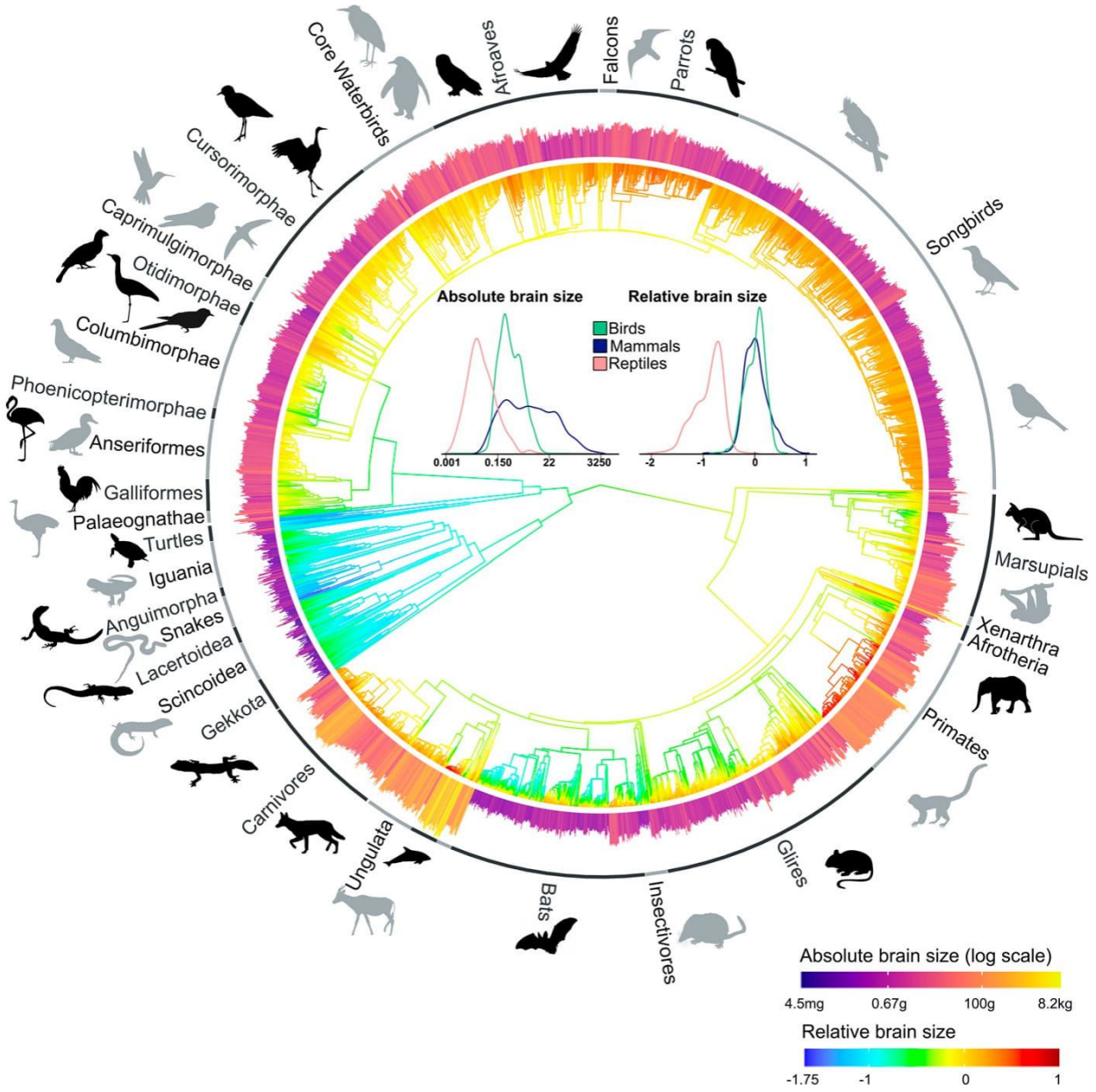

Fig. 1. Absolute and relative brain sizes mapped to amniote phylogeny. Relative brain size expressed as residuals from PGLS regression of log-transformed brain mass on log-transformed body mass is mapped on the tree, with the internal nodes showing relative brain sizes based on an ancestral reconstruction of brain and body mass. The outer bars represent log-transformed absolute brain mass. The *Inset* graphs show the density distribution of absolute and relative brain sizes in birds, mammals, and nonavian reptiles. Silhouette illustrations are from phylopic.org (see *SI Appendix* for detailed credits).

groups (in the 56 species with OB available, 0.02 to 15%; mean, 5%). Nevertheless, we repeated the analysis with data including estimates of OB mass and neuron number and estimates for the striatum added to the telencephalon and subtracted from the rest of brain. These corrections resulted in an average 5.5% increase in estimated telencephalic neurons and a 26% decrease in estimated rest of brain neurons in mammals. This only strengthens the conclusion that the rest of brain contains a minor fraction of brain neurons in mammals and does not change the distinct grades identified by PGLS (*SI Appendix*, Tables S4 and S5). To further demonstrate that different brain division in mammals does not significantly affect the results, we compared numbers of neurons in the avian pallium (comprising the hyperpallium, mesopallium, nidopallium, arcopallium, and hippocampus) with its homolog—the mammalian pallium (comprising the neocortex, hippocampus, olfactory cortices such as piriform and entorhinal cortex, and pallial amygdala). This comparison confirms the convergences in neuron–body mass scaling between anthropoid primates and core land birds and between other birds and nonprimate mammals (*SI Appendix*, Fig. S8).

To measure the evolutionary flexibility of the scaling rules, we assessed the Brownian motion rate of evolution (σ^2) of residuals from PGLS regressions and compared them among the allometric grades and brain parts. The stronger the allometric integration, the lower the residual variation and hence the

