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THE CIRCADIAN SYSTEM: PLASTICITY AT MANY LEVELS

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Abstract—Over the years it has become crystal clear that a variety of processes encode time-of-day information, ranging from gene expression, protein stability, or subcellular localization of key proteins, to the fine tuning of network properties and modulation of input signals, ultimately ensuring that physiology and behavior are properly synchronized to a changing environment. The purpose of this review is to put forward examples (as opposed to generate a comprehensive revision of all the available literature) in which the circadian system displays a remarkable degree of plasticity, from cell autonomous to circuit-based levels. In the literature, the term circadian plasticity has been used to refer to different concepts. The obvious one, more literally, refers to any change that follows a circadian (*circa* = -around, *diem* = day) pattern, i.e. a daily change of a given parameter. The discovery of daily remodeling of neuronal structures will be referred herein as structural circadian plasticity, and represents an additional and novel phenomenon modified daily. Finally, any plasticity that has to do with a circadian parameter would represent a type of circadian plasticity; as an example, adjustments that allow organisms to adapt their daily behavior to the annual changes in photoperiod is a form of circadian plasticity at a higher organizational level, which is an emergent property of the whole circadian system. Throughout this work we will revisit these types of changes by reviewing recent literature delving around circadian control of clock outputs, from the most immediate ones within pacemaker neurons to the circadian modulation of rest–activity cycles.

Key words: circadian plasticity, circadian network, PDF, structural plasticity, clock neurons, rhythmic behavior.

Contents

CIRCADIAN CIRCUITS

The behavioral and molecular characterization of numerous clock mutants, together with the unraveling of the molecular mechanisms underlying the circadian clock, have been the main focus of circadian research in the last decades (for a thorough review of the current understanding of the molecular clockworks, see Ozkaya and Rosato (2012)). Although by no means a closed topic, the challenge is now to understand how different clock neurons connect to each other and establish a network that is able to integrate environmental clues, culminating on a coherent and adaptive behavioral output. *Drosophila* provides an ideal model organism to study clock neuron connectivity because of its anatomically dispersed network, comprising defined clock neuron clusters that are becoming more and more molecularly distinct (Fig. 1, adapted from Peschel and Helfrich-Forster (2011) and Table 1). Compared to studying the connectivity of the densely packed clock neuronal network that the mammalian suprachiasmatic nuclei (SCN) represent, this task seems more feasible. It is this anatomical layout and its scarcity (150–200) of neurons (Kaneko et al., 1997; Helfrich-Forster, 2003) compared to 20,000 in the rat SCN (Van den Pol, 1980), together with the versatile genetic tools available in *Drosophila* (Venken et al., 2011), which makes this model organism an ideal choice to study this biological question.

Given that membrane properties are essential to neuronal function, it seems logical to begin by asking what kind of electrical signals clock neurons generate. In that regard early work on tissue islands containing rat SCN was pioneer showing that, under free-running conditions, electrical activity increased during the

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Abbreviations: AVP, arginine vasopressin; BRP, bruchpilot; CRY, CRYPTOCHROME; DD, constant darkness; DN, dorsal neuron; lLNvs, large ventral lateral neurons; LD, light–dark; LL, constant light; LN, lateral neuron; LNds, dorsal lateral neurons; LPNs, lateral posterior neurons; PDF, pigment dispersing factor; PDH, pigment dispersing hormone; PER, PERIOD; SCN, suprachiasmatic nuclei; sLNvs, small ventral lateral neurons; TIM, TIMELESS; VIP, vasoactive intestinal peptide.

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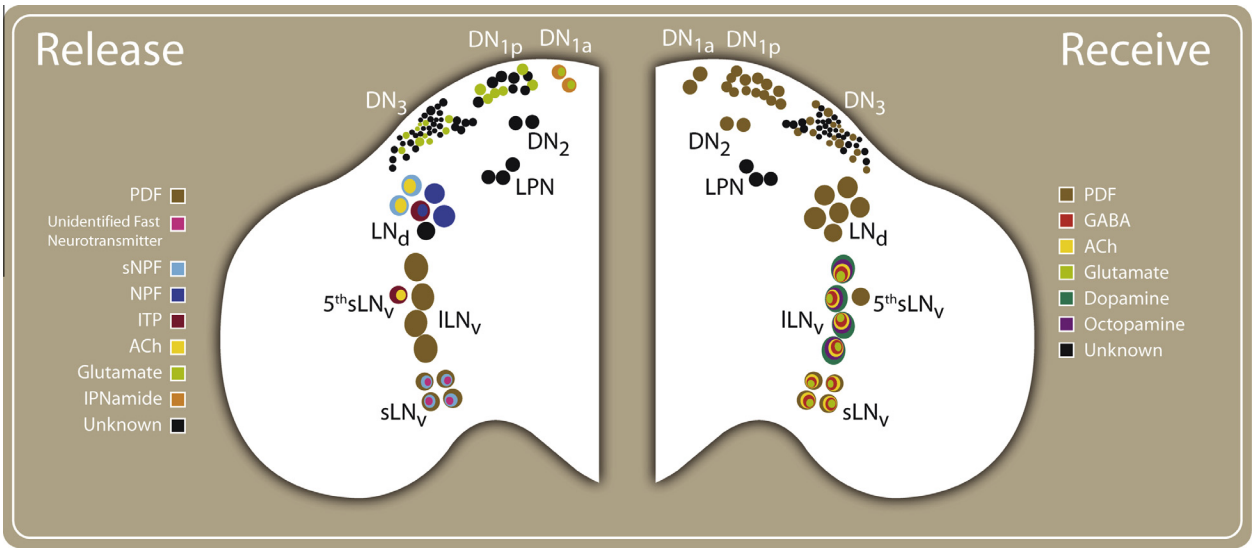

