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REVIEW

# Molecular genetics of the fruit-fly circadian clock

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**The circadian clock percolates through every aspect of behaviour and physiology, and has wide implications for human and animal health. The molecular basis of the *Drosophila* circadian clock provides a model system that has remarkable similarities to that of mammals. The various cardinal clock molecules in the fly are outlined, and compared to those of their actual and 'functional' homologues in the mammal. We also focus on the evolutionary tinkering of these clock genes and compare and contrast the neuronal basis for behavioural rhythms between the two phyla.**
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## Introduction: clocks and disease

The number of reviews written on biological rhythms in the past 15 years has been enormous, particularly those on the molecular aspects. So, why are we writing another one on *Drosophila,* and why for a readership of human/medical geneticists who must care little or nothing for such a subject or such an organism? After all, 24 h circadian rhythms, on which most 'chronobiologists' focus, have little or nothing to do with human disease, and *Drosophila* are of no interest to such an audience anyway given their vast phylogenetic distance from mammals. The answer to the question is two-fold. One is that the editors used their arsenal of weapons, flattery and charm, to convince the reluctant senior (reflecting age) author (CPK) to do this piece. The second reason is more interesting, in that we believe that medical geneticists *should* know about circadian rhythms and their links to disease, and *should* know that *Drosophila* has provided the model organism on which almost all the major clock genes were first identified. Within this latter sentence lies the implicit understanding that the genes that control the fly circadian clock are the same ones that determine the corresponding human 24 h cycle.

Is there a relationship between circadian clocks and disease? In Western societies, about 20% of the population, perhaps more, work in shifts. There are various types of shift-work programmes, but all have the effect of desynchronising the workers internal clock to the outside world. The effects of chronic shift-work have been documented in numerous studies, and in terms of general health and life expectancy, there is nothing to commend it. Shift workers suffer elevated levels of almost everything negative that has been investigated, including sleep, gastrointestinal and cardiac problem, even cancer.[1] While some of these investigations have yet to be duplicated, there is clearly an anxiety about the effects of shift-work on health.

However, there are other reasons for human/medical geneticists to be interested in fly clocks. Over the past few years, a number of papers have appeared revealing that flies appeared to sleep in a manner that was similar to mammals.[2–4] This immediately opened up the traditional area of sleep research to mutational analysis, and at least two clock-related transcription factors, CREB and CYC, have been shown to play important roles in sleep homeostasis.[3,5] In addition, another area in which fly clock genes, and a subset of the neurons in which clock genes are expressed, is important, is the behavioural response to cocaine administration.[6,7] Thus, the molecular genetic analysis of the fly's circadian system may play a useful

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role in understanding one clearly clock-related phenotype, and another in which the relevance of clock phenomena is not quite so immediately obvious. Nevertheless, there is one particular human syndrome, where circadian clocks have been directly linked.

## Familial advanced sleep phase syndrome (FASPS)

There are individuals within families that apparently carry a dominant gene that causes them to wake up very early, and go to bed early[8]. This is reminiscent of the classic $per^s$ mutant in *Drosophila,* where flies have a 19 h rhythm[9] and their locomotor activity occurs a few hours earlier than in the wild-type fly, when they are placed in a 12 h light: 12 h dark (LD12:12) schedule. The $per^s$ mutation is a serine-to-asparagine change in that part of the PER protein that interacts with a kinase called Doubletime[10,11] (aka casein kinase 1$\varepsilon$, CK1$\varepsilon$, in mammals). When the *FASPS* gene was identified in an extensive single-family pedigree, imagine the excitement when it was discovered that the mutation mapped to the human *Per2* gene.[8] *Per2* is one of four *Per* genes in mammals, the first three being transcribed and translated, the fourth being a pseudogene.[12,13] The human *Per2* locus is the one that is closest to the fly's single *per*, suggesting it is the ancestral version from which the others duplicated.[12] Imagine the further excitement when the lesion in *hPer2* was discovered to be within a serine codon, which was replaced by glycine in the FASPS individuals, in the CK1$\varepsilon$-interacting region. In other words, the first identified clock mutation in human and fly was in the same gene, in a target site for CK1! However, more recently, a second set of families with FASPS has been discovered. This time, the lesion is not in *hPer2,* but in *CK1*$\delta$,[14] which is also the homologue of fly DBT, as flies only have one version of this kinase gene. Both *per* and *doubletime* (*CK1*$\varepsilon$) were first identified via mutant screens in flies,[9,15] revealing how *Drosophila* can lead the way in human gene discovery, and giving 'fly guys' like us, a further means to justify our grant proposals.