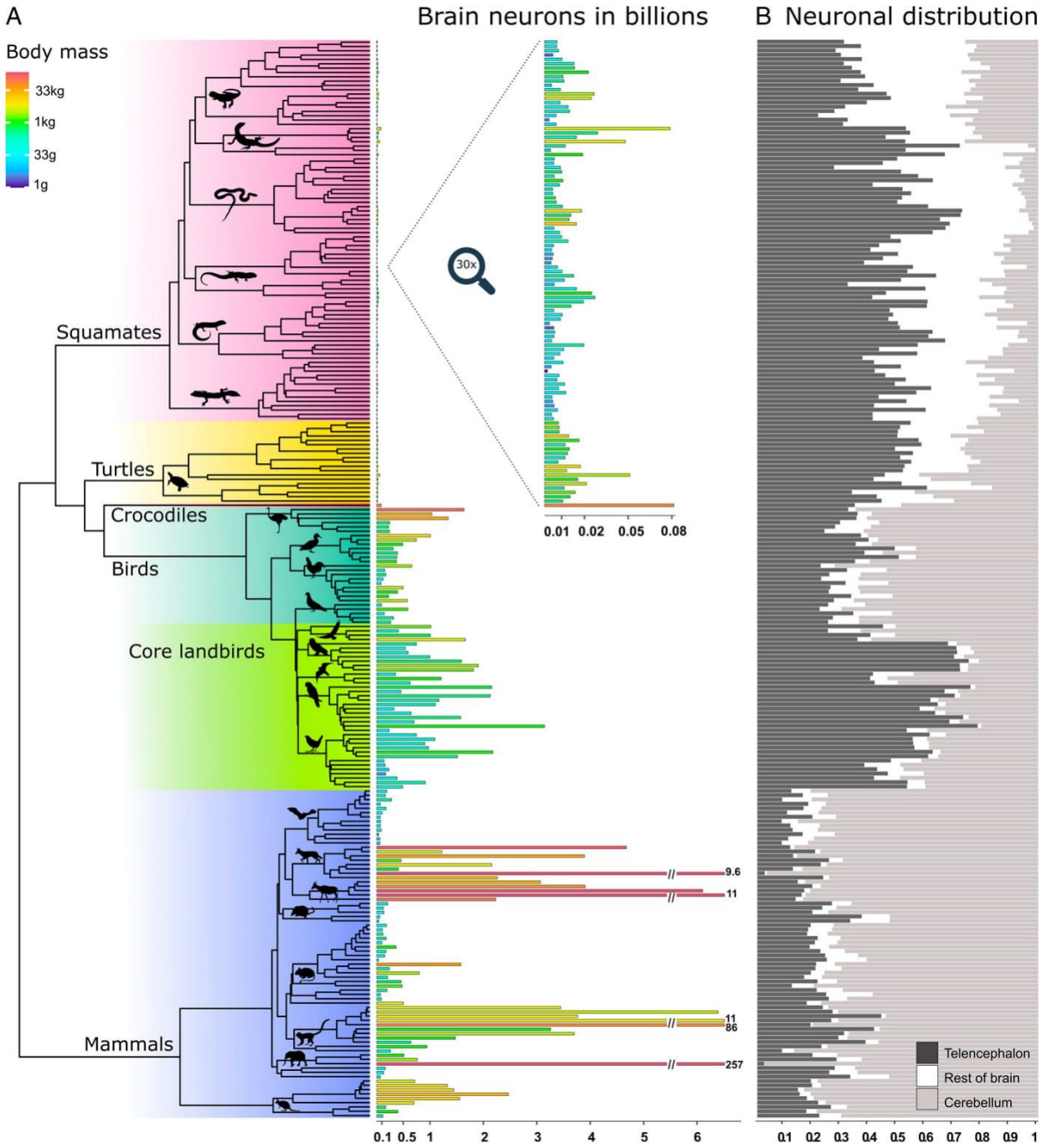

Fig. 2. Absolute brain neuron numbers in amniotes and their allocations to major brain parts. (*A*) Absolute brain neuron numbers are plotted on the amniote phylogenetic tree. Bar lengths correspond to neuron numbers (note that the bars for the five species with the highest numbers of neurons have been truncated), while bar color indicates body mass on a logarithmic scale. The bars for reptiles have been enlarged 30 times in the *Inset*. (*B*) Allocation of total brain neurons to major brain parts. The gray-scale bars indicate the proportion of brain neurons found in the telencephalon, rest of brain, and cerebellum. Cerebellum is the dominant fraction in all mammals, while there are two distinct patterns in birds, with cerebellum being predominant in basally diverging birds and telencephalon in core land birds (Telluraves). In reptiles, the cerebellum generally contains fewer neurons than the rest of brain, which accounts for a negligible fraction of brain neurons in endotherms. An evolutionary trend of increasing cerebellar neuronal fraction is seen in turtles and crocodiles. Silhouette illustrations are from phylopic.org (see *SI Appendix* for detailed credits).

rate of evolution. In other words, a high rate of evolution means that the scaling is not very tight and species can easily deviate in either direction. The strength of allometric integration was generally similar in all the analyzed clades, suggesting quick shifts between the different optima. Primates, however, show accelerated rates of evolution (*SI Appendix*, Tables S6 and S7), which is indicative of relaxed constraints or strong selection. The allometric integration in the cerebellum is strongest in birds, possibly due to the constraints of active flight, requiring a high number of cerebellar neurons, but

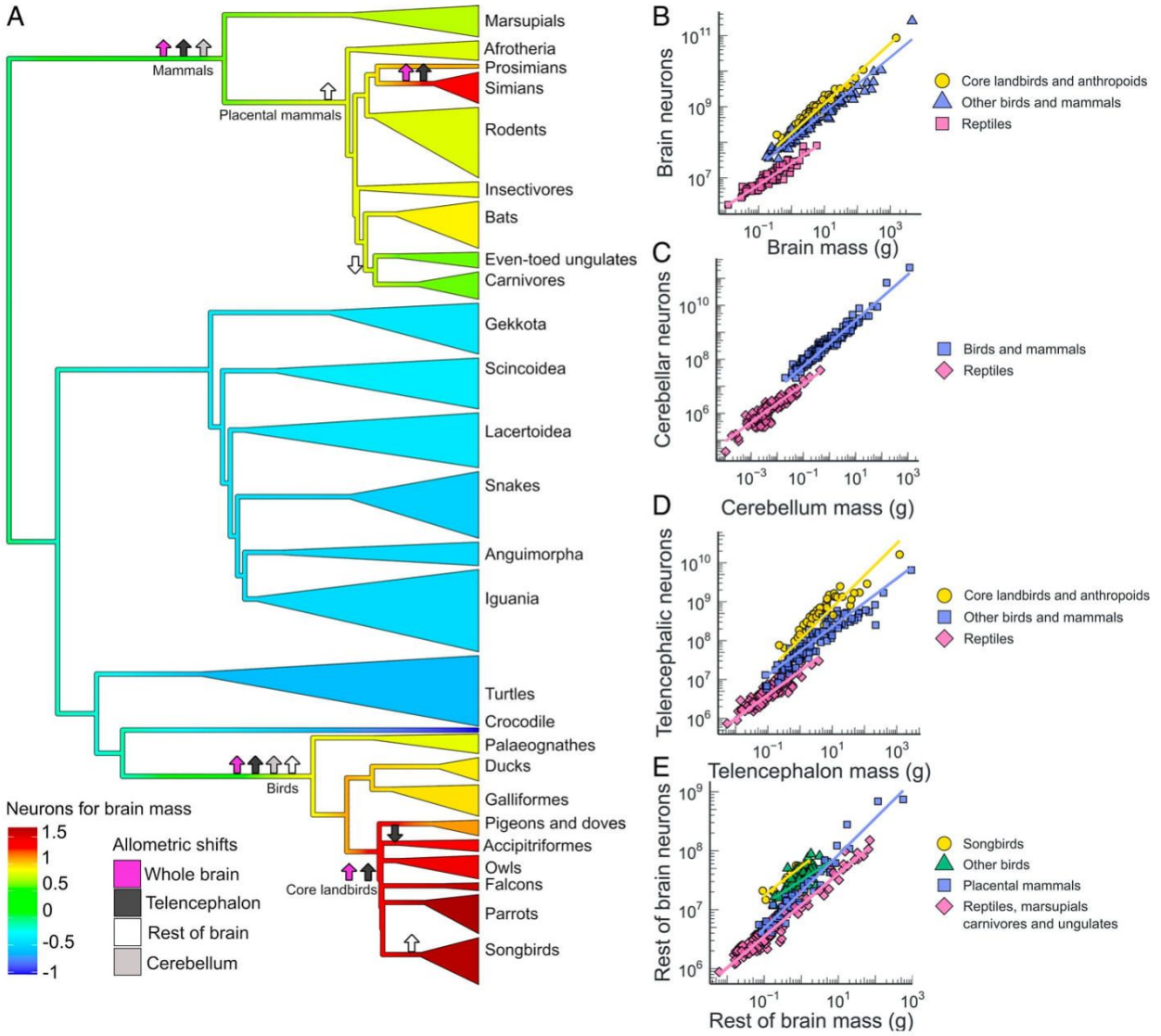

Fig. 3. Shifts in neuron–brain scaling in amniotes and scaling of convergent allometric regimes. (*A*) Tree colors correspond to neuron density in the whole brain, with blue colors indicating low density and red colors high density. The arrows indicate the branches with shifts in allometric relationship between structure mass and neuron number (resulting in either an increase in neurons, arrow up; or a decrease in neurons, arrow down) for the whole brain, telencephalon, cerebellum, and rest of brain, identified by reversible-jump Markov chain Monte Carlo analysis with posterior probability of >0.7 for clades including more than three species. (*B*–*E*) Log-log plots of neuron number for structure mass with regression lines for the distinct regimes identified by PGLS analysis.

precluding substantial brain enlargement. The rates of evolution are the same for telencephalon and cerebellum in mammals, but the cerebellum has a 1.5-fold higher rate in reptiles, whereas in birds, the telencephalon has a 3-fold higher rate than the cerebellum (*SI Appendix*, Table S8).

Another general pattern emerged, revealing a significant positive correlation between relative brain size and relative neuron density (Fig. 5). This holds not only across amniotes (Fig. 5*B*), as a result of the differences between reptiles and endotherms, but also within birds (Fig. 5*C*) and reptiles (Fig. 5*D*), when examined separately. Mammals in general do not follow this pattern (Fig. 5*E*) but primates do (*SI Appendix*, Fig. S9). While absolute neuron density on its own is not a meaningful proxy of brain processing capacity, as it predictably goes down with increasing brain size, relative neuron density (higher or lower than expected for a given brain size) is more informative. Just as animals with a large relative brain size will have larger brains for a similar body size, animals with higher relative neuron density will have more neurons in a similarly sized brain. These effects then compound in the taxa that exhibit a positive association between relative brain size and relative neuron density, leading to disproportionately higher numbers of neurons in species with relatively large brains. The same relative brain size can result from different processes, which do not necessarily involve selection for larger brains (3). Therefore, a simultaneous increase in relative brain size and neuron density might reflect selection on brain processing capacity (absolute number of neurons) and differentiate from passive changes due to body size. This association also suggests that if relative brain size is to be used, it might be a more appropriate proxy for cognitive capacity in birds than in mammals because, in birds, it might be an indirect measure of neuron numbers.

