Department of Nuclear Physics, Research School of Physical Sciences and Engineering, The Australian National University, Canberra, ACT 0200, Australia.
e-mails: david.hinde@anu.edu.au
mahananda.dasgupta@anu.edu.au

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Circadian rhythms

Sunrise and sunset in fly brains

William J. Schwartz

Fruitflies can time their morning and evening activities to the day–night cycle. The basic circadian oscillatory mechanism is intracellular, but networks of cells, now being identified, are what make a working clock.

Animals have an internal timekeeping mechanism that precisely regulates 24-hour (circadian) rhythms of body function and behaviour, and synchronizes them to the day–night cycle. A constellation of 'clock' genes lies at the core of this timepiece, and these genes interact in complex intracellular feedback loops to produce oscillations in their own expression[1]. But how are such molecular cycles translated into the adaptable temporal programmes that are characteristic of whole organisms? In fruitflies (*Drosophila melanogaster*), for example, how does a daily intracellular molecular oscillation drive a rhythm of rest and activity that is overtly bimodal, with pronounced bouts of activity around morning and evening that can anticipate the times of lights-on and lights-off? On pages 862 and 869 of this issue, Stoleru *et al.*[2] and Grima *et al.*[3] show that such behaviour arises at an intercellular (tissue) level of organization, with discrete sets of clock-gene-expressing brain cells being differentially involved in the response to dawn and dusk.

One well-known clock gene in fruitflies is *period* (*per*), which, in the brains of adult flies, is expressed in photoreceptor cells, glial (non-neuronal) cells and in a few clusters of neurons[4]. These neuron clusters lie in specific areas of the brain: there are three groups of dorsal neurons (DN_1, DN_2 and DN_3), and two groups of lateral neurons, on each side of the brain (Fig. 1). One group of five to eight lateral neurons lies towards the top of the brain (that is, dorsolaterally; these are called LN_d neurons). The other group, the LN_v neurons, lies towards the bottom of the brain (ventrolaterally); it includes four to six large cells and five small cells. The LN_v neurons (except for one of the five small cells) express the neurotransmitter molecule known as pigment-dispersing factor (PDF), whereas none of the LN_d or dorsal neurons does.

Attention has focused on the LN_v neurons as the essential circadian 'pacemaker' cells that set fly activity rhythms, especially so because removing them leads to defective behavioural rhythmicity in constant darkness[5,6]. But data from work on flies lacking PDF and on other mutants, as well as studies in which flies were engineered to express neuronal genes that block electrical activity or synaptic transmission, suggest that a multi-neuronal network is also somehow involved[7–11].

Stoleru *et al.*[2] and Grima *et al.*[3] sought to dissect this network, using mutant flies and clever genetic crosses to target specific genes to specific cells. Thus, Stoleru *et al.* succeeded in delivering a cell-death gene to the LN_v or LN_d neurons in flies, killing these cells. Tests of the flies' cycles of rest and activity showed that the insects' behaviour was still rhythmic in a light–dark cycle — but the rhythms were different. The LN_v-lacking flies anticipated only lights-off in the evening, not lights-on in the morning. Meanwhile, the flies nominally lacking LN_d anticipated lights-on but not lights-off. In constant darkness, the strains showed unimodal evening- or morning-phased rhythms, respectively (although rhythmicity could not be sustained in the LN_v-less flies).

Grima *et al.* used a different, non-ablative strategy. They started with flies that were deficient in *per* and therefore arrhythmic, and then forced the re-expression of *per* only in the LN_v neurons, or in both the LN_v and LN_d neurons. LN_v-restricted *per* expression rescued behavioural rhythmicity, but only lights-on was anticipated (actually, expression in the small LN_v cells seemed to be sufficient for this, even in constant darkness). Lights-off was also anticipated when *per* was also expressed in about half of the LN_d cells. Thus, two independent strategies have led to the same conclusion: morning and evening bouts of activity are differentially controlled by the LN_v and LN_d cells, respectively.