Fig. 1. Molecules released (pictured on the left hemisphere) or received (pictured on the right hemisphere) by clock neuron clusters of adult *Drosophila*. In several cases no physiological evidence exists on the biological function of these substances in the specific groups of cells; in those cases their release is implied by the fact that a specific vesicular transporter or a synthesizing enzyme is expressed there. The objective of the schematic diagram is to give an overview of the state of the art on the chemical substances putatively involved in neurotransmission in the *Drosophila* circadian network, for further information the reader should start by revising references included in Table 1.

Table 1. References to the chemical substances released or received by clock neuronal clusters of adult *Drosophila*

Cluster	Release	Receive
sLNvs	PDF[a,b]; sNPF[c]; Unidentified Fast Neurotransmitter[d,e]	PDF[i]; GABA[j]; ACh[j]; Glutamate[h]
lLNvs	PDF[a,b]	GABA[k]; ACh[k]; Glutamate[k]; Octopamine[l]; Dopamine[l]
5th sLNv	ITP[c]; ACh[c]	PDF[i]
LNds	Some cells NPF[c]	PDF[i]
	Some cells sNPF[c] and ACh[c]	
	One cell NPF[f] and ITP[c]	
LPNs	N/A	N/A
DN1as	IPNamide[g]; Glutamate[h]	PDF[i]
DN1 ps	Some cells Glutamate[h]	PDF[i]
DN2s	N/A	PDF[i]
DN3s	Some cells Glutamate[h]	Some cells PDF[i]

N/A: no information available about neurotransmitter and neuropeptides received or released by that particular cluster. Not included in the table but worth noting is the information relevant to neurotransmission in the larval circadian clusters, which includes the substances PDF (Renn et al., 1999), ACh (Wegener et al., 2004), GABA (Hamasaka et al., 2005), Serotonin (Hamasaka and Nassel, 2006), Glutamate (Hamasaka et al., 2007) and sNPF (Johard et al., 2009).

[a] Helfrich-Forster (1995).
[b] Renn et al., (1999).
[c] Johard et al. (2009).
[d] Yasuyama and Meinertzhagen (2010).
[e] Umezaki et al. (2011).
[f] Lee et al. (2006).
[g] Shafer et al. (2006).
[h] Hamasaka et al. (2007).
[i] Shafer et al. (2008).
[j] Lelito and Shafer (2012).
[k] McCarthy et al. (2011).
[l] Shang et al. (2011).

subjective day compared to the subjective night, therefore proving for the first time that electrical activity of clock neurons is circadianly regulated (Inouye and Kawamura, 1979). Moreover, thanks to the development of SCN slice preparations, mammalian models have been extremely useful in determining many electrophysiological characteristics of clock neurons (Kuhlman and McMahon, 2006; Ko et al., 2009; Colwell, 2011). Although very informative to study electrical activity and the properties of clock neurons under conditions that may render them synchronized/desynchronized, SCN preparations are not that practical, due to their complexity, in the unraveling of specific neuronal connectivity within a circadian neuronal network (Vansteensel et al., 2008; Welsh et al., 2010).

Anatomically, the circadian network of *Drosophila* has been thoroughly described (Helfrich-Forster et al., 2007), with each brain hemisphere containing three dorsal

