Whatever you were doing just before reading this, walking to work, drinking a coffee after lunch or winding down in the evening in your favourite armchair, it is likely you will do it again tomorrow, more or less at the same time. This daily cycle is a reflection of the fact that we live in a 24-hour environment that, through rhythmic changes in light, temperature and social interactions, imposes temporal constraints on our lives. However, we do not simply react to those daily changes; rather, we anticipate them and behave accordingly.

It is all about good timing

We now know that the reason why we predictably allocate different activities to different times of the day is that we have evolved an endogenous biological timer that ticks with a period of about a day (circa diem in Latin) (Figure 1).

There is abundant evidence suggesting that this circadian clock has adaptive value. Laboratory tests using different experimental systems (animals, plants, fungi and cyanobacteria) have shown that fitness is highest when organisms are placed in an environment that cycles with a rhythm that is the same as their inner rhythm. Additionally, field studies demonstrated that natural selection sculpts the clock; by sampling natural populations, we can identify clock variants that are best adapted to their local rhythmic conditions. Since this synchrony between internal and external rhythms has been shown to confer benefits, we could argue, in contrast, that living in an environment that does not align with our inner clock might have consequences that are worse than not having a clock at all.

This prediction holds true; people exposed to continuous circadian insult, such as shift workers, have a higher incidence of psychiatric conditions, cardiovascular disease and cancer. Further, as industrial and post-industrial societies move inexorably towards a ‘24-hour life’, where the difference between day and night is less defined, we observe an emerging epidemic of sleep and behavioural disturbances.

Clearly, ‘circadian hygiene’, or the harmony between external and internal rhythms, has real-life benefits. To ensure we implement it (both personally and at the level of society as a whole) and to suggest remedial measures for a broken clock, we must first learn, in detail, how the circadian clock works.

The fact that life on earth evolved from a common ancestor makes it possible to investigate the architecture of complex phenomena in simpler, more malleable, model organisms. On this premise, we discuss the circadian clock of the fruit fly Drosophila melanogaster, a small insect with a complex nervous system. Studying the circadian

Figure 1. Daily rhythms. Our lives are marked by a constellation of activities that happen every day more or less at the same time. For instance, in the morning, our body temperature is at its lowest. Eating is under strong circadian control, and every day we feel hungry at about the same times, depending on when we normally have lunch and dinner. After lunch, we reach the trough of our attention, and many of us resort to caffeine to keep awake. A few hours later, our muscular tone is at its best, and we perform better in sports in the late afternoon. In the evening, our body temperature is at its warmest. Also in the evening, our ability to metabolize alcohol increases, and this is the time when we preferably consume alcoholic drinks. Night is our favourite time to sleep, but at night our sensitivity to pain is highest, so feeling pain may keep us awake.
The clock of the fly has paved the way to understanding our own clock and, more generally, to unravelling how the nervous system works.

**A cell-autonomous molecular clockwork**

In 1971, through a mutagenesis screening in *Drosophila*, the first clock gene was identified. It was named *period* (*per*) and became the first-ever recognized component of the circadian clock. This started a concerted effort across many laboratories working on different species to use genetics to identify other genes involved in the clock and use biochemistry and molecular biology to describe the mode of action of their protein products.

Early efforts were specifically directed at identifying the cellular machinery of the clock, based on the assumption that the mechanism must be cell-autonomous since single cells, whether independent organisms or isolated mammalian cells in culture, show 24-hour rhythms in their physiology.

Multiple clock genes were identified in several model systems. This showed that the general design of the clock is conserved across kingdoms although the molecular components have evolved independently in animals, plants, fungi and cyanobacteria. (Other bacteria do not seem to have a recognizable clock.)

The general principle on which the clock is based is that of a negative feedback loop. Essential clock molecules change their status progressively across the 24 hours, thereby providing time information to the cell. As part of this process, they increasingly inhibit their own maturation or production until they either revert to their original state or are degraded so that a new cycle can begin. Since 24 hours is a particularly long time in terms of single chemical reactions, many biochemical processes come together to provide the necessary ‘delay’ in reaching the 24-hour period. This ‘cooperation’ provides robustness and increases the amplitude of the cycle, which is required for it to be able to influence the overall physiology of the cell.

In animals, the negative feedback loop is built around the control of key transcription regulators, such as PER (the protein product of the *per* gene), that can impart 24-hour rhythmicity to their own transcription and to that of downstream effector-encoding genes (Figure 2; for more information, see the article by Smith and Sassone-Corsi, this issue).