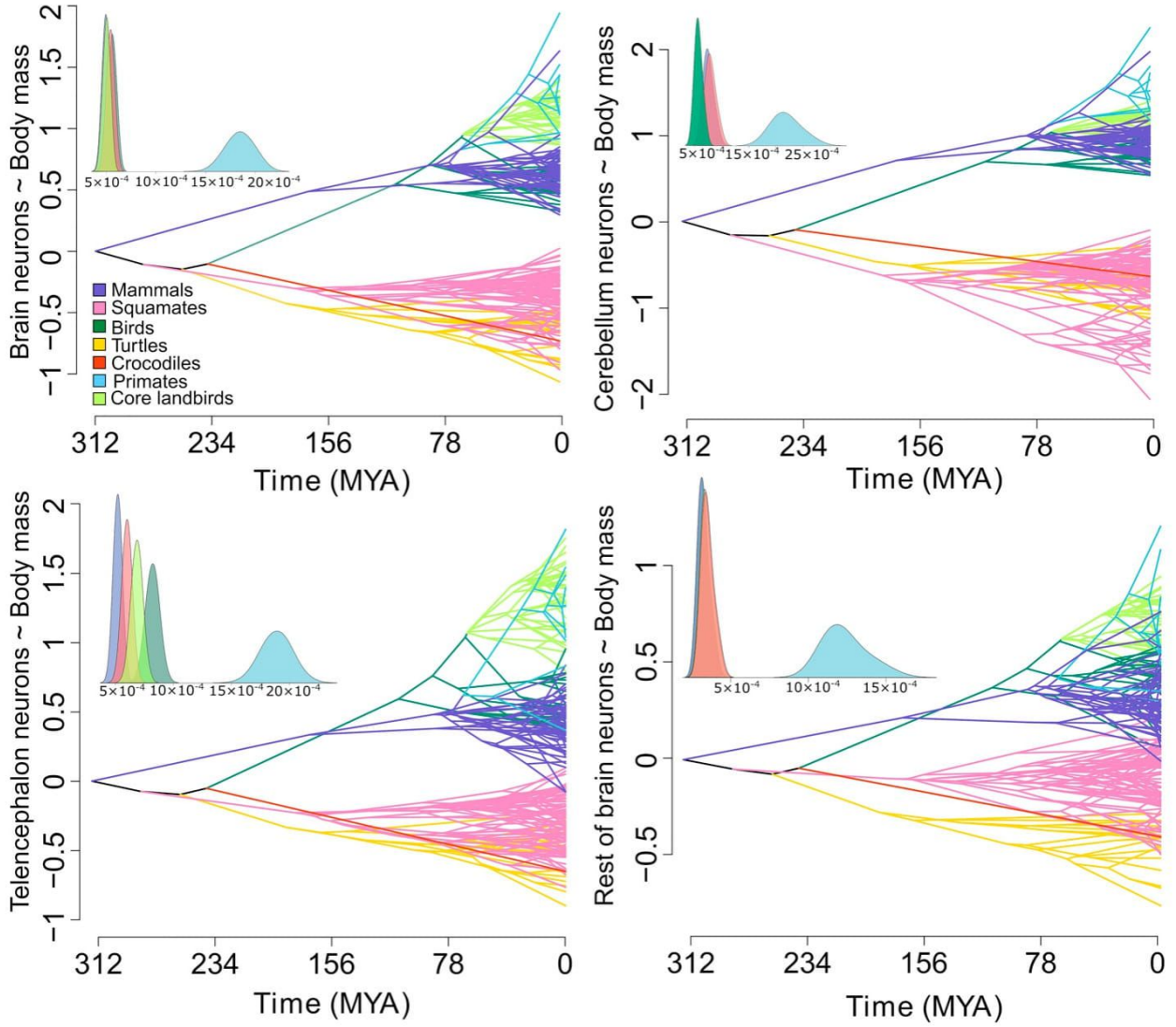

Fig. 4. Phenograms showing the evolution of brain neuron numbers relative to body mass over time. Colors in phenograms correspond to major reptile lineages (squamates, turtles, crocodiles), primates and core land birds (the groups identified as having experienced significant shifts in allometric scaling), and other birds and mammals. The *Inset* graphs show the σ^2 for residuals from PGLS as estimated by a multiple-variance Brownian motion model, corresponding to the strength of allometric integration. All nonavian reptiles are grouped in red color. Primates are characterized by weaker allometric integration of the number of neurons with body mass relative to all other groups.

Discussion

Phylogenetic analyses performed in this study have identified only four major shifts in neuron–body scaling in over 300 million years of amniote evolution. These occurred independently with the appearance of birds, mammals, core land birds, and primates. We suggest that these convergent increases in neuron numbers represent stepping stones in the evolution of avian and mammalian intelligence. No major shifts in the numbers of brain neurons were observed within nonavian reptiles, but they may have happened in other vertebrate groups. Relatively large brains have evolved several times in some cartilaginous and ray-finned fishes, while newts and salamanders have reduced brain size (23). It remains to be seen whether these changes in brain size are also accompanied by changes in neuronal density. At the moment, sufficient data on the numbers of neurons in amphibians and fishes are lacking; brain neurons have been quantified in two miniaturized species of ray-finned fish (24, 25) and two species of amphibians (26).

It is recognized that the energetic cost of neural tissue is an important constraint in the evolution of large brains (27). However, limited evidence exists for an association between relative brain size and metabolic rates within mammals or birds (28, 29). It has also been suggested that larger brains in endotherms compared to ectotherms can be attributed to their higher body temperatures and that increases in nonneuron cell numbers play a critical role (30). Here, we provide a different point of view. The massive increase in neuron numbers relative to body size in birds and mammals might have been enabled by actually relaxing the metabolic constraints due to the transition to endothermy. Since brain metabolism scales linearly with the number of neurons (31) and the brain carries a high energetic cost even at rest and in the absence of active signaling (32, 33),

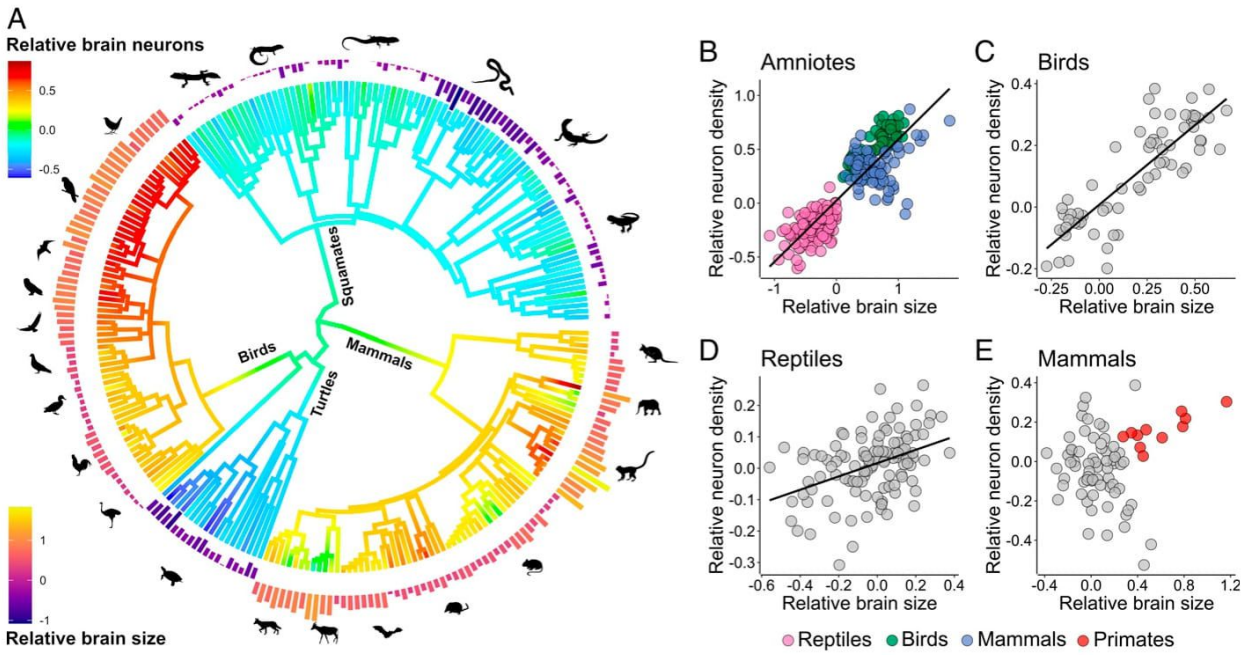

Fig. 5. Large relative brain size tends to co-occur with high relative neuron density across amniotes. (*A*) Ancestral reconstruction of the relative number of brain neurons for brain mass is mapped on a phylogenetic tree. The outer bars represent relative brain size (calculated as residuals from PGLS regression of brain mass on body mass across the amniote dataset). (*B*–*E*) Plots of relative neuron density against relative brain size calculated across amniotes (*B*), birds (*C*), reptiles (*D*), and mammals (*E*). When analyzed across all amniotes, there is a significant positive association between larger relative brain size and higher relative neuron density (*B*) (PGLS;t_{249} = 5.85, $P < 0.001$, $\lambda = 0.92$). This pattern holds also within birds (*C*) (PGLS;t_{63} = 6.01, $P < 0.001$, $\lambda = 0.52$) and reptiles (*D*) (PGLS;t_{108} = 3.74, $P < 0.001$, $\lambda = 0.37$), but not within mammals (*E*) (PGLS;t_{74} = 1.15, $P = 0.25$, $\lambda = 0.96$). However, primates show a positive association between the analyzed traits (PGLS;t_9 = 2.77, $P = 0.02$, $\lambda = 0.3$; *SI Appendix*, Fig. S9). Silhouette illustrations are from phylopic.org (see *SI Appendix* for detailed credits).

a high number of neurons constitutes an energy drain that cannot ever be truly turned off. Reptiles that rely heavily on energy conservation thus cannot afford this expensive tissue beyond a certain point, hence their low allometric exponent for the relationship between the number of neurons and body mass. With the adoption of endothermy, which is inherently metabolically expensive and requires a higher energy intake (34, 35), this constraint might have become relatively less important, enabling the rise in neuron numbers with a smaller percentage increase in total energy expenditure. The resulting increase in brain processing capacity, in turn, may have paid for itself in terms of improved foraging efficiency and other fitness benefits. Thus, the transition to endothermy might have tipped the balance of the cost/benefit ratio of neural tissue. Interestingly, one mammalian species in our dataset, the naked mole-rat, known for its low metabolic rate, weak ability to maintain stable body temperature, and high hypoxia tolerance (36, 37), also has a much lower than expected neuronal density in the telencephalon, which is comparable to those of reptiles.

