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Review

Communication between circadian clusters: The key to a plastic network

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ABSTRACT

Drosophila melanogaster **is a model organism that has been instrumental in understanding the circadian clock at different levels. A range of studies on the anatomical and neurochemical properties of clock neurons in the fly led to a model of interacting neural circuits that control circadian behavior. Here we focus on recent research on the dynamics of the multiple communication pathways between clock neurons, and, particularly, on how the circadian timekeeping system responds to changes in environmental conditions.**

It is increasingly clear that the fly clock employs multiple signalling cues, such as neuropeptides, fast neurotransmitters, and other signalling molecules, in the dynamic interplay between neuronal clusters. These neuronal groups seem to interact in a plastic fashion, e.g., rearranging their hierarchy in response to changing environmental conditions. A picture is emerging supporting that these dynamic mechanisms are in place to provide an optimal balance between flexibility and an extraordinary accuracy.

1. Introduction

Oscillations between day and night conditions are dominant, at times neglected, evolutionary driving forces. To cope with such challenges, life on Earth has developed biochemical timers that run with periods similar to the Earth's rotation, altogether known as circadian clocks. The fruit fly *Drosophila melanogaster* has been instrumental in understanding how these timekeeping systems work at the molecular level [1], and to demonstrate that multiple layers of interconnected cellular mechanisms are recruited by the clock to ensure its function [2,3].

Recent work on the anatomical substrates of the clock has extensively been reviewed elsewhere [4–6]. In short, the fly head contains 150 clock neurons, i.e., neurons that express core clock proteins in an oscillating pattern. They are classically grouped according to their anatomical position and relative size into Dorsal Neurons, subdivided into 3 clusters (DN1, DN2 and DN3), and Lateral Neurons, subdivided into 4 clusters (LN dorsal, large LN ventral, small LN ventral and LN posterior). Further subdivisions stemming from the expression pattern of key proteins within each cluster will be discussed below.

How does a group of neurons in the fly brain orchestrate the animal's activity pattern throughout the day? In principle, presumably identical molecular clocks running at the cellular level are assembled into clusters that could play specific roles. Most of them receive environmental inputs, process information and organize activity of target regions through output pathways. We are beginning to dissect the logic of these properties in the circadian system, and molecular manipulations of different neuronal populations along with clever environmental setups that have been instrumental to this effect will be discussed herein. However, the increasing amount of experimental approaches and genetic manipulations used, together with the inherent complexity of the system, challenges previous interpretation of results, making room for new models of how the circadian system operates at the circuitry level.

In this review we describe the current knowledge on the communication among neuronal clusters. We examine the repertoire of neuropeptides, neurotransmitters and signalling molecules that impact on the clock along with their hypothesized functions. We then describe how differences within distinct clusters, initially considered to be homogenous, define how the network operates. Finally, we highlight how intra-network signals contribute to building an efficient and dynamic multicellular clock that remains plastic to adapt to a changing environment.

2. Communication pathways employed by the circadian network

2.1. Neuropeptides

Both ventral Lateral Neuron clusters express the neuropeptide PIGMENT DISPERSING FACTOR (PDF), the most potent circadian communication signal described in insects. Due to its relevance

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in circadian regulation, PDF functions have been recently revisited [7]. Originally, PDF was described as a non-photic input to the clock contributing to bilateral synchrony [8] and general rhythmicity under constant conditions [9]. Later on, its role as pacemaker coordinator was established [10], as well as its function as a promoter of wakefulness and arousal [11–13]. Thus, PDF serves as an internal signal for the circadian network and also as an output pathway to downstream arousal centers (Fig. 1) [14–17]. A clear evidence of its multiple functions is the distribution of the PDF receptor (PDFR). Within the circadian network all five sLNvs, 2 lLNvs, 3 LNds, both DN2, both DN1a and several DN1ps and DN3s express this receptor (Fig. 1) [7,17–19]. Interestingly, the presence of PDFR in PDF+ cells points to an autocrine role of the neuropeptide, probably ensuring the synchronization of the cluster [10,20]. Beyond the circadian network, PDFR is found in the ellipsoid body (EB) [12], a prominent locomotor center that shows physiological responses to PDF stimulation [21]. In addition, there is anatomical evidence for PDFR expression at the boundary of the lamina and retina, where a role in the modulation of input pathways of clock clusters has been hypothesized [18].