There was in fact already evidence that morning and evening bouts of locomotor activity in flies are each governed by their own circadian oscillators[12], but the neural mechanisms involved were unknown. In 1976 it was proposed[13] that circadian rhythms in rodents are generated by a complex pacemaker consisting of two mutually coupled circadian oscillators. These consist of a morning oscillator (*M*) accelerated by light and synchronized to dawn, and an evening oscillator (*E*) decelerated by light and synchronized to dusk. The circadian pacemaker that regulates rodent locomotor rhythmicity — the suprachiasmatic nucleus in the hypothalamus of the brain — contains a mixed neuronal population[14], and there is some evidence that it is composed of two oscillating *M* and *E* components[15]. The neural elements

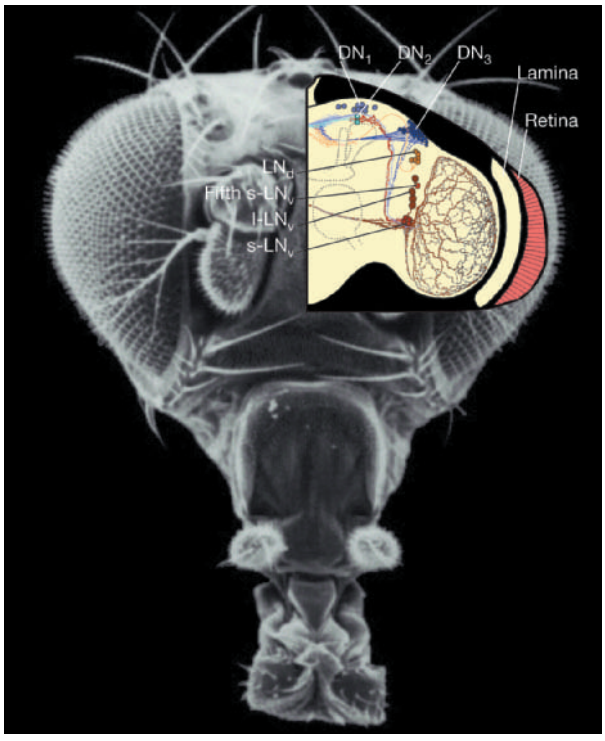

Figure 1 **Clock-gene-expressing neurons and their outputs in the fruitfly brain. These nerve cells include, on each side of the brain, three groups of dorsal neurons (DN_1, DN_2 and DN_3) and two groups of lateral neurons, one dorsolateral (LN_d) and the other ventrolateral (LN_v: either large, l, or small, s). The fifth small LN_v cell is different from the other LN_v neurons in that it does not express the neurotransmitter molecule pigment-dispersing factor. The new papers[2,3] show that the LN_v and LN_d cells differentially control morning and evening bouts of locomotor activity, respectively. (Diagram courtesy of C. Helfrich-Förster, Univ. Regensburg, Germany.)**

involved are unknown, but the LN_v and LN_d neurons are now plausible candidates for two analogous oscillators that control the morning and evening bouts of activity in flies.

With the flies engineered by Stoleru, Grima and their colleagues, clock researchers are in a position to rigorously test dual-oscillator hypotheses. For example, the phase shifts shown by the insects in response to differing light pulses can be analysed, as can their period changes in constant light of varying intensity, and their response to altered day length (photoperiod). There should be much to learn about the network interactions of clock genes, cells and outputs. For instance, the molecular oscillations within LN_v and LN_d cells seem to exhibit a similar phase despite the cells' differing roles, and it seems likely that additional neuronal groups, such as the DN cells, are also involved[16]. Furthermore, a network of interconnected neurons could generate an oscillatory output without requiring every neuron to be independently rhythmic. Indeed, Stoleru and colleagues[2] found that driving *per* re-expression in *per*-deficient flies in all putative clock neurons except the PDF-expressing LN_v cells (that is, in DN, LN_d and the fifth small LN_v cells) could still restore the anticipation of both lights-on and lights-off.

