Staring at the clock face in *Drosophila*

Ezio Rosato and Charalambos P Kyriacou

Dept Genetics & Genome Biology, University of Leicester, LE1 7RH, UK

We live in a world of relentless daily light and temperature cycles and so most eukaryotes and some bacteria have evolved an endogenous circa-24h (circadian) clock that synchronises the processes of life with its rhythmic environment. In animals, behaviour is rhythmic thanks to the action of clock neurons in the brain. In the fruit fly *Drosophila melanogaster*, 75 clock neurons per hemisphere are distributed into six main clusters, three laterally (s-LNv, l-LNv and LNd) and three dorsally (DN1, DN2 and DN3). The molecular components of the clock cycle in unison in all of them. For instance, under both light-dark cycles (LD) and constant darkness (DD) the clock protein PERIOD (PER) reaches a peak at the end of the night and a trough at the end of the day. However, under LD and for the first few days in DD the rest/activity cycle of flies is bimodal, with a peak of locomotor activity in the morning (the 'M' component) and another in the evening ('E'). Genetic dissection of these clock neurons revealed that the s-LNv cells are typically responsible for early morning activity (hence 'M' cells), and likewise, the LNds generate the evening component, so called 'E' cells'. But how can a unimodal and synchronous molecular clock inform the different cells of their different M or E duties?

Liang and co-authors began addressing this issue in a previous publication (Liang et al., 2016). They used light-sheet microscopy (Holekamp et al., 2008) and a genetically encoded Ca^{2+} sensor GCaMP6s, to measure intracellular levels of Ca^{2+} in all circadian neuron groups over a 24h period. They observed that the different groups show maximal Ca^{2+} levels, hence neuronal activity, at different times of the day. The s-LNv neurons peak around dawn, the LNd around dusk, the l-LNv around midday and DN1 and DN3 around midnight. In simple terms, although all circadian clusters agree on what time it is *via* their canonical molecular clock, the timing of their peak excitabilities are distinct and sequential. While this was an important and highly significant step in the right direction, it still did not illuminate the mechanisms that sequentially time neuronal activation.

For the fly, light is the major entraining cue and the neuropeptide Pigment Dispersing Factor (PDF) produced by the s- and l-LNv cells has long been recognised as a major synchroniser of the molecular clock in circadian neurons. In a new paper in this issue Liang et al (2017) have now investigated the effects of light and PDF on the sequence of excitability within the network. They examined mutants that eliminate the production or the response to PDF under LD or DD. They observed that the LNds respond to the light-dark regime and to PDF by delaying their Ca^{2+} rhythm. In contrast only PDF signalling was able to delay the DN3 while l-LNv and DN1 were insensitive to the neuropeptide. The phase of s-LNv was not affected by these manipulations but their Ca^{2+} 'wave' became wider. When PDFR, the PDF receptor, was re-introduced specifically in those cells in an otherwise PDFR-mutant background, the normal width of the Ca^{2+} 'wave' was restored. Hence PDF signalling delays activation of the LNds and contributes to curbing the activation of the s-LNvs. Bath applications of synthetic peptide showed that in both cell types, the effects of PDF can be explained by a reduction of Ca^{2+} levels and that those effects require the presence of PDFR. Ectopic expression of PDFR in the naturally PDFR-null l-LNv further suggested a cell-autonomous mechanism.

Moving back to the effect of light, the authors investigated how light pulses delivered at the beginning or the end of the night influenced the Ca^{2+} waves on the following days. A light pulse in the early night generates a delay in the subsequent start of the locomotor activity cycle whereas a light pulse in the late night results in an advance. This is an almost universal circadian phase response to

light. An early-night light pulse delayed Ca^{2+} rhythms in all clock neurons on the first day, caused desynchrony in the DN1 and DN3 and a further delay in the s-LNvs and LNds on day 2 with a return to normal phase on day 3. A late-night pulse caused a pronounced anticipation (advance) of the Ca^{2+} rhythm in the s-LNvs on day one, whereas on day 2 the advance became smaller and extended to all the other neurons with the same pattern persisting on day 3. This is quite a different picture from the one obtained by examining the cycling of a PER-LUCIFERASE fusion protein in whole-brain explants of transgenic flies. (Roberts et al., 2015). In this preparation a late night light pulse induced the most rapid and coherent phase advance in the LNds while it quickly desynchronised the other circadian groups. Clearly there is not a simple relationship between the activity of neurons and the cycling of their clock molecules.