## Clock genes in fly (and mouse): a very brief historical perspective

Flies do not always lead mammals however. Joe Takahashi's group identified the *Clock* mutant in the mouse in a mutagenesis in 1994,[16] and cloned it in 1997.[17,18] A little later, the gene, *Jerk,* was identified in *Drosophila,* again by forward genetics, and the DNA sequence revealed it to be the homologue of mammalian *Clk*.[19] The fly mutant has since been renamed $Clk^{Jrk}$. However, apart from this single case, most of the core clock genes, which are shared between flies and mammals, were identified initially in the fly.

The first, and most famous study was that of Konopka and Benzer,[9] in which a chemical mutagenesis of the fly *X* chromosome identified $per^s$, and two other alleles $per^l$ (29 h rhythm) and $per^0$ (arrhythmic). The initial character for the screen was pupal–adult eclosion of fly populations, taking advantage of the fact that wild-type flies eclose around dawn. This reflects their adaptation to their ancestral homeland of sub-Saharan Africa, where dawn has the most humid conditions for pumping haemolymph into the wings. Eclosing in the dessicating conditions of the afternoon could be disastrous (ie these late eclosing fruit-flies would be 'fruit-walks'). Indeed, *Drosophila* means 'dew-lover' in Greek, so whoever named them was a genius. However, this population circadian assay, which gives bursts of eclosion every 24 h, even in constant conditions, is of little use where the investigator is interested in the genotype of individual flies. For this, Konopka and Benzer moved to locomotor activity assays, as individual flies show circadian patterns of sleep and wakefulness, just as in humans. In retrospect, it can be argued that Konopka and Benzer identified the first dedicated 'behavioural' gene in any higher organism, as without the *per* gene, flies seemed not to suffer serious viability problems, they are simply arrhythmic. However *per* does have pleiotropy associated with it which includes other effects on temporal programmes that are not circadian,[20–22] as well as phenotypes that do not seem to have anything to do with the dimension of time.[23]

DNA around the *per* locus was cloned in mid-1984, but putative *per* sequences were functionally assayed by transformation in December of that year.[24,25] The first behavioural gene identified by forward genetics had been isolated……a big deal in those days. It took 10 more years for the next clock gene to come on board, courtesy of Michael Young's group, and that was *timeless,* an autosomal locus.[26] In 1995, *tim* was cloned,[27] and it was simultaneously discovered that TIM protein could physically associate with PER,[28] via the PER PAS domain, a sequence bearing two imperfect 51-amino-acid repeats separated by a long stretch of >150 residues.[29] In fact, PER is one of the three founder members of the PAS domain family, the other two being transcription factors, ARNT (Aryl hydrocarbon nuclear translocator) and SIM (single-minded). Since the discovery of PAS, a huge family of PAS proteins have been discovered, from bacteria to eukaryotes,[30,31] a subset of which are responsive to light, oxygen and voltage giving rise to PAS LOV proteins.[32] As the ancestral clock proteins must have responded to light cycles, could PAS be the protein module that links extant clock proteins with the PASt? Maybe, but Cryptochrome, the dedicated circadian blue-light photoreceptor in flies (see below), which is related by ancestry to the photolyases, ancient DNA-binding proteins that repair UV-induced damage, may also have played a starring role in the evolution of the clock mechanism below the oceans, where blue light can penetrate.[33]

## Cycling gene products and negative feedback

The products of the *per* and *tim* genes, RNA and proteins, cycle in abundance in the fly's head in constant conditions with a few hours lag between the RNA peak and the protein's.[34–37] As RNA goes up, protein comes down and *vice versa*, suggesting a negative feedback loop. Consistent with this, both PER and TIM proteins are seen at night, but in a limited number of neurons in the brain. When the proteins are at their peak of abundance late at night, the two proteins move into the nuclei of the various subsets of clock neurons,[37–40] where PER (but not TIM) is believed to act as the major negative regulator.[41] In all, there are about 150 of these master clock neurons, and they provide a much simpler model system than the mammalian suprachiasmatic nuclei (SCN), which houses the corresponding neuronal clock and contains about 20 000 clock cells.[42]