The finding that cerebellar neurons account for most of the difference in neuron numbers between reptiles and endotherms is interesting in light of the mounting evidence that the cerebellum plays a key role in the evolution of sensorimotor and cognitive control of complex behaviors (38–41).

Despite their long independent evolution and distinct anatomical organization of the brain (42, 43), birds and mammals converged on similar numbers of telencephalic and cerebellar neurons, with yet another increase in telencephalic neurons seen in anthropoid primates and core land birds, which might provide the neural substrate for their remarkably similar cognitive feats (44). However, in contrast to mammals, where tight functional coupling and coordinated neuronal scaling of telencephalon and cerebellum are well established (20, 45), the evolution of a large, neuron-rich telencephalon in core land birds is not accompanied by a matching gain of cerebellar neurons. Given that the increase in neurons in the "smartest" birds is limited to the telencephalon, the cerebellum may turn out to be less important for cognitive functions in birds, although little evidence is available at the moment.

Interestingly, a higher relative brain size in birds is accompanied by a higher relative neuron density, whereas in mammals, no such relationship exists. This means that if we take two birds with equivalent absolute brain sizes, the one with the larger relative brain size will also likely have more neurons. This is in line with abundant evidence that relative brain size predicts intelligent behavior in birds (46–48). Ultimately, the cases where an increase in relative brain size is coupled to an increase in neuron density might indicate selection on the brain, as opposed to selection on body size. Primates are an exception among mammals in that they also seem to follow this pattern, suggesting the large brains of anthropoid primates are the result of selection on the neural substrate mediating their remarkable cognitive abilities. Moreover, primates show a weaker integration between neuron numbers and body size than other amniotes, a feature that likely contributed to the rapid evolution of their brains by increasing the variation that selection can act on (49).

This study highlights that encephalization trajectories, neuron densities, and neuron distribution to different brain parts can all be clade specific. Because of that, comparative studies of brain evolution should consider that changes in absolute and relative brain size might not translate directly into changes in brain processing capacity across different clades. A fruitful approach to the study of the evolution of cognition might be to

combine the data on brain size, which are available for a broad range of living and fossil taxa, with data on neuron numbers and scaling, which give a more accurate picture of brain computing power.

Methods

Animals. A total of 132 individuals of 107 species of reptiles and 91 individuals of 37 species of birds were used in this study. We aimed to cover the major lineages of squamate reptiles (Gekkota: 14 species, Scincoidea: 14 species, Lacertoidea: 15 species, Anguimorpha: 7 species, Iguania: 20 species, and Serpentes: 18 species) as well as the two major lineages of turtles (Pleurodira: 14 species, Cryptodira: 5 species) and include a wide range of body sizes. The bird species added in this study were from the following groups: Paleognathae (5 species) Galliformes (8 species), Anseriformes (7 species), Columbiformes (4 species), Accipitriformes (4 species), Strigiformes (6 species), and Falconiformes (3 species). Animals were preferentially wild caught, with those unavailable from the wild acquired from breeders and zoos. All animals were sexually mature or at least had adult-like size and coloration. The sex of all animals was determined upon dissection. Where possible, we preferentially collected animals of both sexes or males in the case of single individuals. However, based on previous findings, there are no significant sex differences in neuron numbers in either squamate reptiles (18) or birds (14), and intraspecific variation is negligible compared to the large scale of body and brain sizes in the sample.

Ethical Approvals. All procedures were approved by the Institutional Animal Care and Use Committee at Charles University (UKPRF/28830/2021), Ministry of the Environment of the Czech Republic (permission no. 53404/ENV/13-2299/630/13), Ministry of Culture of the Czech Republic (permission no. 47987/2013), and Ministry of the Environment of the Czech Republic (permission no. 53404/ENV/13-2299/630/13) and in compliance with the applicable legislation in the Czech Republic implementing the European guidelines (European Union directive no. 2010/63/EU) regarding the protection of animals used for scientific purposes.

Perfusions. The animals were killed by anesthetic overdose (intramuscular administration of ketamine and xylazine for reptiles; inhalation of halothane for birds except for ostrich, rhea, and emu, which were overdosed by intramuscular injection of anesthetics containing midazolam, detomidine, medetomidine, butorphanol, and ketamine). They were weighed and immediately perfused transcardially with warmed phosphate-buffered saline containing 0.1% heparin followed by cold phosphate-buffered 4% paraformaldehyde solution. Skulls were partially opened and postfixed for 30 to 60 min, after which brains were dissected and weighed. Brains were postfixed for additional 7 to 21 d and then dissected into parts and either processed immediately or transferred into antifreeze (30% glycerol, 30% ethylene glycol, 40% phosphate buffer) and kept frozen at $-20\,^{\circ}C$ until processing.

Brain Dissections. Brains were dissected into major parts using the Olympus SZX 16 stereomicroscope. The cerebral hemispheres were detached from the diencephalon by a straight cut separating the subpallium from the thalamus. The cerebellum was cut off at the surface of the brainstem. The rest of brain refers to the remainder after separating the telencephalon and cerebellum, i.e., the diencephalon, mesencephalon, and medulla oblongata. For most individuals, only one cerebral hemisphere was processed since in our previous studies we detected negligible differences between left and right hemisphere mass and cell numbers. In birds, for one individual per species, the second hemisphere was dissected into pallium and subpallium. The hemisphere was embedded in agarose and sectioned on a vibratome at 300 to 500 μm (depending on the size of the hemisphere) in the coronal plane. Under oblique transmitted light at the stereomicroscope and with the use of a microsurgical knife (Stab Knife Straight; 5.5 mm; REF

7516; Surgical Specialties Corporation), we manually dissected the pallium from the subpallium on each section by cutting along the pallial–subpallial lamina, as defined by Reiner et al. (50). All the dissected parts were weighted to the nearest 0.1 mg using a Kern ALJ 120-4 balance (Kern & Sohn GmbH).

Isotropic Fractionator. We estimated the total numbers of cells, neurons, and nonneuronal cells following the procedure of isotropic fractionator, as described earlier (10). Briefly, each dissected brain division was homogenized in 40 mmol sodium citrate with 1% Triton X-100 using Tenbroeck tissue grinders (Wheaton). When turned into an isotropic suspension of isolated cell nuclei, homogenates were stained with the fluorescent DNA marker 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich), adjusted to a defined volume, and kept homogenous by agitation. The total number of nuclei in suspension, and therefore the total number of cells in original tissue, was estimated by determining the number of nuclei in 10-μL samples drawn from the homogenate. At least four aliquots were sampled and counted using a Neubauer improved counting chamber (BDH) at the Olympus BX51 equipped with epifluorescence and appropriate filter settings; additional aliquots were assessed when needed to reach the coefficient of variation among counts of ≤ 0.1. Once the total cell number was known, the proportion of neurons was determined by immunocytochemical detection of the neuronal nuclear marker NeuN (51). This neuron-specific protein was detected by a mouse monoclonal anti-NeuN antibody (clone A60, Chemicon, Temecula; dilution, 1:800) in birds and by a rabbit polyclonal anti-NeuN antibody (Merck Millipore; dilution, 1:800) in nonavian reptiles; the binding sites of the primary antibody were revealed by Alexa Fluor 546-conjugated goat anti-mouse immunoglobulin G (IgG) (Life Technologies, Carlsbad, CA; dilution 1:500) or Alexa Fluor 594-conjugated goat anti-rabbit IgG (Life Technologies, Carlsbad, CA; dilution 1:400), as appropriate. An electronic hematologic counter (Alchem Grupa) was used to count simultaneously DAPI-labeled and NeuN-immunopositive nuclei in the Neubauer chamber. A minimum of 500 nuclei was counted to estimate the percentage of double-labeled neuronal nuclei. Numbers of nonneuronal cells were derived by subtraction. Neuron density was calculated as the number of neurons in a given brain part divided by the brain part mass.