Importantly, the finding that a proportion of *pdf* null mutants or flies with ablated PDF+ neurons retain a rhythmic activity pattern highlights the ability of other circadian molecules and even other neuronal clusters to drive locomotor activity rhythms [9]. In this regard, in addition to PDF, other neuropeptides affect the temporal organization of locomotor activity, although no other signal has such a prominent effect as PDF (Fig. 1).

SMALL NEUROPEPTIDE F (sNPF) is expressed in the PDF+ (immunoreactive) sLNvs and 2 CRYPTOCHROME (CRY+) LNds [22], the latter being strongly coupled to the sLNvs [23]. sNPF has a sleep promoting role [24], probably working as an intra-network communication signal. In addition, sNPF is widely expressed in the nervous system and can work as a hyperpolarizing signal to motor centers [25], conceivably working as an output of the circadian network.

On the other hand, a different molecule named Neuropeptide F (NPF) is expressed in 3 LNds (two CRY− and one CRY+) [22], the 5th-LNv and some lLNvs [26]. NPF receptor has a restricted expression pattern comprised of some DN1 and some LNds [27], supporting the hypothesis that this neuropeptide could work as an additional intra-network communication signal. However, although the function of NPF+ neurons has previously been described as generally relevant for wild type circadian rhythmicity [28,29], its specific role in circadian regulation remains to be established [26,27].

Finally, the fourth neuropeptide used by the network as a communication signal is ION TRANSPORT PEPTIDE (ITP), which is expressed in one LNd that co-expresses NPF, CRY and PDFR, as well as in the 5th sLNv [22] that also express CRY and PDFR. Modulation of ITP levels impacts on the timing of the evening activity peak, and has a subtle period-shortening effect [30]. Thus, ITP might complement PDF activity, which controls the morning peak and lengthens the endogenous period [30]. Unfortunately, the ITP receptor pattern has not been described; therefore, its role as an intra-network pathway or as an output signal has not yet been determined.

2.2. Neurotransmitters

The identification of the classical neurotransmitters employed by the circadian network has proven to be challenging in the fruit fly, despite anatomical and functional evidence of their relevance (Fig. 2). On the one hand, the presence of clear small vesicles together with the PDF-containing dense core vesicles at the dorsal protocerebrum underscores the presence of fast neurotransmitters in the sLNvs projections [31], although their identity has not yet been determined. On the other hand, impairing synaptic transmission through expression of the tetanus toxin light chain in all clock neurons renders flies arrhythmic [32], clearly proving an involvement of fast neurotransmission in the control of overt rhythms.

