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Sleep: A Neuropeptidergic Wake-Up Call for Flies

Endogenous circadian rhythms exert strong effects on sleep, but the neuronal mechanisms that produce these effects have remained obscure. New work implicates neuropeptidergic signaling in a subset of circadian clock cells in the regulation of sleep late at night.

Christine M. Dubowy[1] and Daniel J. Cavanaugh[2,*]

The two-process model of sleep, which posits that homeostatic and circadian influences interact to determine sleep amount and timing [1], has been influential in providing a theoretical framework with which to understand sleep regulation. In this model, the homeostatic system reflects sleep need, which increases with prior wakefulness and dissipates with sleep. The circadian system contributes oscillatory control, and is usually modeled as driving wakefulness at specific times of day. Studies in humans have provided empirical support for this model by demonstrating that alertness and sleep propensity vary both according to time spent awake and circadian phase. For example, during prolonged sleep deprivation, alertness and cognitive performance exhibit spontaneous improvements in the early morning, likely reflecting an increase in the circadian alerting signal even in the face of increased homeostatic sleep drive [2].

The attractiveness of the two-process model derives from its simplicity in describing a complex physiological process. However, while this model has proven conceptually useful, its molecular and neuroanatomical correlates have remained largely undefined. In mammals, the circadian clock cells of the suprachiasmatic nucleus are anatomically connected to sleep-promoting brain regions such as the ventrolateral preoptic area (VLPO) through a multisynaptic hypothalamic circuit [3]. However, details of the output circuitry are lacking, and the identities of both the molecules that signal sleep need and the molecules released by the circadian system to control sleep timing have remained elusive.

Recently, researchers have turned to *Drosophila* to answer fundamental questions regarding sleep regulation. *Drosophila* exhibit sleep with characteristics similar to that of humans, including increased arousal threshold, homeostatic regulation, and response to drugs such as caffeine, and research in *Drosophila* has yielded important findings regarding the genetic regulation and function of sleep [4]. Consistent with the two-process model, studies in *Drosophila* have assigned a sleep-inhibitory role for circadian clock cell populations [4–6], but as in mammals, little is known about the neuronal circuitry connecting clock cells to the regulation of sleep. In this issue of *Current Biology*, Kunst, *et al.* [7] contribute a significant advance towards understanding this circuitry by identifying a novel wake-promoting peptide in the fly that is released by a population of circadian clock cells to promote wake in a time-of-day specific manner.

In both vertebrates and invertebrates, neuropeptide signaling plays a prominent role in the regulation of sleep and circadian rhythms. The authors, who have a longstanding interest in neuropeptidergic signaling, began this study by investigating DH31, a neuropeptide homologous to mammalian CGRP. The authors found that DH31 loss-of-function mutants exhibit elevated sleep specifically during the last 6 hours of the night. Conversely, pan-neuronal overexpression of DH31 reduces sleep, with the effect again restricted to the same time period.

DH31 is found in multiple neuroanatomic loci throughout the fly brain. To determine which of these loci are relevant for the wake-promoting effects of DH31, the authors turned to a large library of GAL4 lines recently generated by the Rubin laboratory at Janelia Farms [8]. In these lines, spatial expression of the yeast transcription factor GAL4 is controlled by short fragments of genomic DNA that serve as enhancer elements. Unlike older GAL4 libraries, which were generated by enhancer trap methods and were often broadly expressed, the Janelia GAL4 lines are often exquisitely specific. These GAL4 lines can be used to drive expression of transgenes placed downstream of an upstream activating sequence (UAS), making them a powerful tool for manipulating neuronal function and activity.

Using this resource to their advantage, Kunst *et al.* demonstrated that DH31 expression in a specific population of clock cells is responsible for the wake-promoting effects of this peptide. In *Drosophila*, the core clock is made up of a network of interconnected cell groups that are defined based on clock gene expression [9] (Figure 1A). Based on initial experiments in which GAL4 lines generated from DH31 enhancer regions were used to drive DH31 expression, the authors hypothesized that a subset of the DN1 group of clock cells may be the relevant sleep regulatory neurons. This was confirmed with a different GAL4 line, which drives detectable DH31 expression exclusively in the subset of DN1s that endogenously express this peptide. DH31 expression in these cells is sufficient both to decrease sleep in a wild-type

background and to rescue the elevated sleep of DH31 mutants.