Finally, as the DN1 do not require PDF for the phasing of their Ca^{2+} cycling, might another neuropeptide be involved? Mutant and mosaic analyses revealed that small Neuropeptide F (sNPF) is necessary for rhythmicity and phase of Ca^{2+} cycling in DN1, though it is not required for the cycling of PER. The s-LNv and two of the LNd neurons produce sNPF. Either group is sufficient for conferring rhythmicity but the s-LNv are necessary for correct Ca^{2+} phasing in the DN1. Similarly to PDF, sNPF operates by suppressing Ca^{2+} activity.

The emerging model (Fig 1) is that of a network where the mismatch between the cycling of the molecular clock and that of neuronal activity is due to the delaying effects of light and neuropeptides on Ca^{2+} cycling. The default condition in the absence of the PDF receptor is a Ca^{2+} peak at dawn in s-LNv, DN3 and LNds. During the time of their maximal activation the s-LNv secrete neuropeptides. PDF curbs the sLNv activation and delays the peak of activity of the LNd till dusk, with a further delay contributed by light under LD cycles. PDF is also responsible for delaying the maximal activation of the DN3 to the middle of the night, although it is not clear how the difference in phase between LNd and DN3 is generated under DD. The DN1 also peak in their activity in the middle of the night. However, unlike the DN3 they do not respond to PDF but to sNPF. According to the model there is a double wave of sNPF, first from the dawn-active s-LNv and then from the dusk-active LNd, although the latter seems redundant as it is not required for cycling nor is it sufficient for phase. The model cannot explain why the peak of Ca^{2+} of the l-LNv occurs in the middle of the morning. Figure 1 illustrates the clock neuronal groups and their expression of PDF and sNPF. Their intercellular communications, dissected by Liang et al (2017) are shown in addition to the time of maximal Ca^{2+} activation for each group under LD (shown as ZT = *Zeitgeber* time, which corresponds to lights on at ZT 0 and lights off at ZT12). If one follows the timing of the clock dial, it is very interesting perhaps that cellular time roughly follows the relative anatomical locations of the cells generating a wave of activation, and of course it is clockwise! (At least in this brain hemisphere). It is almost like looking at the face of a clock.

Clearly the model is still incomplete but it accounts for several of the findings we have described above. Importantly, it suggests that PDF acts as a repressor of neuronal activity rather than as an activator, something that the *Drosophila* circadian community had long assumed. In addition, a very recent twist to this story is that a previously uncharacterised rhodopsin, Rh7, has been identified within the s-LNv and l-LNv and in some cells lying very close to the DN1 (Ni et al., 2017). Rh7 has been shown to mediate an additional light–sensing pathway. As Li and coworkers have used blue light to periodically interrogate the Gcamp reporter might this have activated Rh7 with unforeseen downstream effects on the clock cells? It could be worthwhile to see whether reducing the expression of Rh7 affects the temporal regulation of neuronal activation.

In conclusion, Liang, Holy and Taghert's innovative and sophisticated neurogenetic approach consolidates and extends their previous game-changing discovery of differentially timed clock neuron

activation while also challenging current ideas of how the system might work (Liang et al., 2016). Yet over the past two decades, the fly has also provided a superb model for the mammalian clock with its simpler 150 cell *Drosophila* circadian neuronal network compared to the suprachiasmatic nucleus, with its 20,000 neurons and comparable number of astrocytes. Recent work by Brancaccio et al (2017) using Ca^{2+} reporters has shown how astrocytes and SCN neurons are active during the night and day respectively. Furthermore the astrocytes inhibit the dorsal SCN neurons via releasing glutamate into the extracellular space, and, remarkably, molecular manipulation of the astrocytes significantly alter the mouse free-running locomotor period. This antiphasic functional relationship between astrocytes and neurons in the SCN echoes the E and M cell groups in the fly circuit. Similar logic with different cellular actors…