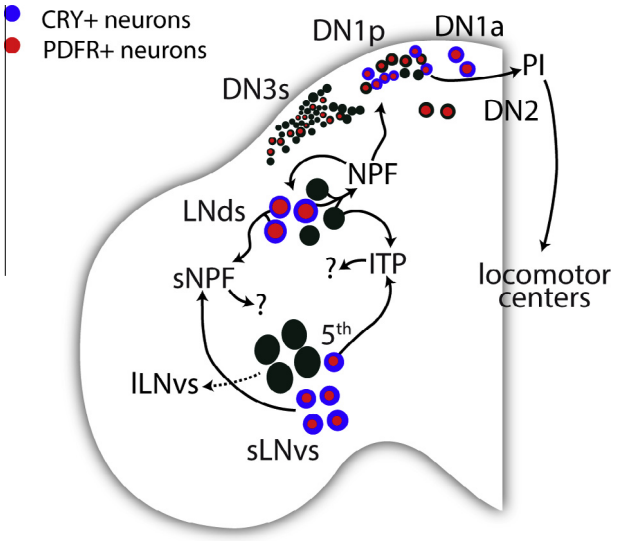

Fig. 1. The peptidergic paths in the fly circadian network. Schematic representation of a single brain hemisphere. The neuronal population that express high levels of the core clock protein PERIOD are depicted. Arrows represent the paths between clusters that communicate through neuropeptides. Four neuropeptides have been associated to the circadian network: PDF, sNPF, NFP and ITP, red arrows highlight PDF's, while black arrows describe the role of the other neuropeptides. While PDF has the most prominent role and well defined targets, those of the remaining signals are still not completely understood. Blue and black cells represent CRY+ and CRY− neurons, respectively. PI: *Pars Intercerebralis*. Note that the LNds represent a diverse group of neurons; in fact, this previously considered homogenous cluster comprises at least four different types of neurons: (1) two CRY+, PDFR+ and sNPF+; (2) one CRY+, PDFR+ and NPF+; (3) one CRY−, PDFR− and NPF+; and (4) one CRY−, PDFR− and ITP+. Interestingly, a group of two LNds employs acetylcholine as neurotransmitter, in addition to the expression of CRY, PDFR and sNPF, exemplifying the complexity of this heterogeneous cluster. So, most of the roles assigned to this cluster will surely be re-interpreted in light of this more complex subdivision.

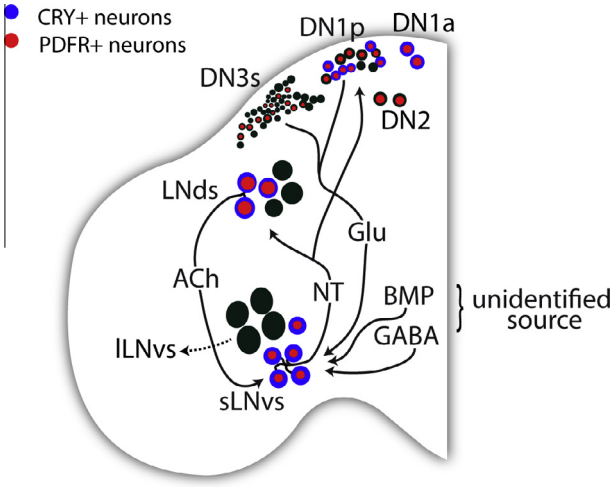

Fig. 2. Fast neurotransmitters and other signalling molecules are instrumental for the circadian network. In addition to the neuropeptides, the circadian system employs several fast neurotransmitters and ligands from the BMP signalling family. The scheme is similar to the one in Fig. 1, but, in addition, PDF target cells (PDFR+) are indicated by red central dots. Although the exact source of BMP ligands is not clear yet, there is indication that, at least in part, it is originated within the network. NT: the unknown neurotransmitter released by PDF+ sLNvs.

Interestingly, the same treatment restricted to PDF+ neurons has a mild effect on locomotor rhythms [32], highlighting that other clock clusters beyond the LNvs exert their function through fast neurotransmission, which is crucial for coordinated activity around the day.

In fact, several fast neurotransmitters have been described to affect circadian organization of activity (Fig. 2) [33]. In particular, the expression of *choline acetyltransferase* (*cha*) in the sNPF+ LNds and in the 5th s-LNv (both also PDFR+) strongly suggests that acetylcholine is employed by these neurons [22]. Interestingly, the sensitivity of the sLNvs to this neurotransmitter [34], together with neuroanatomical and behavioral evidences (see below), supports a bidirectional communication between these clusters.