The PER and TIM proteins, therefore, wax and wane in a circadian cycle, and once inside the nucleus, they interact with two bHLH PAS proteins, CLOCK, which we mentioned before, and CYCLE, both defined by mutagenesis.[19,43] CYCLE is the homologue of BMAL1 in mammals, and both play similar roles in the clock. The CLK-CYC dimer activates transcription by binding to *CACGTG* E-boxes in the *per* and *tim* promoter regions.[44] Thus, the CLK-CYC dimer forms the positive limb of the feedback loop and when PER enters the nucleus, it interacts with the dimer (incidentally, not via PER PAS as had been commonly assumed, but via a C-terminal region), and represses transcription.[45] PER eventually degrades via the phosphorylation of DBT[15,46] (although phosphorylation by CK2[47,48] is also important for shifting PER from the cytoplasm to the nucleus), and transcription is reactivated. The delay between *per* mRNA and protein accumulation is also due to DBT.[49] However, DBT protein does not cycle, but the dephosphorylation of PER does, and is mediated by cycling expression of the relevant phosphatase, PP2A.[50] As PER is translated in the cytoplasm in the early night, it is phosphorylated by DBT as a prelude to degradation, in which the product of *slimb* (*supernumery limbs*), a ubiquitin ligase, becomes critical.[51,52] It is only with the build-up of TIM, itself phosphorylated by the product of *shaggy,* aka *GSK3*,[53] that this premature degradation of PER ceases, and PER stabilises, and moves into the nucleus late at night with the help of TIM.[54] PER and TIM mutually stabilise and cooperate to ensure each other's movement and retention within the different cellular compartments.[55] However, they do not appear to be obligatory partners in *vivo* for nuclear localisation,[56] as initially believed based on *ex in vivo* experiments with cell lines.[57]

This feedback loop of two positive, two negative and a number of modulatory components (DBT, SGG, SLIMB) is coupled to a second feedback loop via CLK. CLK, unlike CYC, oscillates in abundance.[58] CLK/CYC activates the transcription of two genes *vrille*, and *Pdp1ε* (*Par domain protein 1ε*) which encode transcription factors, with VRI (negative) and PDP1ε (positive) acting in opposite ways (see Figure 1) to regulate the transcription of *Clk*.[59] Coupling two loops in this way enhances the stability of both cycles, and it remains to be seen whether there are further feedback loops entrenched within this basic system.

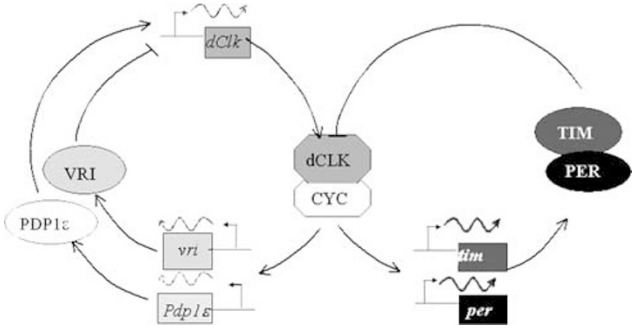


**Figure 1** Two interlocked feedback loops in the *Drosophila* clock. The *per* and *tim* genes are negative regulators of their own transcription via the CLK/CYC transcription factors. The *Clk* gene is interlocked with the *per/tim* loop via the positive and negative actions of the transcription factors PDP1ε and VRI, respectively.