Compilation of Data on Neuron Numbers. In addition to data obtained in this study (Dataset S1), we included additional published data on neuron numbers in the Nile crocodile (17) and 2 species of anoles (19), 28 species of birds (14, 15), and 76 species of mammals (11–13, 52–60). The number of brain neurons and telencephalic neurons includes the OBs, except in 26 species of mammals, where they were not available (*Ursus arctos*, *Canis lupus familiaris*, *Mungos mungo*, *Hyaena hyaena*, *Felis cattus*, *Panthera leo*, *Cynomys* sp., *Macaca fascicularis*, *Macaca radiata*, *Papio cynocephalus*, *Homo sapiens*, *Sapajus apella*, *Saimiri sciureus*, *Amblysomus hottentotus*, *Dendrohyrax dorsalis*, *Dendrolagus goodfellowi*, *Macropus rufogriseus*, *Macropus parma*, *Macropus fuliginosus*, *Wallabia bicolor*, *Chaerephon pumilus*, *Coelura afra*, *Cardioderma cor*, *Hipposideros commersoni*, *Triaenops persicus*, *Miniopterus schreibersii*).

Imputing OB and Striatum Values for Mammals. For the analysis with corrections for missing OB and striatum included in the telencephalon, data were imputed in the following way: data on OB volumes and neurons were estimated using the appropriate scaling rules for the given clade (using data from 61 for volumes in carnivores, where OB were missing in most species). Data on striatum volume were available for 41 species in the dataset (12, 62–64), and for the remaining species, they were estimated from brain volume based on the average proportion in the respective group. Species-specific neuron densities were used to derive the number of striatal neurons, based on the fact that at least in mice (65) average cortical and striatal neuron densities are similar.

Data on Brain and Body Mass. We collected data on brain and body mass for 149 species of reptiles and supplied data on 3 additional species from the literature (66–68) (Dataset S2). We combined these with previously published datasets including 183 species of reptiles (5, 16), 1,989 species of birds (2), and 1,534 species of mammals (69). Endocranial volume was converted to brain mass by multiplying by the density of brain tissue ($1.036\ g/cm^3$) (70).

Phylogeny. For phylogenetic analyses, we adopted a phylogeny constructed from previously published species-level trees. We used recent published species-level time-calibrated phylogenies for squamates (71), birds (2), and mammals (72). For turtles and crocodiles, we used the Timetree of Life (73). We then stitched the trees together manually, using the divergence times from the Timetree of Life, and pruned them to match the brain size and neuron numbers

datasets, substituting closely related species in a few cases that were not present in the published phylogenies.

Data Analysis. Analyses were performed in R version 4.0.3 (74) using average values for each species, and the variables were log10 transformed. Where appropriate, statistical significance was evaluated at an α level of 0.05.

Absolute and relative measures. Absolute measures represent the species average value of the trait, while relative measures represent the residuals from PGLS regression across the group of interest (e.g., relative brain size in amniotes refers to the residuals from the PGLS regression of brain mass on body mass across amniotes with one slope and one intercept; relative neuron density in primates refers to the residuals from the regression of neuron number on brain mass across primates).

Ancestral reconstruction of brain and body sizes. Ancestral reconstructions of continuous traits were performed using the function fastAnc in the package phytools v0.7 (75) and the function mvBM in the package evomap (76). Both methods gave very similar results, and only the values from fastAnc are used in the paper. The values were mapped onto phylogenetic trees using the R packages ggtree 2.4.0 (77) and phytools 0.7 (75).

Detection of significant shifts in allometric scaling. We used the Bayesian reversible-jump bivariate multiregime Ornstein–Uhlenbeck modeling approach as implemented in the R package bayou 2.2.0 (21) to detect changes in the slope and intercept in neuron scaling in the whole brain and the three brain compartments with brain structure mass (effectively changes in relative neuron density) and body mass (reflecting changes in both neuron density and brain/structure size). This approach enables the identification of shifts in intercept and slope without specifying their location a priori.

We ran at least four chains with different random starting points for 10 million iterations, sampling every 100th iteration, and discarded the first 0.2 samples as burn-in. We used the following priors: half-Cauchy distribution with scale factor 0.1 for α (the strength of attraction toward an adaptive optimum) and σ^2 (change of the trait per unit time), Poisson distribution with a mean equal to 2% of the total number of branches in the tree and a maximum number of shifts equal to 20% for the number of shifts, normal distribution $\theta \sim N(\mu = \text{mean (trait)}, \sigma = 1.5 \times \text{SD(trait)})$ for the intercept, and normal distribution $\beta \sim N(\mu = \text{PGLS } \beta, \sigma = 0.3)$ for the slope. We assessed the convergence of the run by inspecting the diagnostic plots and convergence of the chains using Gelman's R-statistic (78) and by comparing the uncovered shift locations. We then combined the chains to summarize parameter estimates. All parameters had effective sample sizes greater than 150 (typically several thousand). Only shifts in clades containing more than three species were reported and included in further analysis.

PGLS analysis of allometric scaling. We tested the models including the shifts identified in bayou in a PGLS framework, using the gls function in the R package nlme 3.1 (79), with Pagel's lambda estimated using restricted maximum-likelihood. Separate slopes and intercepts were considered for the putative grades, i.e., the model was in the form Dependent~Independent*Group. Model selection was carried out in a top-down fashion. Starting with the full model, the putative grades were consecutively merged with the ancestral grade to confirm they are significantly different, and the identified unrelated grades were then merged together to identify convergence. The models were compared using the core R function ANOVA, and the simplified model (with fewer factor levels) was adopted if the *P* value for the full model was >0.05. Shifts identified by bayou with a posterior probability of >0.7 were supported in all cases, except for the change in neuron density in the telencephalon of Accipitriformes.

Fold change estimates. We used the following approach to quantify the fold change in the number of neurons for body size between reptiles and birds and mammals: as the regression lines are not parallel, differences in intercepts do not accurately capture the distance between the lines. We calculated the difference between the PGLS regression lines at every data point (body size of all included species) and then took the average. This corresponds to the average distance between the lines, i.e., the average fold change. See also *SI Appendix*, Fig. S10 for a visual representation.

Evolution rates. To compare the evolutionary rates among the different brain parts (*SI Appendix*, Table S5), we used an approach devised specifically for comparing rates of evolution for multiple phenotypic traits on a phylogeny (80). The likelihood of a model with distinct evolutionary rates for each trait is compared to the likelihood of a model where all traits evolve at a common rate. We used the implementation in the function compare.multi.evol.rates in the R package geomorph 4.0.0 (81). The evolutionary rates of the groups with identified allometric shifts were compared using the function compare.evol.rates in geomorph 4.0.0. Here, we only tested primates as a whole, as they include anthropoid primates and the sample size is larger. The *P* values were calculated by bootstrap simulation with 10,000 iterations. The rates of evolution plotted in Fig. 3 are estimated using the multiple-variance Brownian motion framework (82) implemented in the mvBM function in the R package evomap (76) with Markov chain Monte Carlo sampling. Both methods agree in identifying primates as having significantly higher rates (weaker allometric integration) than the other groups.

Data Availability. All study data are included in the supporting information and have also been deposited in Figshare, https://doi.org/10.6084/m9.figshare.

ACKNOWLEDGMENTS. We thank J. J. M. Massen, T. Fitch, L. Lefebvre, S. Brusatte, and M. Osvath for reading of the manuscript and discussions; S. Herculano-Houzel and A. Ngwenya for providing neuron counts for individuals of the Nile Crocodile; T. Hajek for logistic support; R. Lučan, P. Frýdlová, L. Kratochvíl, M. Rovatsos, and J. Pluháček for providing or help with collecting the experimental animals; and P. Benda and J. Mlíkovský for providing access to dissection facilities of the National Museum of the Czech Republic. This research was funded by the Czech Science Foundation (Grant 18-15020S to P.N.) and the Grant Agency of Charles University (Grants 810216 to M.K. and 1438217 to B.S.).

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SUMMARY OF THE INCLUDED PUBLICATIONS

Chapter 1

To establish the extent of individual variation in neuron numbers in a species with extensive adult neurogenesis and assess the effect of sex and age, we used the Madagascar ground gecko (*Paroedura picta*). We examined brain size, neuron and glial cell numbers and densities in the telencephalon and the rest of brain in 14 hatchlings, 10 young adults and 10 fully grown adults from one population and 10 fully grown adults from another population to assess interpopulation differences. Surprisingly, the variation in brain size and neuron numbers across both populations was similar to that of laboratory mice of the same age and sex from one population (Herculano-Houzel et al., 2015b). There were no differences between the sexes in any of the parameters, but the two populations differed significantly in everything but neuron density, most notably in the number of non-neuronal cells. The hatchlings had significantly smaller brains and fewer neurons than either of the adult groups and the fully-grown adults had larger brains but not more neurons overall than the young adults. In the telencephalon, however, fully-grown adults housed significantly more neurons, suggesting that substantial neurogenesis leads to the addition of new telencephalic neurons throughout life. Including adult, but not fully grown animals in comparative studies thus may not significantly affect the results in terms of absolute numbers of brain cells, but might slightly skew the results in terms of neuron densities. Neuronal density seems to be the most conserved feature in this species, in contrast to rodents (Herculano-Houzel et al., 2015b; Kverková et al., 2018).