Three pioneers in this field, Jeffrey Hall (Brandeis University, USA), Michael Rosbash (Brandeis University, USA) and Michael Young (Rockefeller University, USA), were awarded the 2017 Nobel Prize in Physiology or Medicine for their discoveries of how *per* is regulated.

**Many ‘clock cells’**

Although clock cells conform to a general design, animal clocks are made of cells that are not identical. For instance, there are molecular components that are expressed at a much lower level, if at all, in some clock cells compared to others in the same organism.

Experiments in mammals have shown that some isolated cells are more rhythmic than others *in vitro.*
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Surprisingly, neurons derived from the suprachiasmatic nucleus (SCN), a brain centre that in mammals exerts control on the rhythmicity of the whole organism, are poorly rhythmic in dispersed culture. Conversely, SCN slices that maintain extensive communication among neurons are strongly rhythmic when grown ex vivo. This suggests that cellular communication is an additional, fundamental characteristic of animal clocks, at least in the brain.

In flies, about 150 clock neurons carry out functions similar to the approximately 20,000 neurons that constitute the SCN in mammals. This reduced complexity and the availability of tools to manipulate groups of clock neurons separately explain why our understanding of the circadian neuronal network is particularly advanced in Drosophila (Figure 3).

Figure 3. The anatomy of the circadian clock in Drosophila. The figure shows a representation of the clock neurons in the brain of the fly. The right hemisphere is a composite image obtained by combining several pictures of the same specimen acquired with a confocal microscope at different focal planes. The left hemisphere shows a diagrammatic representation. The clock neurons are named lateral neurons (LN) and dorsal neurons (DN), which are indicative of their relative position in the brain. The lateral neurons are divided into dorsal (LNd) and ventral (LNv). The latter are further distinguished into small (s-LNv), large (l-LNv) and 5th-LNv (because originally it was considered related to the four s-LNv). The dorsal neurons are numbered consecutively, e.g., DN1, DN2 and DN3. According to the dual-oscillator model (see text), the s-LNv form the morning oscillator while the 5th-LNv plus three LNd (the LNd are a heterogeneous group) constitute the evening oscillator. Additionally, the s-LNv function as a pacemaker. In flies, the oesophagus passes through the brain; Oe indicates the oesophageal foramen (opening).

The 'clock network'

Since we can manipulate different groups of clock neurons independently, we can ask two fundamental questions:

1. Are different neurons in charge of the different features of the 24-hour rhythm?
2. Which neurons are necessary and/or sufficient for self-sustained rhythmicity?

Note that these questions assume a division of roles and a hierarchical relationship among the clock neurons; but how does it happen? One possibility is that roles and hierarchy are pre-defined to accommodate the features of different environments. Another is that the role and hierarchy adopted by different neurons depend on the configuration that the network assumes in the present condition and following its experience of previous environments.

The difference between the two hypotheses is fundamental. The first assumes that the functioning of the circadian system is predominantly stereotyped and that the characteristics of the clock as a whole reflect the characteristics of discrete neurons according to their leading role. The latter assumes that plasticity has a more important function and that the overall characteristics of the clock do not belong to any specific neuron but emerge from the configuration of the ensemble.
Rhythms in locomotor activity and rest are the preferred circadian phenotype in *Drosophila*. Using small commercial devices, this phenotype can be monitored for hundreds of flies in parallel with little effort. Flies are crepuscular, meaning they are far more active in the morning and in the evening compared to the middle of the day or the middle of the night. Therefore, their activity profile shows a morning (M) and an evening (E) peak separated by a 'siesta' in the middle of the day and a phase of consolidated sleep at night. In the laboratory, light is usually presented as a symmetrical rectangular signal (either ON or OFF, 12 hours apart) under constant temperature (often at 25°C). Under such conditions, both M and E peaks reach their maximum at the time of the switch. The increase in activity before the switch is known as 'anticipation' and denotes the function of the circadian clock (Figure 4).

Flies carrying a mutation in the *per* gene, called *per*², have no functional PER protein. These flies cannot anticipate M or E peaks and are arrhythmic under constant darkness and temperature. We can test the function of different groups of neurons by reintroducing PER into those neurons only, while the rest of the animal still lacks functional PER. Such manipulations showed that different groups of neurons rescued the 'anticipation' of the M and E peaks. Thus, we could suggest that the clock is composed of two connected oscillators: one morning oscillator controlling the M peak and one evening oscillator controlling the E peak.