Of course, flies can do much more than merely walk about in a tube, and activities such as olfaction and reproduction (in individuals), as well as hatching (in populations), also show circadian rhythmicity[1]. Other studies have found that peripheral tissues that display molecular oscillations are part of the underlying circadian circuitry, and that their organization is complex, with some tissues (such as the prothoracic gland in hatching[17]) depending on LN_v input, and others (such as the antennae in olfaction[18]) being apparently autonomous. A truly integrative circadian biology is close at hand, as researchers learn about an adaptable, layered system that has emergent properties at many levels of organization. *Drosophila* workers, who have been so effective at taking the clock apart, are now succeeding in putting it back together. ■

William J. Schwartz is in the Department of Neurology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA.
e-mail: william.schwartz@umassmed.edu

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Neurobiology

Accessing a transporter structure

Michael P. Kavanaugh

Information processing in the brain requires the neurotransmitter glutamate. Hence the importance of today's publication of the structure of an archaeal relative of the transporter controlling glutamate's levels.

In the brain, neurons communicate with each other primarily through the use of neurotransmitters. These chemical signals are released by presynaptic neurons in response to electrical impulses, then detected and converted back into electrical signals by receiving (postsynaptic) neurons. For this process to allow recurrent and selective signalling, the neurotransmitter must be efficiently removed soon after release[1]. On page 811 of this issue, Yernool and colleagues[2] describe the structure of an ancestral counterpart of the membrane transporter that is responsible for clearing glutamate — the predominant excitatory neurotransmitter in the mammalian central nervous system. This structure is the first to be obtained for a neurotransmitter-transporter-related protein. It is also the first amino-acid-transporter structure to be solved.

In a sense, this work marks the end of a chapter that began decades ago, with the discovery of a transport system that uses the gradients of ions across the plasma membranes of brain cells to concentrate glutamate within them[3–5]. The molecular species responsible for this activity were subsequently identified as members of a mammalian family of five genes that encode glutamate transporters[6]. Now, with the structure and model presented by Yernool *et al.*[2], we receive some tantalizing new clues to how these proteins work — and so this publication also marks the beginning of a new chapter in the study of their structure and mechanism.

The glutamate-transporter family had previously been recognized as being distinct from the larger family of transporters for other neurotransmitters, such as dopamine, noradrenaline, serotonin and γ-aminobutyric acid. Proteins in the latter family, and in the very large 'major facilitator' superfamily of transporters (MFS)[7], each have a topology that consists of 12 membrane-spanning α-helical domains (transmembrane domains, TMDs), connected by intracellular and extracellular loops. But the topology of glutamate transporters has been more controversial, complex, and difficult to elucidate. Although they seemed to have eight TMDs that behave as membrane-spanning α-helices, biochemical analyses[8] suggested that the carboxy-terminal region also contains two hairpin loops — located between TMDs 6 and 7 and between TMDs 7 and 8 — that partly re-enter the membrane from opposite sides (Fig. 1a).

This carboxy-terminal region — including the 're-entrant' loops and TMDs 7 and 8 — has been a focus of study because it contains interesting functional determinants, including sites that interact with glutamate and competitive analogues, and with key ions such as sodium and potassium (reviewed in ref. 6). A stoichiometric coupling mechanism, in which three Na^+ ions

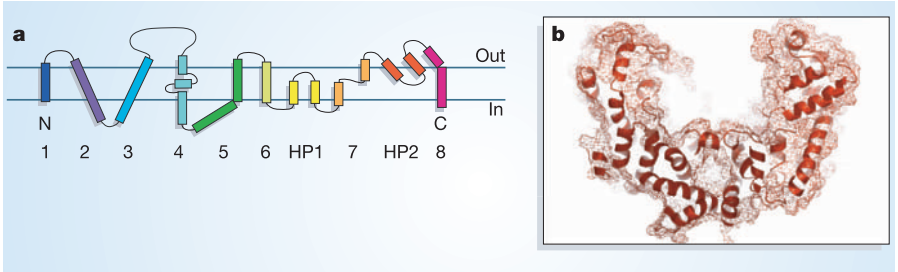

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Figure 1 **Glutamate transporters in two and three dimensions.** a, **Schematic topology of the archaeal glutamate-transporter-related protein Glt_{Ph} (ref. 2) — showing the transmembrane domains (1–8) and the two 're-entrant' hairpin loops (HP1 and HP2). N, amino terminus; C, carboxy terminus.** b, **Side view of a Glt_{Ph} trimer in the membrane plane. One of the three Glt_{Ph} subunits is removed in this view to reveal a bowl-like structure.**