Chapter 2

It has previously been shown that guppies *Poecilia reticulata* selected for large or small relative brain size differ in performance in a number of cognitive tasks (Buechel et al., 2018; Kotrschal et al., 2015, 2013), with the larger-brained group generally outperforming the smaller-brained one. To get to the bottom of this effect, we looked at the cellular composition of brains of 53 adult female guppies that had

been selected for either small or large relative brain size over 5 generations. The large-brained guppies had substantially more neurons in both the whole brain and the telencephalon, but they did not differ in neuron densities (i.e. followed the same neuron-brain scaling). At the same time, there were some pronounced individual differences in neuronal density within both groups. Unlike mice (Herculano-Houzel et al., 2015b), larger-brained guppies thus exhibit a matching increase in the number of neurons, potentially explaining their enhanced cognitive abilities. In terms of neuron number variation, guppies are similar to geckos and mice. Interestingly, absolute neuron densities in the guppy brain are the highest reported to date in any vertebrate, suggesting that the previously established pattern of decreasing neuron densities with increasing brain size (Herculano-Houzel et al., 2015a) holds for all vertebrates. This high neuron-packing density might be part of the reason why small-bodied animals with tiny brains are able to solve surprisingly complex problems.

Chapter 3

The social brain hypothesis (SBH) posits that animals with complex social lives require complex brains to deal with the associated cognitive demands. It has been very influential, especially when it comes to theories about primate, and specifically human, brain evolution. However, the empirical evidence for this hypothesis is equivocal, with numerous studies finding support and more than a fair share finding none (e.g. DeCasien et al., 2017; Dunbar, 1992; Powell et al., 2017; Shultz and Dunbar, 2007; Vidal-Cordasco et al., 2020). These studies are usually heavily confounded by differences in ecology but also by differences in the type of social complexity. To avoid these pitfalls, we tested the hypothesis in an ecologically uniform clade – the African mole-rats (Bathyergidae). These subterranean rodents are all well adapted to underground life, with reduced eyes, powerful incisors for digging their tunnels, and multiple physiological adaptations, including lowered metabolic rates and high hypoxia tolerance (Begall et al., 2007; Ivy et al., 2020; Yap et al., 2022). They feed mostly on underground plant parts. Despite being very similar in all other aspects, they exhibit a wide array of social systems. Some

are strictly solitary and territorial, the male and female meeting only for a short time to mate and the young leaving upon weaning. Others are cooperative breeders with colonies consisting of the breeding pair and their offspring who act as helpers. Some have been classified as eusocial with life-long philopatry, even though these definitions are somewhat blurry. The social species show complex vocalizations, division of labour and dominance hierarchies (Begall et al., 2007).

African mole-rats thus represent a good model group to test the effect of sociality on brain evolution without the confounding effects of differences in a host of other factors. We measured absolute and relative brain size, neocortex ratio (the ratio of neocortical volume to the rest of brain volume, a proxy for "intelligence" previously used in testing the social brain hypothesis), numbers of neurons in 5 brain parts, and volumes of 8 brain parts in 11 species of African mole-rats. We then used phylogenetic Bayesian generalized linear mixed models to look for associations between these brain measures and sociality, either as a categorical variable or as mean and maximum group size. We found no support for the social brain hypothesis; there was either no effect of sociality or the effect found was in the opposite direction, i.e. solitary species having more forebrain neurons, and the neocortex ratio and neuron numbers going down with increasing group size. The demands of social interactions clearly do not promote the evolution of larger brains in these rodents, weakening the general support for the SBH. There might be two reasons behind this, even if the SBH generally holds. First, competition rather than cooperation might be the driving factor behind the previously reported increased brain size in social species. Competitive "Machiavellian" interactions are not likely to be selected for in species with extreme reproductive skew and cooperative breeding, because they would decrease inclusive fitness. Second, metabolic constraints might be too strong for any potential benefits of increased cognitive capacity to outweigh the considerable cost of neural tissue. Sociality in mole-rats might have evolved to deal with unfavorable environmental conditions and patchily distributed food resources (Faulkes and Bennett, 2013), and, whether or not that is the case, social species tend to occupy harsher environments (Burda et al., 2000), which would exert more pressure to conserve energy at the expense of costly brains. In

any case, this highlights the point that energetic constraints play a crucial role in brain evolution and should always be taken into account.

Chapter 4

To uncover macroevolutionary patterns in brain cellular composition across amniotes, we compiled a large dataset of brain sizes for almost 4000 amniote species and estimated numbers of neurons in three main brain parts in 144 species of birds and non-avian reptiles, more than doubling the number of vertebrate species with known neuron numbers. We then combined these with previously published data and analysed the resulting dataset of 251 species. Using reversible-jump Markov chain Monte Carlo analysis, we were able to detect significant shifts in neuron-brain and neuron-body scaling along the amniote phylogeny with no prior assumptions about the location of these shifts. It turns out that birds and mammals independently increased the number of neurons for brain mass, arriving at similar levels, with two other subsequent increases in core landbirds and anthropoid primates. We suggest these convergent increases in neuron numbers coincide with the advent of endothermy, an energetically expensive mode of life. Neurons are metabolically costly and require a steady supply of energy; having large numbers of neurons thus might not be advantageous for animals with low energy intake and expenditure, such as non-avian reptiles. Accordingly, we observed no major increase in neuron-brain scaling within squamate reptiles and turtles in over 300 million years of evolution. The scaling of neurons with brain and body mass is rather conserved, with a handful of dramatic shifts, whereas mosaic changes in specific brain regions are more frequent. There was an additional decrease in cerebellar neurons in snakes, likely connected to the loss of limbs, as a similar pattern is evident in all legless lizards. We also detected a secondary decrease in telencephalic neurons in Accipitriformes, a clade within the core landbirds. However, our sample included only four species, so this finding still needs to be confirmed.

The distribution of neurons to the three major brain parts, telencephalon, cerebellum, and "rest of brain", shows distinct patterns in different amniote groups. Mammals and birds outside of Telluraves are characterized by the preponderance of

cerebellar neurons that make up ∼60 to over 90% of all brain neurons. In Telluraves, the telencephalon is the dominant fraction, containing ∼40 to 80% of brain neurons. In non-avian reptiles, the cerebellum is much less developed, and holds typically only about 20-30% of all brain neurons in squamates, with some turtles having a larger cerebellar fraction of about 40%, and the cerebellum becomes dominant in the Nile crocodile (and, presumably, all crocodilians), where it houses 49% of brain neurons. The rest of brain (comprising the brain stem, mesencephalon, and diencephalon) contains only a minor proportion of brain neurons in mammals and birds (often less than 10%) but is more important in non-avian reptiles, where it can be the dominant fraction, with over 50% of brain neurons. The distributions of brain neurons are much more variable than the neuron-brain or neuron-body scaling, especially in non-avian reptiles. It seems the overall "neuronal energy budget" can be flexibly allocated to brain structures depending on species-specific needs, with different regions or circuits having different per-neuron utility. A modest volumetric increase in a neuron-dense structure such as the cerebellum translates into a substantial number of added neurons. In fact, the cerebellum is the region that truly sets apart ectothermic and endothermic amniotes, with the latter having on average almost 60-fold more cerebellar neurons for equivalent body mass but only about 7-fold larger brains.

We additionally calculated the strength of allometric integration between the number of neurons in different brain parts and body size. It was similar in all the identified grades except for primates, who exhibit a weaker allometric integration, that is, they have much higher rates of evolution, and changes in neuron-body scaling happen quickly. Primates also stand out in another aspect. Across amniotes, there is a clear positive relationship between relative brain size (brain mass for a given body mass) and relative neuron number (number of neurons for a given brain mass). This is despite the fact that absolute neuron density goes down with absolute brain mass. When we break the relationship down to look at reptiles, birds, and mammals separately, it turns out that the trend is very strong in birds, somewhat less pronounced in non-avian reptiles and non-existent in mammals. However, primates are an exception among mammals and

they show a clear significant relationship. Cases where increases in relative brain size are coupled with increases in relative brain neuron numbers might represent evolutionary "signatures" of selection for higher brain processing capacity. When the brain is larger than expected for a given body size while neuron density is simultaneously higher than expected for a given brain size, it seems likely that selection favoured increased neuron numbers and therefore information processing capacity.

DISCUSSION AND PERSPECTIVES

In this thesis, I aimed to bridge the gap between the traditional study of brain size evolution, which spans large phylogenetic scales but works with rather crude data, and modern comparative neuroscience, which generates detailed data about specific brain structures or circuits but only in a handful of model species. Using the isotropic fractionator, I obtained novel data that go beyond brain size and bring important insights to the study of vertebrate brain evolution. However, with every progress made, new unforeseen issues emerge and often we are left with more questions than answers. Clearly, there is still much that we do not understand about the neural substrate of cognition and its evolution.