Glutamate represents another potential signal between clusters. Glutamate may be released by some DN1s and DN3s, which express a vesicular glutamate transporter, DvGluT [35]. Interestingly, LNvs dendrites stain positively for DmGluRA, and knockdown of this receptor has a mild lengthening effect on the endogenous period in constant darkness (DD) suggesting a potential pathway that reinforces rhythmicity [35]. More recent work supports the role of glutamate in the control of wild type rhythmicity, specifically, glutamate released from CRY+ non-LNvs is required for robust locomotor rhythms [36], impacting on the synchronization of the sLNvs via mGluRA at least in the larva [37]. In addition, DN1s receive light information and sustain some degree of rhythmicity under constant light conditions (LL) [38–40]. Thus, it is clear that the glutamatergic system could couple DN1s and sLNvs to provide synchrony to the network.

In terms of classical neurotransmitters, GABA appears to act through $GABA_B$ receptors on the sLNvs for the correct determination of 24 h rhythms [41]; however, the source of this neurotransmitter is yet unclear [34,42], preventing further interpretations of its role in the network.

2.3. Beyond peptides and transmitters

In addition to neuropeptides and fast chemical neurotransmitters a variety of other ligand–receptor couples are employed by neurons to communicate with each other. For instance, two independent groups showed that PDF+ neurons require a functional endocytic pathway to function properly [43,44]. Overexpressing a dominant negative version of the *Drosophila* dynamin *shibire* leads to a clear lengthening phenotype (about 3 h) that does not affects the overall rhythmicity. This phenotype is likely accounted for a deficient regulation of PDFR signalling [43]; however, other membrane receptors and second messenger cascades could concomitantly be affected by this perturbation. In this regard, our laboratory has recently shown that the components of the BMP signalling pathway are expressed in the sLNvs and that activation of the BMP pathway slows down the pace of the clock [45]. Interestingly, its constitutive activation leads to a long period phenotype of 3 h, similar to the one achieved by blocking the endocytic pathway. Moreover, despite the identity of the ligand/s source was not addressed, it is clear that, at least in part, the signal is produced within the network and that at least some members of this ligand family function as intra-network signals [45].

The dorsal terminals of the sLNvs undergo daily remodelling, in which neuronal contacts change along the day, in a clear example of circadian regulation of neuronal plasticity [46–50]. Considering the role of the BMP pathway in retrograde regulation of synaptic strength in central and peripheral nervous system [51,52], it is tempting to consider that remodelling of the sLNv terminals may be in part driven by their postsynaptic target through retrograde BMP signalling. Such dynamic network remodelling could in turn impinge upon the pace of the molecular clock, ultimately affecting the temporal distribution of activity, since this mechanism could provide a potential synchronizer and/or a regulator of the coupling strength among clusters. In sum, the BMP signalling pathway could provide signals from the network to the sLNvs to fine tune the pace of the molecular clock in the sLNvs.

3. Focusing on the clock network

3.1. Complex rhythms as a window to the clock

The mammalian circadian pacemaker residing within the suprachiasmatic nucleus (SCN) is composed of different neuronal clusters with slightly different endogenous periods [53,54]. Synchronization among clusters is ruled by several cues such as neuropeptides, nitric oxide, and synaptic contacts and gap junctions, which ensure the coherence observed at the behavioral level [55]. Under particular environmental circumstances, i.e., surgical interventions or genetic perturbations, circadian outputs display more than one rhythmic component with a defined circadian period for each component, a phenomenon termed "complex rhythms". These rhythms arise as a result of desynchronization within neuronal clusters and are observed in several circadian outputs, i.e. locomotor activity, melatonin release, corticosterone regulation, core body temperature and sleep. Importantly, the analysis of complex rhythms has been fundamental to understanding the interaction of the clock components [56–61]. Moreover, analysis of complex rhythms in locomotor activity has also been crucial to locate and understand the interaction between paired pacemakers in crickets [62–65]. In particular, these seminal reports showed that in insects both bi-lateral pacemakers have their own pathway to control behavior. Interestingly, contralateral pacemakers exert a phase-dependent inhibition of activity, ensuring correct synchronization.