Moreover, when PER expression was limited to the morning oscillator, flies were rhythmic under constant conditions. This was not the case for the evening oscillator, which led researchers to propose that the morning oscillator is the 'pacemaker'. This interpretation that agrees with a deterministic model of the clock resonates well in the circadian field. Many years ago, researchers observed that small mammals placed under constant illumination split

**Figure 4.** Circadian phenotypes in *Drosophila*. **Left panel.** One-day locomotor activity profile of several wild-type male flies averaged together (the activity profile of females is different) under 12-hour light and 12-hour dark conditions (LD). The temperature was constant at 25°C. The height of each bar corresponds to the amount of activity (measured by a commercial device that counts how many times a moving fly interrupts an infrared beam). The width of each bar is constant and represents 30 minutes. White bars denote the light phase and black bars the dark phase. M indicates the morning peak of activity and E the evening peak. Both peaks display 'anticipation' shown by an arrow. The phase of rest during the middle of the day is referred to as 'siesta'. During the night, there is a prolonged phase of consolidated sleep. **Right panel.** Average locomotor activity profiles of male flies. Three days under LD conditions followed by 4 days under constant darkness (DD) are shown. The temperature was constant at 25°C. The activity profiles are double-plotted (e.g. day 1–day 2, day 2–day 3, etc.) to help visualize the rhythm. White bars denote the light phase and black bars the dark phase. Grey bars correspond to subjective light. This means that the flies experienced dark but at a time when they would have been in light under LD conditions. Note that the flies are still rhythmic under DD, showing they have an endogenous clock.
their locomotor activity into two components: one with a long and the other with a short period. This led them to hypothesize that the clock is a dual-oscillator system.

Today, many investigators suggest that the results of the fly experiments provide further evidence for such a model as they map the morning and the evening oscillators to two distinct groups of neurons. This is why, in many circadian articles, the *Drosophila* clock is described using terms such as ‘morning cells’, ‘evening cells’ and ‘pacemaker neurons’.

However, other researchers (including the authors) argue that such an interpretation is too simplistic. There is evidence of functional plasticity that should be taken into account in a model of the clock. For instance, it is possible to shift morning anticipation to an earlier time by ‘speeding up’ the clock of some other neurons (i.e. not the ‘morning cells’). Additionally, increasing the excitability of these neurons under constant conditions makes the behavioural outcome worse (i.e. a higher proportion of flies are arrhythmic) than increasing the excitability of the ‘pacemaker neurons’. These results do not fit the expectations of the dual-oscillator model, suggesting instead a functional switch in the role of neurons according to experimental conditions.

**From ‘clock network’ to ‘internal state networks’**

In recent years, *Drosophila* researchers have described several neuronal circuits related to internal states. For instance, some circuits provide an internal representation of homeostatic needs such as sleep, hunger, thirst and sex drive. Others reflect social experiences, such as assuming a dominant/subordinate position after winning/losing several aggressive encounters or after multiple acceptance/rejection events following courtship. Some others depend on health/sickness status.

Internal states modulate how animals perceive and interpret the environment and motivate the selection of appropriate actions. Superficially, those circuits appear anatomically invariant, but they are functionally plastic. Some neurons respond to internal needs and changes in environmental variables by releasing neuromodulators. These molecules modify circuits by varying the excitability of neurons and/or the strength of synaptic connections, resulting in the physiological flexibility that is required for behavioural variability.

Internal state circuits interact with each other and sometimes even physically overlap. Yet, they regularly compete to take control of the overt behaviour. For instance, hungry flies search for food even at times when they should be asleep. Conversely, well-fed but sleep-deprived flies sleep at times when they should be alert.

Under this perspective, the concept of a distinct and almost ‘self-contained’ circadian network seems restrictive, and new questions are beginning to emerge. Can we physically separate the circadian network from other networks defining internal state? Is the presence of the molecular clock (i.e. the components of the negative feedback loop) necessary and/or sufficient to qualify a neuron as part of the clock network? Are there any conditions under which non-clock neurons (those that do not express clock genes) could take control of the circadian rhythmicity of the animal?

We suggest that unravelling how ‘internal state networks’ interact under ‘good’ or ‘bad’ timing (i.e. under conditions of harmony or disharmony between the inner clock and the external environment) is the new ‘frontier’ of circadian research. The main challenge will be to abandon a comfortable experimental and theoretical framework that considers the clock a highly stereotyped network built by ‘clock neurons’, and instead to embrace the unknown.
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Further reading