First of all, while we now have a better idea of how brain neuron numbers and densities evolved in amniotes, we know very little about how this (or any) measure of processing capacity even translates into cognitive capacity. There is an assumption that any "spare" capacity can be used for what we call higher cognition. The very concept of higher cognition is inherently anthropocentric in that we consider human-like behaviour to be intelligent. And, more importantly, we do not have a good idea of how costly it is in terms of neural tissue. As an example, vision is extremely computationally demanding (up to 50% of primate cerebral cortex is involved with image processing (Essen, 2004)), but somehow we do not consider it to be the main driver of brain processing power (even though, coincidentally, most species we consider intelligent are visually oriented). Associative areas occupy a comparatively small percentage of the brain, whether in terms of volume or neurons, suggesting animals do not need particularly large brains to be capable of complex cognition. Given that even insects with miniature brains and modest neuron counts are capable of impressive cognitive feats (Chittka and Niven, 2009), or, if we want to stay in the realm of vertebrates, tiny-brained guppies perform surprisingly well in a supposedly complex detour task (Lucon-Xiccato et al., 2017), it begs the question of why should animals have big brains at all.
Yet, the association between more neurons and perceived "intelligence" seems pretty

robust across species. And larger brains clearly do confer some benefits, otherwise they would not have evolved repeatedly in many lineages. Perhaps, we are neglecting the fact that sensory inputs are necessary for any complex behaviour. While the neural circuitry underpinning the cognitive processing itself might not need to be extensive, dealing with the streams of sensory information can be a limiting factor. After all, a blindfolded person would not fare very well on visual tasks. And assembling puzzle pieces with tied hands would not go well either; motor control is equally important (as proposed by the concept of embodied cognition (Foglia and Wilson, 2013)). Another possibility is that more neurons afford better parallel processing, larger capacity for memory and also redundancy. The longer you live, the more you potentially have to remember and the higher the likelihood that some neurons will be lost to damage during your lifespan. This might be partially behind the association between longevity and brain size reported in mammals and birds (González-Lagos et al., 2010; Jiménez-Ortega et al., 2020; Minias and Podlaszczuk, 2017; Smeele et al., 2022), even though learning speed (another potential driver of brain size) is theoretically predicted to be more important in short-lived organisms (Liedtke and Fromhage, 2019). At the same time, some reptiles are particularly long-lived and they seem to do just fine with limited neuron numbers.

Disturbingly, we do not have a good grasp of "general intelligence" or know if such a thing even exists in non-human animals. The evidence is mixed and taxon-specific; mammals and birds seem to have it (Bastos and Taylor, 2020; Burkart et al., 2017), while it is not present in a fish species capable of solving rather complex tasks (Aellen et al., 2022). The tangled net of different facets of cognition that are being used as proxies for intelligence in comparative studies complicates things further. It would help to have at least a composite score of some kind or maybe stop trying to treat cognition as one-dimensional and incorporate multiple proxies at once. Another issue is the discordance between the obvious and robust correlation of the number of telencephalic neurons with cognitive abilities at the interspecific level (Herculano-Houzel, 2017; Sol et al., 2022) and the apparent lack of one at the intraspecific level. This is still very much an uncharted territory, but one study

examined the association between performance in a battery of tests and neuron numbers in several brain parts in mice and found no links (Neves et al., 2020) and neither did we between learning in spatial tasks and brain cellular composition in geckos (Neves et al., 2020, Polonyiová et al., in prep). It is possible that the tasks were not designed well to actually reflect what they sought to measure or that the individual variation in the lab populations is not enough to translate into measurable cognitive differences. In contrast to the above-mentioned studies, in Chapter 2 we found that the guppies selected for larger brains and showing enhanced cognitive performance did have more neurons (although the differences in cognitive tasks were assessed at group level, not at individual level), suggesting a within-species effect. This clearly deserves further study. It would be particularly interesting to turn the experiment around and select for some sort of "intelligent" behavior, to see whether there are any corresponding changes in the brain.

It is possible that we focus too much on brain parts associated with "higher cognition" (although as mentioned before, the brain ultimately functions as a unit and even the "non-thinking" parts provide some input for decision-making). The cerebellum has been traditionally considered of low importance for cognition, labeled instead a "center of coordination and balance" (although for arboreal creatures such as our primate ancestors that is rather crucial for survival). Lately, it is becoming clear that, at least in mammals, the cerebellum is directly involved in cognitive functions (Beaton and Mariën, 2010; Barton, 2012; Smaers et al., 2018). There is less evidence in birds, probably because it has not been studied as much, but it seems likely that the avian cerebellum handles some cognitive processes as well (Day et al., 2005; Spence et al., 2009). The cerebellum is capable of high-throughput processing of structured information, especially useful in detecting sequences of any kind (Molinari et al., 2008), and can in principle be used to off-load some expensive computations. This is analogous to modern computing GPUs. Although originally designed with a very specific purpose of processing graphic information, they have found use in computationally intensive tasks such as machine learning. As a case in point, mormyrid fishes that rely on

active electroreception for orientation and communication possess an extremely enlarged cerebellar valvula that has been co-opted to process electrosensory stimuli (Finger et al., 1981). New clues from comparative studies keep coming: the most dramatic changes in neuron numbers throughout amniote evolution happened in the cerebellum, the number of cerebellar neurons relative to body size is the best predictor of innovativeness in birds (Sol et al., 2022), and apes are characterized by rapid cerebellar expansion relative to the cortex (Barton and Venditti, 2014). All of this suggests the "little brain" might play a bigger role than we currently recognize. Another example of distributed processing is the modification of brain stem structures in many fish species. Mammal-centric textbooks profess that the brain stem is highly conserved and go as far as claiming it is rarely good for anything but keeping you alive. It also generally contains very low neuron densities. Yet various fishes have evolved elaborate modifications of the brain stem, most strikingly the vagal lobes of cypriniform fishes, associated with their highly sensitive sense of taste, but also the electric lateral line lobe in active electric fishes, or the bizarre spiral vagal lobe of *Heterotis niloticus* receiving input from its special epibranchial organ (Meek and Nieuwenhuys, 1998). Clearly, the telencephalon is the structure most involved in what we classify as "thinking", but maybe we should consider that specific brain parts reflect adaptations to species-specific needs and are needed for sensorimotor processing that cannot really be separated from cognition. Going further, we would benefit from a more balanced approach with less focus on the forebrain and more recognition of the mosaic-like evolvability of different brain parts.

Regardless of what the relationship between neural substrate and cognition turns out to be, some argue that neuron numbers are not the best proxies for brain processing capacity and that numbers of synapses should be used instead. Of course, no single number is likely to adequately capture the complexity of the brain, but we can try to come up with the best approximation. More neurons do not necessarily mean more synapses, although it is reasonable to assume these two numbers might be related. The number of connections probably scales with the number of neurons, but this can substantially differ across brain structures or species and also in the

degree of redundancy. So should we aim to count synapses instead?

At face value, this does seem reasonable. Individual neurons only work as a part of a neural network and connections are of the utmost importance. But again, the paucity of empirical data prevents any concrete claims. There is a theoretical framework, however, that shows redundant synaptic connections enhance learning (Hiratani and Fukai, 2018). Nevertheless, the sheer number of synapses might not be a better proxy of processing capacity for the purposes of evolutionary analyses. First of all, it might be difficult to get a representative figure for the species, as individual differences due to plasticity can be expected to be substantially higher than in neuron numbers. Larger sample sizes would therefore be required, which is already the limiting factor for broad comparative studies.

More importantly, not all connections are beneficial. In fact, a recent study comparing connections in the visual cortex of mice and macaques found that the monkeys have two to five times fewer synapses per neuron (Wildenberg et al., 2021). This should not be surprising and has been shown before in a larger sample of species (Colonnier and O'Kusky, 1981), since the dendrites in larger brains have to be thicker just to maintain passive cable properties (Bekkers and Stevens, 1990). As a result, there might simply not be enough space to fit in more connections, especially in an already densely packed primate brain. Given that macaques clearly outperform mice when it comes to vision, this goes to show that just having more synapses might not be particularly advantageous. Indeed, one of the crucial processes in postnatal brain maturation is synaptic pruning, where excess interneural connections are being removed (Chechik et al., 1998). While expanding neural circuits up to a point can result in faster and more precise learning, hyperconnectivity might actually impair performance due to inherent synaptic noise (Raman et al., 2019). Moreover, the sheer number of connections in the brain is not very informative without accounting for where these connections are, which is a whole new challenge altogether. Even in the nematode *Caenorhabditis elegans* with its simple nervous system, over 40% of all cell-cell connections are not conserved between adult individuals with the same

genetic makeup (Witvliet et al., 2021). Neuronal circuitry can also be altered with profound functional implications without necessarily manifesting as a change in the quantity of connections; for example, autistic individuals exhibit a pattern of both increased and decreased connectivity in certain brain areas (Hahamy et al., 2015). In any case, there is simply no easy way to quantify synapses in whole brains in a meaningful way and it seems out of reach in the foreseeable future. Moreover, synapse complexity might add another dimension when comparing across large phylogenetic scales (Emes et al., 2008; Grant, 2016).