References

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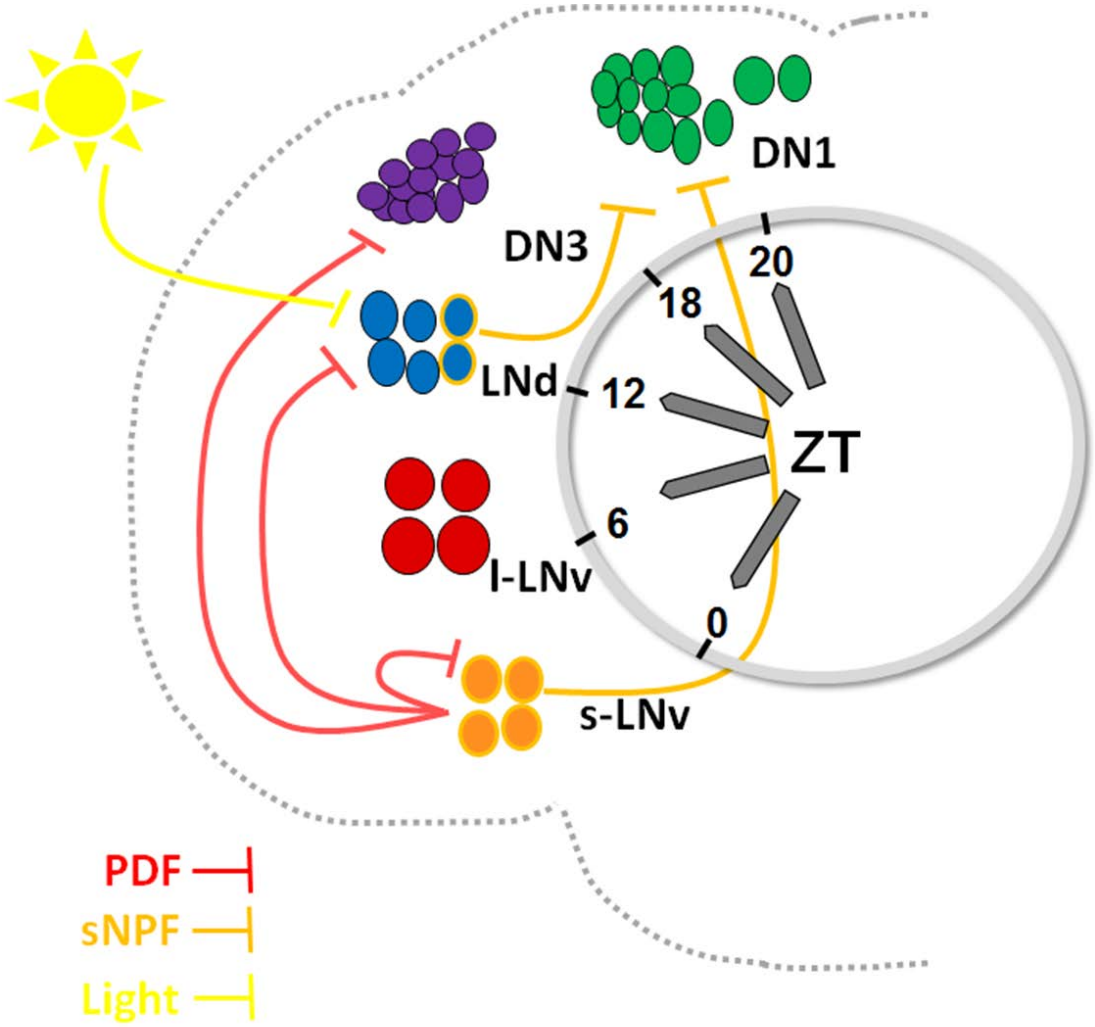
Fig 1. The *Drosophila* neuronal clock face

Left hemisphere of a fly brain showing the position of the main clock neurons (s-LNv in orange, l-LNv in red, LNd in blue, DN3 in purple and DN1 in green). The clock dial gives the time of peak Ca^{2+} activation for all neuronal groups under light-dark conditions (LD), with lights-on at Zeitgeber Time ZT0 and lights off at ZT12. The inhibitory effects of PDF (from the s-LNv), sNPF (from the s-LNv and two LNd) and light (sun symbol) are shown by red, orange and yellow lines, respectively.


DN1
DN3
20
18
LNd
12
ZT
6
l-LNv
0
s-LNv
PDF
sNPF
Light