Although anatomically distinct, connectivity between groups of clock cells is thought to be important in generating time-of-day specific behaviors. DN1s are known targets of another wake-promoting group of clock cells, the LNvs, which express the neuropeptide PDF [10–12]. Loss of PDF leads to increased sleep [6], consistent with an arousal-promoting function. This effect is likely due to release of PDF from l-LNvs, which have been shown to promote daytime arousal and are silenced via GABAergic mechanisms at the beginning of night to determine the timing of sleep onset [4–6]. The authors wondered if PDF provides a wake-promoting input to DH31-expressing DN1s to drive wake late at night. To test this, a new GAL4-driven tool was introduced: membrane-tethered PDF, which can elicit a postsynaptic response when expressed in the same cells as its receptor [13]. In support of a role for PDF in this circuit, expression of tethered PDF in DH31-expressing DN1s produces a decrease in late night sleep that is similar to the decrease seen with DH31 overexpression (Figure 1B). LNvs are thus implicated as a presynaptic input to DH31-expressing DN1s.

The authors also employed membrane-tethered DH31 to test which postsynaptic cells are relevant for the wake-promoting effect of this peptide; however, the neurons targeted by DH31 proved harder to identify. Although pan-neuronal expression of the tethered DH31 does result in late night sleep loss, none of the 33 more restricted GAL4 drivers tested elicited the same response. This might not be particularly surprising; sleep regulatory genes have been notoriously difficult to map to specific neuroanatomic loci. Failure to map DH31 to a specific set of postsynaptic neurons may indicate that many different targets are required for its effects.

Curiously, almost all of the manipulations employed result in an effect on sleep that is specific to the late night, whether upstream or downstream of DH31 release. Even when the heat-activated ion channel TrpA1 is used to depolarize DH31-expressing DN1s throughout the day, the sleep-inhibiting effect remains restricted to late night and early morning. The same is true for the

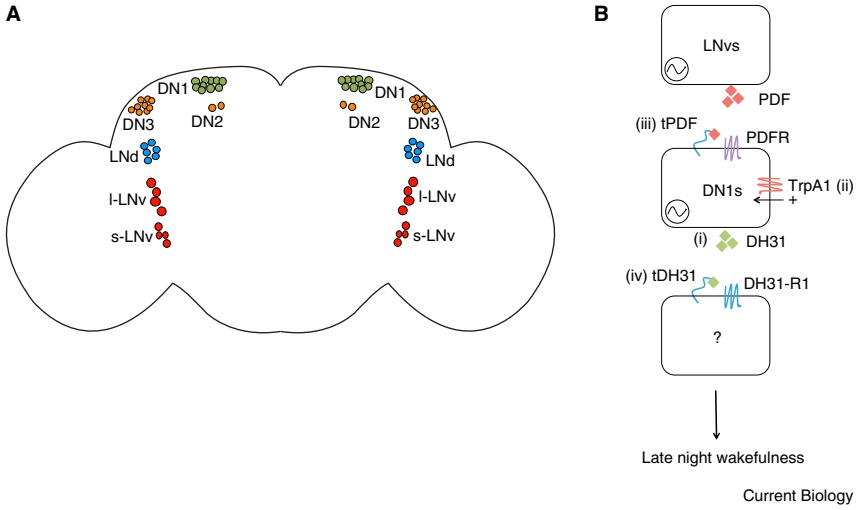

Figure 1. Circadian clock cells and sleep regulation in the *Drosophila* brain.

(A) Distinct, albeit interconnected, groups of cells in the *Drosophila* brain express the molecular components of the circadian clock. These include the large and small ventral lateral neurons (l-LNvs and s-LNvs, respectively, red), the dorsal lateral neurons (LNds, blue), and three groups of dorsal neurons (DN1, green; DN2 and DN3, orange). (B) A schematic of work performed by Kunst *et al.* [7]. The authors propose that DN1s function downstream of PDF-expressing LNvs, and promote wake through release of DH31 onto unidentified DH31-R1 expressing cells. The authors observed decreases in late night sleep following manipulation of several parts of this circuit, including (i) overexpression of DH31, (ii) TrpA1-mediated activation of DH31-expressing DN1s, (iii) expression of tethered PDF peptide in DH31-expressing DN1s, and (iv) pan-neuronal expression of tethered DH31 peptide. tPDF, tethered PDF peptide; PDFR, PDF receptor; tDH31, tethered DH31 peptide; DH31-R1, DH31 receptor.

responses elicited by tethered PDF and tethered DH31. This raises an intriguing question: what is responsible for the time-of-day specificity in this wake-promoting circuit? The data suggest that the neuropeptides PDF and DH31 are only part of the story. Perhaps the effects of DH31 require co-release with an unidentified small-molecule neurotransmitter, the production or release of which is gated by the time of day. Experiments Kunst *et al.* performed with the voltage sensor ArcLight [14] do suggest a difference in basal electrical activity of DH31-expressing DN1s between late night and late day. Alternatively, the time-of-day specificity might be set downstream of the postsynaptic response to DH31, or mechanisms that provide specificity might exist at multiple steps of the pathway to confer robustness to the system.