A similar argument can be extended to neurons. They are by no means all the same. Depending on whom you ask, hundreds to over a thousand of neuron types exist; more than a hundred have been described in the rodent hippocampus alone (Wheeler et al., 2015). One can conceive how certain types of neurons might have different impacts and also put different constraints on other neuron types, depending on their connectivity. For example, the number of granule cells in the cerebellum depends on the number of Purkinje cells but not vice versa and their ratio is species-specific (Lange, 1975; Wetts and Herrup, 1983). We can imagine that increasing the number of one or the other would not have the same effect. But again, at the moment it would not be technically achievable to quantify individual neuron types at scale. Although new studies using single-cell transcriptomics provide insightful data to infer the evolutionary origins of certain brain parts and circuits (Colquitt et al., 2021; Woych et al., 2022) and also about intraspecies variation in neuron type distribution (Li et al., 2022), these methods are not suitable for quantifying the numbers of neuronal populations across more than a few species. One way to proceed could be to look at more specific brain areas and start with broader categories based on neurotransmitters to see if any patterns emerge. More generally, it makes sense to venture into exploring connectomes if we are after functional implications. This is becoming increasingly feasible, with a recent pioneering study analysing connectomes of over 100 mammalian species derived from diffusion MRI data. Not surprisingly, it showed that species differ mainly in local network topology while the global architecture is much more conserved, and that connectome similarity

corresponds to phylogenetic distance (Suarez et al., 2022).

Neuron density itself might have some interesting implications. While, as we show, it predictably scales with brain size across vertebrates, it is not without further consequences. However, these are not straightforward and deserve more study. Lower neuron density implies larger somata and more elaborate dendritic trees (Beul and Hilgetag, 2019), with potentially finer modulation, whereas higher neuron density implies smaller neurons with potentially shorter local connections and better efficiency in terms of cost per bit (Niven et al., 2007). Intraspecifically, neuron density is the best morphological predictor of several aspects of cortical connectivity (Beul and Hilgetag, 2019). Interspecifically, the ability to discriminate quantities was found to be associated with higher cortical and cerebellar neuron density (rather than brain size or neuron numbers), but this is based on a very small number of species – only nine mammals and three birds were included in the analysis (Bryer et al., 2022).

We also need to consider that brains are not composed solely of neurons. Glial cells have been neglected for a long time, relegated to simple "glue" that holds the brain together. Now, the focus is shifting and we are becoming increasingly aware that glial cells play more important roles than previously thought (Chung et al., 2015; He and Sun, 2007; Khakh and Sofroniew, 2015). In an experimental study, mice with grafted human astrocytes experienced enhanced long-term potentiation and consequently better learning, while presumably having the same neuron numbers (Han et al., 2013). This also highlights that functional properties of brain cells might be as much as or more important than raw numbers. The diversity of glial cell types and the glia-to-neuron ratio seem to increase predictably in the *scala naturae* sense, from invertebrates to "higher vertebrates" and humans (Herculano-Houzel, 2014; Verkhratsky et al., 2019). However, we showed that densities of non-neuronal cells are much more conserved than neuron densities across amniotes. This means that non-avian reptiles actually have higher glia-to-neuron ratios than endotherms, disrupting this apparent pattern (although it has to be noted that we did not

distinguish non-neuronal cell types and they include non-glial endothelial cells). The evolution of glial cells and the implications of altering the glia-to-neuron ratio are certainly worth exploring in more detail.

Another crucial point is that metabolic constraints play an important role in brain evolution. Balancing the energetic budget is the reason for many evolutionary trade-offs and the brain is no exception; a number of such trade-offs have been proposed under the auspices of the expensive tissue hypothesis (Aiello and Wheeler, 1995; Barrickman and Lin, 2010; Kotrschal et al., 2016; Mai and Liao, 2019; Tsuboi et al., 2016). However, there does not have to be an obvious trade-off for this principle to apply. There is no point in having stronger muscles, a more spacious digestive tract or a larger brain, if the benefits do not outweigh the associated cost and this internal "checkbook balancing" can be very complex and opaque. Incidentally, uniquely among mammals, some mole-rats and bats show reptile-like neuronal densities in the telencephalon, strengthening the proposition that energy conservation is a crucial factor in brain evolution.

It is also possible that in some instances, the cost of growing neural tissue might be more restricting than the upkeep cost. The need to grow a fully functional brain within some timeframe available for development might also be a limiting factor. There are correlations between brain size and incubation length and fledging age in birds (Isler and Schaik, 2006; Iwaniuk and Nelson, 2003) and gestation length and weaning age in mammals (Barton and Capellini, 2011; Isler and Schaik, 2009; Weisbecker and Goswami, 2010). The reptile groups in which we observed the highest relative numbers of neurons (Varanidae and Teiidae) also have the longest incubation times among squamates (Birchard and Marcellini, 1996). Incubation temperature itself might have some implications for brain development and function (Amiel et al., 2017; Amiel and Shine, 2012; Clark et al., 2014; Coomber et al., 1997; Siviter et al., 2017), although we did not find any differences in brain size or composition in Madagascar ground geckos incubated at different temperatures (Polonyiová et al. in prep). It might be an interesting line of study in viviparous

reptiles, where the mother can behaviourally manipulate the temperature that the embryos are exposed to. As an aside, avian and mammalian embryos develop at much higher temperatures than any non-avian reptiles (While et al., 2018) and body temperature might directly affect a range of physiological processes, including energy consumption in neurons, which might be reduced in birds compared with mammals (Eugen et al., 2022).

The high early-life cost in fast-maturing species might preclude generating too many neurons; similarly, yolk size imposes growth limits on the embryo. All the animals with exceptionally high neuron numbers that we have identified so far are either mammals (circumventing the yolk issue altogether) with long gestation and extended period of parental provisioning, or altricial birds with telencephalic neurogenesis delayed well into the post-hatching period (Charvet and Striedter, 2011). This does not mean that altricial species necessarily invest more in the brain, just that it is a handy preadaptation. As a case in point, pigeons and doves are highly altricial, yet they do not exhibit the high neuron density observed in Telluraves, and altricial rodents do not seem to have systematically more neurons than precocial ones (Herculano-Houzel et al., 2011).

As discussed earlier, there is substantial mosaic evolution in the brain, especially in non-avian reptiles and fish. The overall energy budget of the brain can thus be flexibly allocated so that there are trade-offs *within* the brain, not observable if we treat it as one unit. In consideration of this, it would be helpful to know more about the relative costs of neurons in different brain parts and how variable it is across species. While the energy budget per neuron is assumed to be conserved (Herculano-Houzel, 2011), this has been studied only in a handful of mammals and there can be substantial between-study variation (Karbowski, 2007). The recent suggestion that birds have a more efficient neuron metabolism (Eugen et al., 2022) should spark further investigation. It might once again turn out that conclusions based on mammalian data do not generalize that well across vertebrates.

This brings us to the obvious but nonetheless pertinent point that more data is needed. Good phylogenetic coverage is integral for understanding the evolution of any system, because to gain a deeper insight we need to observe both the regularities and the exceptions. If we focus on a few "model" species, we cannot even tell which is which. While there has been substantial progress on this front, we can still do much better. The choice of species is understandably limited by practical considerations, but we can try to be smart by focusing on species with the highest potential to yield new valuable information, by considering their phylogenetic position, size, and other relevant characteristics. At this point, we need data on amphibians, as outgroups to amniotes, and the realm of fishes is particularly enticing. Actinopterygians represent an enormous radiation of ectotherms (with very rare exceptions) in the aquatic environment. This somewhat reduces the effect of widely different modes of locomotion and limb use observed in terrestrial vertebrates (although there is still obvious variation), making it potentially easier to identify other factors that lead to significant changes in neuron numbers and distributions.

In this thesis, I presented new findings on several aspects of vertebrate brain evolution that lay the groundwork for future research. While they considerably expanded our knowledge and brought some novel insights, we have barely begun to scratch the surface. To conclude, here are some takeaways for the study of evolution of brain processing capacity and its implications for cognition that I have gleaned from our own and others' studies: we should expand phylogenetic coverage and not assume general validity across clades, strive for a more fine-grained level of analysis, try to integrate data on behaviour with data on the neural substrate, and incorporate a multidimensional view instead of focusing on a single brain part or taking the whole brain as a homogeneous unit. New technologies and capabilities are rapidly emerging that should enable us to put some of these into practice. Luckily for my future career, it amounts to enough work to last a lifetime.

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