In *Drosophila*, studies on complex rhythms similarly clarified how distinct clusters of the circadian system contribute to and interact to drive rhythmicity. Mutants with altered brain development and/or PDF miss-expression clarified PDF function, and shed light on the function and properties of different circadian clusters, namely, that not all clusters share the same free running period [20,66]. Moreover, examining complex activity patterns that emerged as a result of exposure to constant dim light contributed to understanding the role of the sLNvs as pacemaker neurons [67,68], refining the proposed two-oscillator model [28,29] (see below). Additionally, hyper-excitation of the sLNvs by a constant Na+ current also led to complex locomotor activity profiles. These manipulations sustained a role for PDF and electrical activity as synchronizers of multiple independent neural oscillators [69]. So, in depth analysis of activity profiles have been essential in defining the contribution of individual components to the circadian rhythm.

Entrainment is an emergent property of systems based on interconnected oscillators. Among these properties, a particularly relevant one is how oscillators are coupled. In terms of biological clocks a salient aspect of the interaction between oscillators is how they communicate with each other, which defines the coupling rules among cells or clusters. As an example, it was suggested that in wild type animals maintained in darkness the CRY− LNds are not entrained by the network and free run [20], probably because their endogenous clock is outside the entrainment range of the network, i.e., the pacemaker neurons (namely, the sLNvs) are not able to force the clock of those neurons to entrain because the coupling is not strong enough. Thus, genetic manipulations that introduce period differences between the sLNvs and the rest of the network, and in doing so they challenge the coupling between oscillators and lead to complex locomotor behaviors, allowed to conclude that the ability of the sLNv pacemakers to drive oscillations in DD is limited [23,70,71]; whether sLNvs can equally successfully accelerate or lengthen the pace of the other

oscillators is still an open question. In sum, experimental manipulations of the coupling strength between clusters arises as a promising strategy to uncover the signals involved in network connectivity, and define how these signals act together to shape an efficient and flexible network.

Regarding the relationship between specific clusters, sLNvs drive the oscillation of the CRY+ PDFR+ LNds through a strong coupling mechanism [23,71], probably involving PDF but also a putative fast neurotransmitter [31,48]. On the other hand, the CRY− LNds appear to be more loosely coupled [71,72], or probably only indirectly coupled to the CRY+ LNds [23], and run desynchronized with the rest of the network under constant environmental conditions [20]. A recent model of LNd coupling further splits the PDFR+ LNd cluster into two groups. In this analysis, only two PDFR+ sNPF+ LNds are strongly coupled to the sLNvs, while the remaining PDFR+ LNd (that is also ITP+), together with the 5th sLNv, are more loosely coupled to the sLNvs [23]. However, the nature of the coupling and its modulation remains unclear.

Interestingly, there is another cluster whose molecular clock runs with a slightly different period than the rest of the network. The DN2 neurons show a short period oscillation of core clock proteins [69,72,73], and together with other PDF− and CRY− neurons, they likely drive oscillation when PDF signalling is attenuated, thus balancing the sLNv clock in a wild type context [69,72] or in response to environmental stimuli that impact on the sLNv oscillator *per se*.

Regarding other dorsal clusters, the DN1ps appear to be direct targets of the sLNvs (Fig. 3) [16,48,74], and were also shown to contact a subset of neurons of the neuroendocrine *Pars Intercerebralis* [74]. These neurons appear to integrate light and temperature inputs together with circadian signals, and therefore directly impact on the pattern of rest/activity cycles [38,39]. In sum, it appears that each specific cluster sends and receives information from other pieces of the circadian puzzle and the accuracy of the clock relies on this ability. Thus, the role of the sLNvs as central pacemakers not only depends on their precise cell autonomous molecular clock but also on the ability of the rest of the network to provide feedback when relevant internal or external perturbations take place. In fact, despite the growing body of evidence questioning the role of the sLNvs as necessary and sufficient for sustained rhythmic behavior, more experiments are ensured to precisely define the contribution of each cluster to a fully operational network under natural conditions.

