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

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Previously, the authors have demonstrated the presence of sleep in larvae. However, contrary to adults where a minimum time length of inactivity defines sleep, here, from what I understand, as soon as the larvae stops moving it is considered as sleep.
It takes about 5 days at 25°C from an early L1 to enter into pupation at the end of the L3. L3 lasts between 2.5 and 3 days. Hence, while L1 to L2 are mainly for the growth of the larvae, the L3 stage is mainly for accumulating nutrient stock for the metamorphosis that lasts around 5 days at 25°C. At the end of the L3, the larvae stops eating and moves upward out of the food to look for a spot to pupate. Hence, I do not think that we can easily conclude that when a larvae stops moving, it can be considered as a sleep episode. There are so many other reasons. For example, it can stops because the brain is analysing the input it receives in order to drive a response such as continue eating or moving somewhere else where the nutrient content would be more appropriate for the current developmental stage it is at.
I do not know what is the target of this manuscript but I do not think it will loose interest if the authors focused instead on a metabolic point of view which would make more sense. Notably, they found an interaction between the DH44 neurons and the DN1a, which is interesting.

For all the quantifications, the authors measure the fold change. They normalized to the average of a control (chosen depending on the figure). The problem with this is that we cannot compare the raw level of sleep (or bout number or bout length). Is it because the raw level is largely different between experiments and control strains? For example we do not know whether early L3 sleep more or less than early L2.

Fig. 1: The authors are comparing the level of sleep of early L2 or L3 larvae at CT1. While there are no significant differences for the early L2, depending on when in their circadian time, the early L3 have more periods of inactivity or not compared to CT1. Because the average bout length do not change significantly, L3 sleep cannot be considered getting deeper during the subjective night (Sup. 1a).
Fig. S1: h-j) the authors compared for the same time points different ages in the L3 stage. No difference apart from L3 +8h. The authors concluded that it was reflecting perhaps an anticipation to pupariation. However, it is very early, as late L3 only happens at least 48h after molting. It would be interesting to compare sleep of L3+8h between subjective day and subjective night. K-l) The authors compared sleep of the same larvae at different time points. They found that if a L3 emerges at CT21 it will sleep more 4h later at CT25 and vice versa, if a L3 emerges at CT9, it will sleep less 4h later at CT13. This can be sensed as counter-intuitive since in fig. 1 they show that L3 that emerges during the subjective night

sleep more than L3 that emerge during the subjective day. And that a L3 that emerge at CT9 sleep the same amount as a 4h older larvae at the same time point, same for CT21.

Fig. 2: The authors are focusing on Dh44 neurons as the downstream neurons for sleep regulation. This is based on a mini screen (Sup. 2a). Personally, I would have focus on the c929 driver as there L3 sleep 2 times more while activation of the DH44 only reduce by 50% the amount of sleep.
For the DH44 part, two important references are missing. M. Dus et al.,*Neuron***87** :139-151, 2015 and Y. Ohhara et al., *JCN* **526** :1351-1367, 2018. These articles clearly demonstrate a function of the DH44 neurons in feeding behaviour, metabolism and nutrient sensing. They do this through genetic manipulations but they also relate it with a very finely defined projection pattern in both adult and larvae. Therefore, these two articles make the hypothesis of a role of the DH44 neurons in sleep difficult to believe, unless it is indirect and therefore it should involve an effect on the siesta (postprandial sleep) in the adults.
The primary goal of a larvae is to eat, grow and stock nutrients for the metamorphosis period. Hence, it makes more sense that the larval brain clock (much simpler than the adult) is there to orchestrate the metabolism and feeding behaviour of the larvae relative to its development stage.

Fig.2: The authors conclude that activation of the DH44 neurons in L2 reduces sleep, however a more logic interpretation would be they eat more. They counter argue by saying that they don't eat more (Sup. 2e). However, what they are showing is that the amount of mouth hook contraction/sec awake is not changed. If they sleep less (amount of time not moving reduced) then they stay longer awake. Hence, what we need to look is the total amount of hook movement which is necessarily increased. When a larvae moves it also moves its mouth hook.
Sup. 2: a) what is the difference between Dh31 next to dh44 and dh31 next to c929? Are they 2 different Dh31 drivers? e) the mouth hook contractions /sec of wakefulness is not different between activated Dh44 and controls. However, since the activated DH44 larvae spend more time awake they inevitably spend more time moving their mouth hook and therefore spend more time eating.

Fig. 3: Here a dual labelling image between the DN1a and the DH44 neurons would have been nice to see. m) If DH44 activity is under circadian control from the DN1a then the oscillation should be lost when artificially activating them. Therefore, the authors need to compare CT1 to CT13 of DH44>TrpA1.
Sup. 4: a dual labelling would be nice also there.

Fig. 4: b-c) the authors should also compare CT1 together and CT13 together using a Kruskal-Wallis test (not mean comparison but median comparison).

Fig. 5: I am not sure to understand. Activation of Dh44 neurons promotes wakefulness. Decreasing the expression of CCHA1-R in the Dh44 neurons increases sleep (Fig. 4b compare CT1 controls with CT1 experimentals). However, DH44>CCHA1-R RNAi are more aroused and loose their LTM, suggesting that the increase of inactivity periods does not

actually correspond to sleep.