As with any work in invertebrate model organisms, it is important to ask to what degree these mechanisms are shared with mammals. DH31 is homologous to the mammalian neuropeptide CGRP, a vasodilator with diverse roles including pain sensation and anxiety [15,16]. CGRP has been previously shown to increase locomotor activity in zebrafish [17], suggesting that its wake-promoting function may be conserved between fish and insects. It will be interesting to see if such conservation extends to mammals, a finding that could have important implications for the intersection between sleep disturbances and anxiety disorders. It is also possible that DH31 has a functional analog rather than a genetic homolog, much like *Drosophila* PDF and mammalian VIP. Although they lack sequence similarity, these two neuropeptides play similar roles in synchronizing disparate clock cells.

On a circuit level, the biggest challenge remains identifying the neurons targeted by DH31. It follows from the two-process model that there exist brain structures that integrate homeostatic and circadian cues. The VLPO is a good candidate in mammals, but the evidence to support such a role remains circumstantial. Several fly brain regions (the mushroom body, fan-shaped body, and pars intercerebralis) are known to regulate sleep [4,18]; however, the interconnectivity and inputs to these regions have not been fully resolved. Thus, the targets of DH31, although

elusive in this study, will ultimately lend important insight into the nature of circuits that integrate circadian and homeostatic cues to produce changes in sleep behavior.

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Speciation: Frog Mimics Prefer Their Own

Ranitomeya **poison frogs in the Peruvian Amazon are a rare example of Müllerian mimicry in vertebrates. These frogs also prefer to court same-coloured mimics. This suggests that divergence in mimicry plays a role in reproductive isolation.**

James Mallet

Had they been alive today, Henry Walter Bates and Charles Darwin would have enjoyed the recent finding that natural selection for mimicry in poison frogs (Figure 1) is involved in the origin of species, or speciation [1]. To understand why the new result is interesting today but also would have intrigued early Darwinians requires a little history. Darwin's 'Origin' [2] was long on logic and evidence for evolution, but short on convincing evidence for natural selection [3]. Henry Walter Bates supplied a key example: Batesian mimicry was the best and arguably the first clear case of natural selection [3]. Bates argued that edible butterflies in the Brazilian Amazon mimicked the colour patterns of inedible 'model' species avoided by predators. The patterns of both mimic and model switched every few hundred kilometres or so. The multiple convergences and rapid spatial turnover in mimetic colour schemes argued for natural selection on signalling rather than mere chance or inheritance from a common ancestor [4]. Fritz Müller later showed how mimicry between unpalatable butterflies could be mutualistic: similar-looking species benefit by sharing the costs of educating predators. This leads to a lower *per capita* mortality in each species, as predators need to learn to avoid only one colour pattern in several bad-tasting prey [5]. Mimicry between unpalatable species is today termed 'Müllerian mimicry'.

Neither Bates nor Müller noticed that on the mossy floors of the rainforests they knew so well there were tiny jewel-like dendrobatid frogs playing the same Müllerian games as the butterflies. Dendrobatid frogs are often known as 'poison arrow frogs' or 'poison dart frogs' due to their extreme toxicity. Extracts of some species are used by Amazon peoples on the tips of blowpipe darts to kill prey. When I first visited the Amazon of Eastern Peru in search of contact zones between mimicry races of butterflies, Rainer Schulte, a resident of Tarapoto, astonished me by demonstrating a rare case of Müllerian mimicry in a frog he had just described. His new species, the dendrobatid *Ranitomeya imitator* [6] mimics various other *Ranitomeya* species. Some *Ranitomeya*, according to Schulte, are so toxic that a single whiff can lead to a headache. As in butterflies, mimetic frogs in different places switch colour morphs in concert. In contrast to Bates' butterflies, however, these mimicry switches take place over tens instead of hundreds of kilometres. The narrower spatial scale of dendrobatid colour switching is easily explained: butterflies fly further than frogs hop.

In the new study, Evan Twomey *et al.* [1] found that local mimicry switches by *Ranitomeya* correlate with behaviour. Near Tarapoto, five distinct colour morphs of *R. imitator* are known, each mimicking a different model species in a different location. Two of these *R. imitator* morphs meet in a narrow