## How the clock responds to light

The circadian feedback loops must be responsive to the external environment, particularly light and heat. One way that this is achieved for light is through the canonical visual pathway mediated by the rhodopsins. The other way is through the *cryptochrome* (*cry*) gene that encodes a blue-light-sensitive molecule mentioned earlier. CRY is activated by light, and binds to both TIM and PER[60,61] in transient interactions that are difficult to capture *in vivo*.[62] A consequence of the activation of CRY is that it precedes light-induced TIM ubiquitination and degradation.[63] A nearly null mutant, $cry^b$, does not show any alteration in free-running circadian rhythms under DD, but does have drastically reduced circadian phase responses to short light pulses,[64] revealing its function for light signalling to the clock.[65,66] However, with long light pulses, as in LD12:12 cycles, $cry^b$ flies entrain relatively normally, reflecting the underlying function of the rhodopsins.[64] The light response of TIM provides the basis for how a clock entrains to different light regimens.[67,68] Consider that early at night, when *tim* mRNA levels are high, but TIM levels low, the reduction of TIM levels by a light pulse (via CRY signalling) takes the clock backwards a few hours to a phase that had less TIM.[37,69,70] However, the levels of *tim* mRNA restore TIM within a few hours, effectively generating a time delay for the clock. The same light stimulus late at night reduces the high levels of TIM to the level that they will be in a few hours time, that is, low TIM, but as there is little *tim* mRNA, the move to a future time (with less TIM) is permanent, giving a phase advance.[37,69,70] This compelling

(but perhaps superficially understood mechanism), explains beautifully the circadian light phase response, where light pulses early at night give delays and those late at night give advances, and is a universal feature of circadian clocks. The delay between *tim* mRNA and protein provides the pivotal feature for explaining this mechanism.

### Central and peripheral clocks in flies

So far, we have dealt only with clock gene expression in the brain, which controls the rhythmic behavioural output that we observe. However, autonomous light-sensitive clocks are almost everywhere in the fly, as seen by fusing *luciferase* sequences to the *per* promoter region. In these beautiful experiments, the fruitfly becomes a cycling firefly, with rhythms of luciferase observed in isolated legs, eyes, proboscis, wings, antennae, etc.[71] Thus, compared to mammals, there are autonomous, independent light-regulated clocks in the fly. In the mouse, for example, cycling in peripheral tissues depends on the master clock, the SCN, so that in the absence of SCN signalling, peripheral clocks become desynchronised and rapidly dampen.[72,73] However, this traditional idea of the SCN being the master oscillator may need to be amended because recent reporter-clock protein fusions reveal that peripheral tissues can maintain robust rhythms in the absence of the SCN, although synchrony between tissues and between animals may be lost.[74]

In the fly, in at least two of these peripheral clocks, the antennae and the eyes, the $cry^b$ mutation appears to stop the clock,[64,75] whereas it has little effect on free-running circadian locomotor behaviour, which is generated from the clock neurons mentioned above. Thus, CRY is a flexible molecule that plays a light-sensing role that can modulate the central 'behavioural' clock, but does not disrupt it when mutated, whereas in the periphery, it appears to encode a more fundamental clock feature. Interestingly, in mammals, CRY is the major negative regulator of the circadian clock, playing very much the PER/TIM role, so the central clock of mammals is functionally similar to the peripheral clock of flies. This evolutionary tinkering of the role of CRY between mammals and flies is also seen in the fact that CLK cycles in flies,[58] whereas CYC does not, but this role is reversed for mammalian CLK and BMAL1.[76] In addition, the coupling of the PER/TIM and CLK feedback loops mentioned earlier, via VRI and PDP1$\varepsilon$, is also observed in mammals, in which BMAL1 cycling is determined by the balance between Rev-erb$\alpha$ and RORa.[77,78]

## Further evolutionary tinkering: gene duplication and divergence

From the evolutionary point of view, one of the striking differences between mammalian and fly clocks is that vertebrates seem to employ multiple paralogous clock genes, while the *Drosophila* clock usually does not involve duplicated genes.[79,80] For example, the mammalian clock has at least two *cry* genes or paralogues (the zebra fish has six), three functional paralogues of *per*, two of *Clock* and two of *Bmal1*.[80] In contrast, in *Drosophila*, all these genes exist in one copy only. This is apparently a general feature resulting from the genome expansion (duplication) during vertebrate evolution. Once gene duplication occurs, the selective constraints on the new copy are reduced, allowing the new copy (or both) to evolve and to acquire a new function resulting in *functional divergence*.[81] For example, the three functional mouse *Per* paralogues appear to play different roles.[82–84] A recent study suggested that mPER1 is important for prolonged seasonal light adaptation,[85] while mPER2 was previously shown to be important in generating the daily activity cycles.[84]