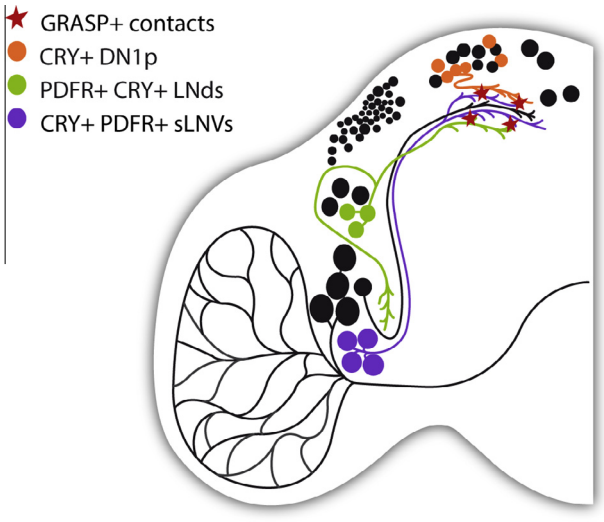

Fig. 3. The potential synaptic contacts between the PDF+ sLNvs and downstream clusters. Most of the communication paths within the circadian network are inferred from the expression of signalling molecules and/or their receptors in the different clusters. Hence, lack of evidence of direct functional connection between clusters is a major issue to model the information flow in the network. Recently, positive GRASP contacts (red stars) between the PDF+ sLNvs (purple) and the CRY+ LNds (green) and CRY+ DN1s (orange) have been described [16,48,74], and constitutes the best available description of the connectivity among circadian clusters.

3.2. The hierarchical organization of the clock network

The seminal work of Konopka and Benzer opened the great adventure of dissecting the genetic control of behavior with the description of three pleiotropic mutations in the same genetic locus [75]. Starting from this elegant and simple work the addition of complexity to the current model of how the circadian clock works has mostly been incremental. Two outstanding papers established that, in the fly brain, there are two separate but mutually coupled clocks devoted to controlling the morning and evening activity [28,29], strengthening the idea of an analogous clock between flies and mammals, but this time at the organizational level of the neuronal network [76,77]. In evident support of this possibility, the clusters were termed morning (M) and evening (E) oscillators. According to this model, the sLNvs were proposed as the M oscillator and the CRY+ 5th LNv, LNds and DN1 as the E oscillator, with the M neurons controlling anticipatory activity in the morning as well as the endogenous period in constant conditions, while the E neurons dominate the anticipatory activity at the end of the day. This model resulted instrumental to interpret existing data and delineate new lines of research, but ten years later a more complex picture on how the circadian network assembles is emerging. Recently, a more complex picture emerged when the central role of the M cells in the control of period in DD was challenged. Manipulating the speed of the PDF− clusters of the circadian network but in a context in which PDF signalling was attenuated (PDFR null mutants) Yao and Shafer showed that overt rhythms could be driven by these PDF− clusters [23], a conclusion also reached by two independent groups [72,78].

Understanding the rules governing the interaction among circadian oscillators is a central goal in the field; and the hypothesis of several independent clocks interacting within circadian networks is conserved across species. With regard to the interaction between M and E oscillators several lines of evidence point to a direct and indirect connectivity between the sLNvs and the LNds, and it is becoming increasingly clear that the communication between theses clusters is bidirectional [23,72,79]. From an anatomical perspective, there is a LNd projection reaching the accessory medulla (aMe) ending nearby the sLNvs somata [29,78] and the sLNv axonal projections pass by the LNd somata [80]. More recently, putative synaptic contacts driving information from the PDF+ sLNvs to the CRY+ LNds were described based on GRASP (Fig. 3) [48]. However, the peptidergic identity of these LNds was not assessed. This anatomical evidence supports the idea that information flows from the sLNvs to the CRY+ and PDFR+ LNds, and the latter group communicates with the CRY− LNds, a concept that also stems from recent empirical and theoretical data [23,71,79].

