Review of: "Reduction of acetylcholine in the hippocampus of hippocampal cholinergic neurostimulating peptide precursor protein knockout mice"

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This is an article that clearly shows that reduction of Hippocampal cholinergic neurostimulating peptide (HCNP) decrease the concentration of acetylcholine (Ach) in the hippocampus using HCNP-pp conditional knockout (HCNP-pp KO) mice. This is an important finding for understanding the function of the cholinergic centrifugal network from the medial septal nucleus to the hippocampus. This is essential for understanding the mechanisms of memory and how diseases such as Alzheimer's occur, as well as for dealing with them.

I would like to evaluate this article in terms of the statistical methods used. The authors performed statistical tests on every comparison. Those were ideal approaches; for example, as required by the journal, the authors have met the basic necessity to avoid multiplicity of tests. But then, I would like to ask a question here. Is statistical testing always the best choice for scientific objectivity? In fact, the overuse of significance tests has been pointed out repeatedly [1-3].

In the first place, are statistical tests as necessary as they seem? Maybe the test becomes important when the difference is not clear objectively. For example, the authors used microdialysis to directly quantitatively compare the amount of Ach. This is the key data in this article, and it shows a very clear difference. Since there were about 200 measurements in 12 cycles, ANOVA and 12 post-hoc analyses were performed, and p-values were noted for each. This is a very objective attitude, but I am not sure that the information on these p-values is very useful to the reader.

How can we present the information more clearly and objectively? Tukey's Exploratory Data Analysis (EDA) [4] recommends boxplots in such cases. In this case, for example, Panel A would consists of 12 rows of 24 boxes. The boxes will probably not overlap at the top and bottom, showing a clear visual indication that the distribution of KOs and wilds is different. Then, in its objectivity, there is no need to perform statistical tests. One advantage of this approach is that classical testing assumes a distribution of the data, whereas EDA can rather show the data as it is distributed. The objectivity would be rather higher with EDA, or at least the quality of information it can provide is higher.

Authors have shown in Fig. 2 that the number of choline acetyltransferase (ChAT) positive neurons in the medial septal nucleus was unchanged, and in Fig. 5 in the stratum oriens and stratum radiatum was unchanged. Of course, those are also important findings for understanding the function of HCNP. Interestingly, the authors performed the test even though there was no clear difference, and concluded that there was no significant difference because P>0.05. Especially in Fig. 2, they try to show that there is no difference by calculating the Bayes factor.

In general, a large p-value in a test does not prove the correctness of the null hypothesis. Rather, it means that the noise in the measurement would be greater than the difference between the two groups. There is nothing that can be claimed when the test was not significant. And this could be a matter of preference, but we have to be very careful about applying Bayesian methods in scientific fields since often unverifiable assumptions [5] are introduced. Rather, to test whether the sampling is randomly drawn from the same population, the run test for randomness of two related samples [6] is a more reasonable choice.

One very sensitive observation is Fig.4. The authors reported that vesicular acetylcholine transporter (VAchT) in the hippocampus was reduced in HCNP-pp KO mice by immunohistochemical analysis. Of course, there is no contradiction between the decrease in Ach and VAchT. However, the quality of the data is different from the values quantified by dialysis, because this is an image. The density of the image can easily change depending on the exposure to which it is obtained, and even though DAPI was used as a control, there must be some variation in the staining. If it is a difference in the number of vesicles, it is easy to summarize, but the shading of the image is difficult to determine. For example, it would be more reliable to collect vesicles biochemically and then quantify Ach. As with Western blotting, it is actually quite difficult to quantify an image, and it is hard to obtain objective data. This is a fundamental problem that cannot be solved by a statistical test. In any case, the decrease in VAchT is not unexpected given the decrease in Ach. I think it is more interesting to note that the number of vesicles seems to be not so different.

In the future, there will be many approaches to learn more about the function of HCNP, such as: how were the motor and learning abilities of HCNP-pp KO mice? Microarray analysis will help to understand which genes were affected. Can we quantify HCNP by HPLC or other means? There should be a receptor for this, and if we can use surface plasmon resonance with it, we can measure it in real-time. What kind of cues would stimulate the synthesis of this? Also, is it possible to administer artificially synthesized HCNP to HCNP-pp KO mice? What would happen then? It would be technically challenging, but it would be possible in a lab with microdialysis capabilities. The interest is enormous.

1 Hurlbert, S. H., Levine, R. A. & Utts, J. Coup de Grâce for a Tough Old Bull: “Statistically Significant”

Expires. *The American Statistician* **73**, 352-357, doi:10.1080/00031305.2018.1543616 (2019).

2 Baker, M. Statisticians issue warning over misuse of P values. *Nature* **531**, 151-151, doi:10.1038/nature.2016.19503 (2016).

3 Wasserstein, R. L. & Lazar, N. A. The ASA Statement on p-Values: Context, Process, and Purpose. *The American Statistician* **70**, 129-133, doi:10.1080/00031305.2016.1154108 (2016).

4 Tukey, J. W. *Exploratory data analysis*. (Reading, Mass. : Addison-Wesley Pub. Co., 1977).

5 Ellis, G. & Silk, J. Scientific method: Defend the integrity of physics. *Nature* **516**, 321-323, doi:10.1038/516321a (2014).

6 Bradley, J. V. *Distribution-Free Statistical Tests*. Chapter 12 (Prentice-Hall, 1968).