In *Drosophila*, only one *per* gene exists, but this does not apply to all insects because in the silk moth, *Antheraea pernyi*, two paralogues are present *perZ* and *perW* on the different sex chromosomes.[86] The rhythmicity of the males, which are the homogametic sex (chromosomes ZZ), suggests that *perW* is not crucial for circadian function. Many additional degenerate *per* sequences exist on the female *W* chromosome including one that generates an intriguing antisense transcript.[86]

In one case, however, a *Drosophila* circadian clock gene, *timeless*, does have a paralogue called *timeout* (aka *tim2*). Although it is not yet clear whether *tim2* has a circadian function in the fly, this sequence is more closely related to the single mammalian *tim* sequence, than is fly *tim*.[87,88] Thus, *tim2* must be the ancestral sequence, which in diptera and lepidoptera, at least, has relatively recently duplicated to generate the clock-relevant *tim*. A role for *mTim* in the mammalian clock has been proposed from the work of Barnes *et al*[89] and Ünsal-Kaçmaz *et al*,[90] the latter suggesting that the role of mammalian TIM is to couple the circadian clock to the cell cycle. This relates well with the observation that *tim* has sequence similarity to cell-cycle-related proteins, for example, the yeast Sw1 (Switching deficient)[91] and Tof1 (Topoisomerase 1-interacting factor),[92] which are involved in DNA damage activation checkpoints. In addition, TIM1 from *Caenorhabditis elegans* is important for chromosome cohesion.[93] However, any role of mTIM in the mammalian clock would appear to be very different from the light-sensitive negative regulator function of fly TIM.

TIM proteins are intriguing, in that they are pioneer proteins and do not look much like anything else in the databases. By using various conformational algorithms, a number of papers have suggested that TIM may contain ARM domains,[93–96] all $\alpha$-helical repeat motifs found in proteins such as fly ARMADILLO (aka $\beta$-catenin in mammals). While the bases for these types of bioinformatic analyses have been hotly disputed,[97] ARMADILLO/$\beta$-catenin is the focal point for the *wingless* (or *Wnt)*

signalling pathway, which converges on the ARM/$\beta$-catenin transcription factor to regulate its stability and subsequent nuclear accumulation/retention. If TIM is an ARM-like protein, could this explain why so many of the wingless pathway members have been recruited into the clock mechanism, DBT, SGG, SLIMB, CK1$\varepsilon$, CK2, PP2A (see above)?

## Role of splicing in the fly clock

Splicing may provide another route to achieve fine-tuning of a system, and may serve a similar function as gene duplication, with the different splice isoforms undergoing functional divergence. Kopelman *et al*[98] found that gene duplication and splicing are inversely correlated in the human and mouse genomes, with large gene families showing little alternative splicing, whereas single-copy genes with no paralogues had high levels of alternative splicing. In *Drosophila*, the *per* 3′UTR undergoes alternative splicing, which is regulated by temperature and light, and determines seasonally adaptive changes in the timing of the fly's locomotor activity.[99–101] Intriguingly, it is the act of alternative splicing *per se* that is important, not the informational content of the two *per* 3′ splice forms that is important. At colder temperatures, splicing stimulates the earlier upswing of *per* mRNA, leading to earlier behavioural activity.[101] The implications of this is that at hotter temperatures, the fly's main burst of activity moves to later, cooler parts of the day, thereby avoiding the 'fruit-walk' dessication scenario outlined earlier. Analysis of the fly genome (www.flybase.org) suggests that other clock genes have multiple splicing isoforms, for example, *vri* has two isoforms, *tim* has five and *Clk* has six. We feel a PhD project or two beckoning us here!