Functionally, the connectivity between sLNvs and LNds has been proposed several times, however, with slightly different conclusions. Originally it was suggested that PDF had a period lengthening effect on the DNs and LNd cells, and that these cells were responsible for the short period phenotype of the rhythmic pdf^{01} flies [70,73,81]. Later on, the role of PDF as an output of the sLNvs was described as dual, lengthening some clusters and shortening others [82]. In subsequent work, Helfrich-Förster and colleagues determined that PDF lengthens the period of sLNvs, the 5th LNv, and the CRY+ LNds and, and shortens the period of the CRY− LNds [20].

In an independent attempt to understand the relationship among clusters, Rosato and colleagues introduced a slightly different paradigm, dividing the network in four clusters based on the expression of PDF and CRY. Among the latter, they defined neurons with strong, weak or null CRY expression [72]. They proposed that PDF+ CRY+ cells have a synchronizing and lengthening effect on the network, while the PDF− CRY− clusters, in particular the DN2 neurons, shorten its period, in contrast with results published earlier [20,82] that do not appear to have been taken into account in the proposed model. In addition, Rosato and colleagues proposed that this opposing "forces" balance each other employing yet undescribed communication signals [72].

Finally, a potentially direct contact between sLNvs and DN1 neurons, specifically the 8–10 posterior ones, has been reported at the dorsal protocerebrum (Fig. 3) [16,48,74]. Functionally, DN1ps respond to focal application of PDF with an acute depolarization and an increase in firing rate, through a cAMP-dependent, PKA-independent mechanism that is different from the one recruited to synchronize the molecular clock [16].

Thus, a picture is emerging in which there is time-of-day dependent information flow from the sLNvs to two different circadian clusters LNds and DN1ps, [48]; the latter directly contact neurons in the *Pars Intercerebralis*, which, in turn, project to the dorsal tritocerebrum, and thus, towards premotor centers [74]. Once the neurotransmitter identity for each independent cluster is addressed more precise experiments will enable to determine the logic underlying this rather complex network.

4. A flexible network for a changing environment

Light is the most prominent *zeitgeber* for most plants and animals. In the fly brain light penetrates clock neurons through a photic path involving the eye, the ocelli and the HB organ [83], and also acts in a cell-autonomous fashion by activating CRY and thereby increasing neuronal firing [84] and triggering TIMELESS (TIM) degradation, which, in turn, leads to a resetting of the molecular clock [85–87]. In a recent study Holmes and colleagues reported an extraordinary effort to address the response of the circuit to light, in particular, to a phase advancing (CT22) 2 h light pulse (LP) in the second day of constant darkness [88]. Through live imaging recordings of whole brains they showed that, upon stimulation with a phase-advancing LP, neurons immediately lost synchrony and only regained coherent oscillations a few days later (in stark contrast to the rapid phase-adjustment of the behavioral pattern showed by animals exposed to phase advances or delays [83,89]). Furthermore, while the sLNvs, DN1s and DN3s recover rhythmicity after receiving the 2 h advancing light pulse, the lLNvs do not and, LNds resynchronize faster than other groups [88]. These ex vivo results are in agreement with previous reports showing that both M and E clusters are involved in phase shift adjustment [90], and data indicating that TIM degradation within s-LNvs is not necessary to respond to a phase advancing stimulus [91]. Together, these findings led to the hypothesis that LNd neurons are leading the resynchronization and phase shift of the network.

