Review of: "Developmental emergence of sleep rhythms enables long-term memory capabilities in Drosophila"

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In the article: Developmental emergence of sleep rhythms enables long-term memory capabilities in Drosophila, Amy R. Poe et al. described the rhythmic sleep behavior during larval development. The rhythmic sleep emerged in the 3rd instar and facilitated long-term memory. The Dh44 neurons receive the circadian signal CCha1 released by DN1as and regulate sleep when the synaptic formation occurred at the early 3rd instar stage.

Compared to adult sleep studies, there are fewer studies on developmental sleep. They utilized Drosophila as a model to elucidate the neural circuits and neural signals involved in immature stage sleep. Overall, the data is solid and the conception they provided is novel. However, for that very reason, I'm afraid that the authors should provide more detailed evidence to exclude the possibility of over-interpret their data.

1.

the authors should further optimize the figures. many labels of the figure are very confusing. For instance, the indicator line to show the different significance is inconsistent in fig 1C and 1D, in fig 1C, maybe you should use two separate lines to show the different significance of CT19-CT1 and CT19-CT7. Another example is in fig 4b-e and fig s4c-f, the same genotype is compared with each other at the same CT time. So, I really cannot understand what the figures show. There are also very limited explanations in the figure legend.

2.

For the genetic tools, the authors didn't mention whether the flies are backcrossed in the same genetic background. for adult flies, backcross is necessary for sleep research because genetic background plays an important role in sleep behavior(Zimmerman et al., 2012). Authors should provide crucial evidence if they think genetic backgrounds don't alter larval sleep. otherwise, they should backcross all the flies into the same genetic background if these flies are conducted sleep behavior test.

3.

The authors should pay attention to the accurate representation or paraphrasing of citations which is the basic scientific

literacy. For example, in the 7th to 9th line of page 6. According to original literature [(Liu et al., 2015)], at the L2 stage, the functional larval clock network is comprised of 4 s-LNvs, 2 DN1as, and 2 DN2s per hemisphere, however, there are more clock neurons emerged at the L3 stage (like LNd and LPN neurons). Especially, in the late L3 stage, DN1p neurons also expressed circadian molecules.

4.

For detecting the rhythmic sleep in the larva. the animal number is below 50. According to fig1c, the data does not conform to a normal distribution. So, an appropriate increase in the number of repetitions would be helping ensure the credibility of the basic experimental paradigm of the article. besides that, according to the former research also conducted by the same lab [(Szuperak et al., 2018)], 6s of stillness is defined as the criterion for sleep based on the possibility of subsequent behavior after a high-intensity blue light stimulus. However, in this paper, they also mentioned that the sleep depth is different across development stages (fig5b). So, if is it possible that the 6s quiescence is not suitable for all stages of larval sleep? In this case, they should reanalyze the sleep data they present.

5.

Fig5d, there is no difference comparison between the mcherry-RNAi and CCha1R-RNAi. For proving the LTM is dependent on the rhythmicCCha1 signal, it is necessary to compare the performance Index among different genotypes. Also, the way to indicate the genotype in fig5 is barely clear. In fig 5a, why only show L3 stage performance Index of CCha1R-RNAi but show both L2 and L3 stage results of mcherry-RNAi?

6.

For the GAL4 and lexA lines that they used to manipulate the gene expression by RNAi strategy, are the expression strength of the GAL4 and lexA promoters relatively consistent? For instance, if the expression strength of Dh44-GAL4 was weaker in L2 stage than L3 stage, the conclusion from fig4 would not be persuasive.

7.

Do Dh44-GAL4 and Dh44-lexA exhibit the same or similar expression pattern? If Dh44-GAL4 and Dh44-lexA label different neurons, the GRASP and GCAMP imaging results can only prove DNa1s synaptically connected to Dh44-lexA labeled neurons, but may not be Dh44 neurons.

1.

Even in adult flies, the CCha1-R seems to be weakly expressed in PI neurons(Fujiwara et al., 2018), authors should give more evidence to confirm the CCha1-R expression in Dh44 neurons. The chemoconnectome genetic tools or the

Mi{MIC}MI03750 strain may be helpful to explain this question.

9.
Under constant light, the circadian behavior is diminished in adult flies, this phenotype is regulated by Cry(Parisky et al., 2016). If Cry is mutated, flies will preserve the circadian behavior in constant light conditions. I wonder whether the larval circadian is also regulated by Cry under constant light. However, Cry is weakly expressed at L2 and early L3 stages according to the Flybase Database. The authors need to explain whether the behavior results shown in fig S1 are regulated by the circadian system.

In summary, exploring the circadian sleep regulation mechanism during development is challenging. The neural circuits are formed gradually during consecutive behavior monitoring. For example, the DN1p neurons emerged at the 12th hour of the L3 stage which may also be partially involved in sleep regulation. I'm not sure if this will affect the conclusion of this article.

The genetic tools may not be as reliable as it is in adult flies. For example, many researchers favor lines with nos-Cas9 instead of vas-Cas9, as nos expression is more tightly confined to the germline(Port et al., 2015). In other words, different promoters may exhibit different expression capacity in different developmental stages. My concern is that the authors should verify the expression of the GAL4 and lexA lines they use in the larval stage to make sure they are reliable.

In addition, I also worry that the conclusion derived from adult flies' research might not be suitable to explain the circadian system at the larval stages. So, they should review whether the conclusions from former research are properly utilized.

Besides that, please optimize the figures.

I believe this paper will provide valuable insight for exploring "infant stage" sleep regulation after proper modification. Good luck!

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