## The 'neuro' revolution in chronobiology: anatomy of the clock

In the last few years, rather than grinding up the fly head and running Western or Northern blots, workers have started to dissect the contributions of the different clock neurons to circadian behavioural output. Figure 2 shows these cells, which constitute the central clock. There are six main clusters that stain strongly with PER and TIM antibodies. These are divided in three groups of lateral neurons (LNs) and three groups of dorsal neurons (DNs).[39,40,102–105] The LNs are further subdivided into dorsal ($LN_d$ – one cluster of $\sim$6 cells) and ventral, the latter further subdivided into four large (l-$LN_v$) and four small (s-$LN_v$) neurons. These $LN_v$ neurons are usually missing in *disconnected* mutants, which are behaviourally arrhythmic.[40,104] Furthermore, these $LN_v$ cells also produce the neuropeptide PDF,[102,105] shown to be essential not for rhythmicity *per se,* but as a synchronising signal among

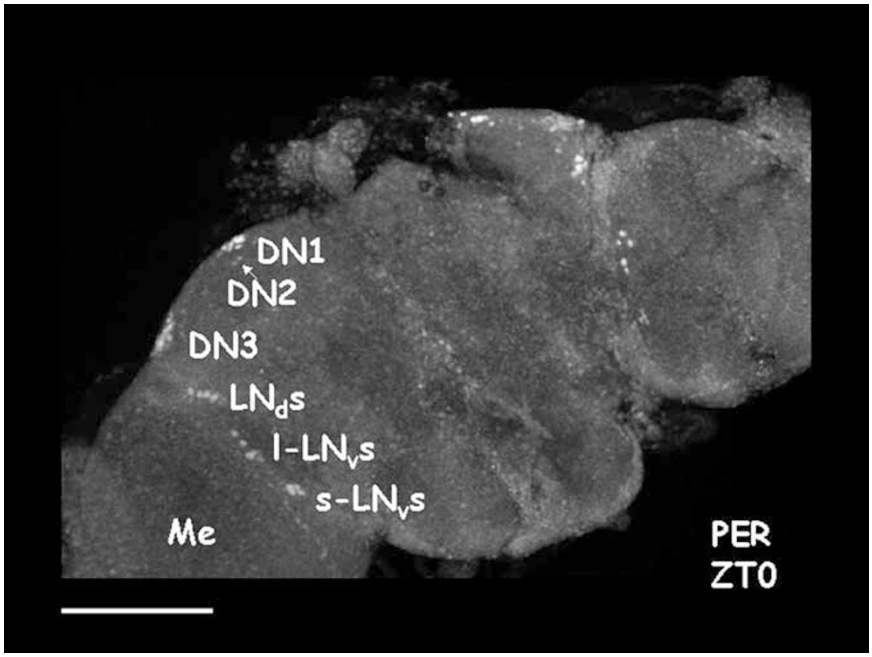


**Figure 2** Central clock cells. Confocal projection of optical slices (*z*-axis) from a *Drosophila melanogaster* brain sampled at the end of the night (ZT 0) and stained with anti-Per antibody. Anti-PER antibody[135] was used at a dilution of 1:15 000. Goat anti-rabbit IgG-Cy3 (Jackson, 1:200) was used for detection. The size bar is represents 100 $\mu$m. The major clock clusters are all visible. l-$LN_v$s = large lateral neurons ventral; s-$LN_v$s = small lateral neurons ventral; $LN_d$ = dorsal lateral neurons; DN1 = dorsal neurons 1; DN2 = dorsal neurons 2; DN3 = dorsal neurons 3; and Me = medulla.

different classes of clock neurons.[106–108] The DNs, which are located more posteriorly, are subdivided into DN1 (~15), DN2 (2) and DN3 (~40 cells in a very dorsolateral position).[109–111]

## The wiring of the circadian network

Evidence that clock neurons are not equivalent and do not act in isolation lies initially in the architecture of their neuronal projections.[109–111] With the exception of the l-$LN_v$s, most of the clock neurons project to a wide area of the dorsal brain where we find most of the fly neurosecretory cells, as well as integration and associative regions for motor behaviour.[110] However, the main circadian centre is probably the accessory medulla, a mass of neuronal processes derived from the larval optic lobe.[112,113] The s-$LN_v$s lay at the edge of the accessory medulla from where they extend their dendritic arborisation.[112] Further input to the accessory medulla comes from the Hofbauer–Buchner eyelet, an extraretinal photoreceptor derived from the visual (Bolwig's organ) system of the larval,[112] and, via the l-$LN_v$s, from the eyes.[110] The s-$LN_v$s project to an area of the brain in very close proximity to the DN1s and DN2s,[110,111] possibly communicating with these cells via PDF and/or some other neurotransmitter. Furthermore, in $Pdf^0$ flies maintained under DD, PER cycling becomes accelerated and dampened in the $LN_d$s, suggesting that the s-$LN_v$s use PDF to entrain the $LN_d$s in LD conditions.[108]