Interestingly, LNd-mediated clock resetting is critical to phase changes, although a signal from the sLNvs is in part required to trigger this response [78]. In addition, in a meticulous analysis of CRY-dependent phase shifts, Yoshii and colleagues showed that cell autonomous photoreception in the sLNvs does not seem to be necessary for re-entrainment to light pulses, suggesting that the signal that mediates sLNvs firing could derive from visual input pathways [92]. This hypothesis would be consistent with the previously described picture of how the network operates. Light pulses could impact widely in the network and the visual system, resetting the molecular clocks of several clusters. Specifically, in the CRY+ LNd neurons the signal could strengthen the coupling with the CRY− LNds (running desynchronized from the rest of the network under normal conditions [20]). In this event, the entire LNd cluster could be in sync, displaying fast synchronization at the gene expression level [88], and thus the LNd cluster could impose a new phase to the rest of the network. Although hypothetical, this interpretation implies that part of the system, in particular the sLNvs, show robust and long lasting oscillations in DD, but the system remains plastic to respond to external stimuli by recruiting neuronal clusters that were out of sync. This is in agreement with data indicating that CRY− LNds could impose their period on the sLNVs, working as fine tuners of the DD period [72], and also with the proposed intra-group synchronization signal from the CRY+ to the CRY− LNds [79].

5. Technical challenges and frontiers

Despite the extensive effort that has been made to shed light on the relevance of individual clusters, we still lack fundamental information regarding basic properties of specific subsets as well as more restricted genetic tools and output reporters that would enable us to interrogate the system in detail. For instance, there is still a technical gap between the recorded activity profiles of overt rhythms and the approaches that report oscillations derived from network function. In general, animals subjected to constant darkness remain rhythmic for several days or even weeks, but most reporters employed show dampening after a few days in constant darkness, which is even the case for core clock component oscillations measured by qPCRs, Western-blots, immunocytochemistry (ICC), luciferase reporters (whole animals, body parts or brain explants) or even electrophysiological properties (Muraro & Ceriani, unpublished). Although it is possible that technical issues could account for this apparent paradox to a certain degree, it could also be used to provide further insight into clock function. Dampening of a signal that involves homogenised tissue is likely stemming from out of phase oscillations from different parts of the head/brain/animal alongside other tissues that retain rhythmicity. However, behavioral patterns do change when the animals are shifted from L:D to D:D. Are the tissues that need entrainment responsible for changes in behavior? For instance, core clock proteins loose synchronization under constant conditions in specific clusters, while others retain rhythmicity [20,70], underscoring that gaining further understanding of cluster-specific loss of synchrony in detail is worth exploring. The recent introduction of an ex vivo luciferase reporter assay from individual clusters [88] or even electrophysiological recordings from single neurons [93,94] opens a new window of possibilities. What are we missing in brain explants that might be responsible for oscillatory gene expression in so many circadian neurons? How is the input from sensory organs, particularly the eye, modulating brain activity? Research devoted to tackle these questions, and other stemming from the same apparent paradoxes, will certainly uncover new exciting findings.

6. Concluding remarks

A biological system evolved to deal with the a changing environment that is a complex combination of predictable and fixed oscillations together with stochastically and variable events needs two main characteristics, (1) the ability to respond to changes in the relevant cues and (2) the ability to avoid hypersensitivity to uninformative signals. The fly circadian clock has developed a series of plastic properties to properly cope with this requirement. Plasticity is observed not only in the connectivity between clusters

but also in the hierarchy of each cluster in response to different stimuli or even further in the refractory behavior of specific subsets of neurons to certain stimuli at specific times of the day. Experiments employing constant conditions (LL or DD), or the different manipulations altering entrainment phase, showed that the coupling and the hierarchy of the clusters within the network is dynamic and necessary to ensure proper responses.

Finally, modern societies have created situations in which circadian clocks are constantly challenged for re-entrainment, namely those encountered by frequent travellers, social jetlag and workers under shifting schedule regimes, situations that have already been associated with major health problems [95–97]. Thus, understanding the rules governing entrainment and network synchronization could help improve everyday situations and drive policy makers to take into account chronobiological aspects.

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