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19 January 2023

To Whom It May Concern:

The thesis submitted by Mgr. Kristina Kverková is an impressive scholarly work. I commend the candidate on an excellent thesis and fully support moving ahead with an oral defense as scheduled on 1 February 2023. Below, I include my formal thesis report.

Summary

The thesis submitted by Kverková addresses some key knowledge gaps in evolutionary neurobiology concerning variability in neuron numbers within and across species. In recent years, there has been an increased emphasis on quantifying neuron numbers as a measure of "cognition" across species, largely due to the development of the isotropic fractionator technique. However, until the publication of the papers within this thesis, data on neuron numbers and densities across vertebrates was limited to relatively small number of species and very few clades. The thesis as a whole therefore represents a significant increase in our understanding of how neuron numbers evolve within and across species and provides much needed data to analyse behavioural and other traits across vertebrates. Based on the submitted thesis, the candidate has clearly demonstrated a deep knowledge of the issues in the field and the publications included in this thesis are both extremely important to the study of brain evolution and foundational for future research. Although all of the papers included are significant contributions to our understanding of how the brain evolves from both micro- and macroevolutionary perspectives, the latest paper on neuron numbers across amniotes is particularly important. In fact, I fully expect it to become both well cited in the primary literature as well as in textbooks on anatomy, evolution, neurobiology, and behaviour.

Weaknesses

I will begin this section by stating that I do not see any major weaknesses in the thesis whatsoever. The comments within this section refer entirely to some minor comments regarding interpretation and future directions for the candidate to consider.

More space could have been devoted to discussing the potential contributions of neuron size and shape to intraspecific and interspecific variation in brain size, brain region size, and cognition. The issue of neuron size/shape is discussed briefly in several of the publications included in the thesis. Often it is only mentioned in the context of explaining residual variation in mass. Neuron size and morphology do, however, play significant roles, albeit not well understood, on computational capacity and speed. The candidate does discuss synapses, but the papers or General Discussion would have benefitted from some discussion of the potential roles of

variation in neuron size and shape. For example, birds lack large, pyramidal-like neurons in the telencephalon and that could play a significant role in the evolution of differences in neuron density.

With respect to the suggestion that synapses could be measured as another neuroanatomical correlate of cognition, the thesis does not suggest a means by which this could be accomplished. Without a proposed means of this quantification, I found it a bit of an empty suggestion as there are multiple ways of quantifying synapses and each of them has specific pros and cons. Even a few sentences would have enriched this part of the discussion.

Similarly, with the breadth of statistical methods used by the candidate, I was a bit disappointed that the final part of the Discussion recommends a "multidimensional view" without suggesting any statistical approaches. Integrating different data sets that vary in what they measure and the absolute range of numerical values can be problematic for many multivariate methods, so it would have been useful for the candidate to provide at least one example of how this kind of analysis might be done.

Last, isotropic fractionation has been a useful in documenting broad patterns in neuron numbers and densities across and within species and is relatively rapid compared to stereological counts of histological sections. However, isotropic fractionation also has several important caveats and these were not discussed sufficiently in the thesis or papers. One caveat is that it remains unclear how many nuclei are damaged during the process of homogenization and if that varies across species. This would not affect very large differences across species, but could affect the accuracy of intraspecific studies. A second caveat is the dependence on gross dissection. Although in this thesis, the dissections were far more precise than in the majority of studies, there will still be error introduced that be lower in histological sections. Further, histological sections allow us to quantify neurons within brain regions that cannot be grossly dissected, providing more detailed information on neuron numbers and densities across brain regions. A third caveat is that by homogenizing the tissue, the specimens cannot be used by other researchers or for other types of labeling. This is not a major issue for the intraspecific studies in the thesis, but for species that can be difficult to obtain, we likely know very little about brain morphology, cytoarchitecture, and brain composition (i.e., relative size of individual brain regions). Overall, I would have preferred to see some discussion of these caveats within the thesis and perhaps some suggestions on how best to maximize the amount of information derived from specimens to further brain evolution research.

<u>Strengths</u>

The writing throughout the thesis is excellent. In fact, this is the most well written thesis I have examined. I did not encounter any significant typos or spelling errors and the grammar and flow of the document made it easy and enjoyable to read.

The analyses of the intraspecific and interspecific data are state-of-the-art for evolutionary biology. I should emphasize that incorporating these kinds of statistics into neuroscience publications has been very slow. This thesis truly stands out because it was one of very few that incorporates sophisticated statistics from evolutionary biology to neural data. I was particularly impressed with the array of statistical methods used in Chapter 4. I am unaware of similar

attempts to be so comprehensive in an analysis of comparative neuroanatomical data. The candidate should be commended for this significant effort.

The breadth of work covered in this thesis is also impressive. I am unaware of similar theses in which a student has incorporated both intraspecific and interspecific variation in neuroanatomy. That is not, however, the only reason that I list this as a strength. Understanding how micro- and macroevolutionary processes can differ from one another requires a deep understanding of neuroanatomy and evolutionary biology and that is relatively rare among PhD students, in my experience.

Further emphasizing the breadth and depth of the candidate's thesis research is the comprehensive bibliography in the individual papers/chapters as well as the General Introduction and General Discussion. In fact, I have taken advantage of the thoroughness of the references cited to add a number of papers to my reading list that I was unaware of previously.

Last, the General Discussion raised several significant issues facing our understanding of brain evolution that have not been articulated by others within the field. For example, the candidate stresses the important of broadening species coverage, particularly with respect to amphibians, and I agree this is truly needed. I also agree that phylogenetic coverage needs to be expanded within all clades and we need to try to develop additional measurements/data that might aid in explaining species and individual variation in cognition.

In closing, this was an exceptional thesis and I look forward to participating in the oral defense.

Sincerely,

Dr. Andrew Iwaniuk
Associate Professor
Canada Research Chair in Comparative Neuroanatomy