## Functional differences in circadian cells

The wiring of the clock cells suggests that the s-$LN_v$s are probably the most important cluster in the circadian network. It follows that the physical or functional ablation of the s-$LN_v$s should result in dramatic behavioural effects at least in terms of locomotor activity. Mutant $Pdf^0$ flies, although rhythmic under LD, lack the morning locomotor activity peak, whereas the evening peak is anticipated by about 1 h.[106] In DD, the mutant flies remain rhythmic for about 2–3 days, suggesting that this is the synchronisation among clock cells, rather than core clock properties that are affected.[106] The physical ablation of the $LN_v$s by overexpression of apoptotic genes has basically the same effect, suggesting that it is via PDF alone that these cells are necessary for sustained rhythmicity in DD and for the morning peak in LD.[106] PDF is packed in dense core vesicles localised in varicosities that lack synaptic specialisation, suggesting a nonsynaptic paracrine release of the peptide.[114] In agreement with these data, suppression of synaptobrevin-mediated synaptic transmission does not result in a rhythmic phenotype.[115]

At this point, one should mention that the mammalian circadian system also has a neuropeptidergic axis that shows similar features to the fly's PDF. Vasoactive intestinal peptide (VIP), and pituitary adenylate cyclase-activating peptide (PACAP), may be important in the light entrainment pathway of the mammalian clock.[116–118] VIP is synthesised in retinally innervated, ventrolateral neurons of the SCN,[119] whereas PACAP is made in the melanopsin-containing photoreceptor cells of the retina that also innervate the SCN.[120] Both peptides use the G-protein-coupled $VPAC_2$ receptor, and receptor knockout mutant mice show various types of circadian behavioural and molecular dysfunction.[121] Subsequent analyses reveal that neurons in the SCN may retain cycling clock gene expression, but the absence of the $VPAC_2$ receptor means that they are desynchronised with respect to each other.[122] Thus, VIP may, as with PDF in the LNs of the fly, represent a paracrine signal that maintains temporal order between SCN neurons.

Recently, using a variety of techniques that allow the cell-specific manipulation of clock genes, or the ablation of subsets of clock neurons, two groups have shown that the $LN_v$s (actually the s-$LN_v$s) are necessary and sufficient for sustained rhythmicity in DD.[123,124] However, only the morning, not the evening activity component is rescued, both under LD and DD by the s-$LN_v$s. Extending the expression of *per* also to the $LN_d$s, additionally rescues the evening peak,[124] and suggests that the s-$LN_v$ and the $LN_d$s, respectively, govern the morning and evening bouts of locomotion.[123,124] This is an important result as it shows that different parts of the neuronal circadian network control different aspects of the circadian programme as in mammals,[125,126] and suggests an anatomical substrate to the morning (M) and evening (E) oscillator hypothesis first proposed nearly 30 years ago.[127,128]

## Future directions

We have already discussed PDF as an 'output' molecule of the clock that serves to synchronise the various clock neurons. However, there are hundreds of clock gene outputs that serve to generate the behavioural and physiological rhythms that can be observed in flies, and these have been detected using microarray approaches.[129–133] Of course, we do not know if mRNA cycling is accompanied by protein cycling, and future studies will inevitably add the proteomic dimension to the transcriptomic. It may be that many genes cycle at the protein level via post-transcriptional mechanisms without having a cycling mRNA. This remains to be seen. However, the systems approach, as such 'omic' programmes are called, have revealed that almost every physiological function has a cycling component in flies and in mammals.[72] Our own feeling is that the molecular and physiological dissection of identified neurons, and the determination of their contributions to rhythmic behaviour and entrainment via light, heat and even social stimuli,[134] will probably provide

the most important growth area for fly clock molecular genetics in the next 5 years.

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