neuron (DN) clusters corresponding to 16 DN1s, two DN2s and around 40 DN3s; and four lateral neuron (LN) groups comprising five small ventral lateral neurons (sLNvs), four large ventral lateral neurons (lLNvs), six dorsal lateral neurons (LNds) and three lateral posterior neurons (LPNs), see Fig. 1. A number of years ago, a model consisting of two oscillators, each one commanding one of the two high-activity periods that *Drosophila* show under laboratory conditions was proposed, with the cellular substrates for these being the sLNvs (the M, for Morning, oscillator) and the LNds, DNs and the fifth PDF-negative sLNv (the E, for Evening oscillator) (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006). This simplified model lost consistency when locomotor activity was analyzed using different paradigms, such as constant light conditions (so called LL) (Murad et al., 2007; Picot et al., 2007; Rieger et al., 2009). Nowadays, the more consented model agrees to consider the sLNvs as the main pacemaker under free-running conditions and regards the M/E oscillators as plastic entities, composed of subsets of clock neurons that change their predominance according to the photoperiod (Rieger et al., 2006; Stoleru et al., 2007) and temperature condition (Dubruille and Emery, 2008; Sehadova et al., 2009; Gentile et al., 2013). Although the sLNvs and their release of the neuropeptide PDF (Pigment Dispersing Factor) is at the top hierarchical position of circadian regulation, the actual connectivity among clusters has not yet been fully demonstrated. See more of both subjects below.

ELECTRICAL ACTIVITY OF CLOCK NEURONS

Neuronal activity, which in most neuronal types takes the form of action potential firing, determines the release of neurotransmitter and neuromodulatory substances allowing neurons to pass on information to downstream targets. The first experiments actively attempting to disrupt such mechanisms within the circadian network *in vivo* came from expressing the tetanus toxin light chain in *Drosophila* clock neurons (Kaneko et al., 2000). As could have been anticipated, disruption of neurotransmission in the whole circadian network resulted in locomotor arrhythmicity; however, restricting tetanus toxin expression only to PDF-positive neurons resulted in a milder disruption of rhythmicity. This weaker phenotype is in agreement with the existence of two different types of vesicles in the dorsal termini of sLNvs axons, some electron-dense larger PDF-containing vesicles and some clear smaller ones (Yasuyama and Meinertzhagen, 2010); the latter were taken as an indication of the existence of a fast (classical) neurotransmitter, whose identity is still unknown. Expression of tetanus toxin impairs only synaptobrevin-mediated mechanisms of classical neurotransmission, sparing neuropeptidergic release such as PDF's, confirming the previously described major role of PDF as an output of sLNvs (Renn et al., 1999). Through a similar approach, that is, interfering with vesicle recycling, it was recently demonstrated that this process is relevant for setting the free-running period (Kilman et al., 2009; Wulbeck et al., 2009), although the mechanism is still largely elusive (Frenkel and Ceriani, 2011). Further studies playing with different light conditions demonstrated that the unknown classical neurotransmitter inhibited by tetanus toxin expression does impact on circadian locomotor behavior but in a more subtle and still not completely understood way (Umezaki et al., 2011).

Silencing of electrical activity would disrupt the release of both types of sLNvs vesicles, the unknown classical neurotransmitter-containing clear ones and the dense PDF-containing ones. Electrical silencing of this kind was achieved in LNvs through the expression of a hyperpolarizing potassium channel, which produced a clear impairment of locomotor rhythmicity under constant darkness (DD; Nitabach et al., 2002). Under these conditions, PERIOD (PER) and TIMELESS (TIM) oscillations were severely affected suggesting that neuronal silencing produces a disruption in the molecular clock. However, these observations are in contradiction with those made in SCN primary cultures treated with tetrodotoxin (which stops cellular communication by preventing sodium action potential firing, providing an alternative means of silencing neurons) where, after a few days of treatment, the phase of electrical activity re-emerged unaltered, suggesting that while electrically silent the molecular clock had not altered its pace (Welsh et al., 1995). That said, it should be noted that processes that impinge upon membrane properties of clock neurons can modulate their molecular clock, as anticipated when taking into account that a property of circadian clocks is their entrainability (Block et al., 1993; Welsh et al., 2010; Colwell, 2011).

The notion that such a fundamental mechanism, i.e. neuronal activity acting either as an output or as a cog of the circadian clock, could have diverged between mammals and *Drosophila* is both intriguing and confusing. It is true that, at the level of anatomy and physiology, differences between mammalian and insect circadian systems are substantial, and reflect the obvious disparities in complexity and function. However, taking gene duplication aside, the fundamental mechanisms governing the molecular clocks of all animals have so far been found to be incredibly conserved (Pegoraro and Tauber, 2011). Why would this aspect differ between mammals and *Drosophila*?

Further investigations trying to shed light on this issue took advantage of an inducible genetic strategy to silence PDF neurons by driving the hyperpolarizing potassium *kir2.1* channel in LNvs but in an adult-specific fashion (Depetris-Chauvin et al., 2011). While behavioral rhythmicity was disrupted by this manipulation, PER oscillations were barely affected, indicating that restricting neuronal silencing to adult stages disrupts clock outputs (such as sLNv dorsal projections' PDF levels and structural plasticity, Fig. 2) but does not alter the pace of the molecular clock. This result is further supported by the observation that the level of hyperpolarization achieved by either the constitutive or the inducible treatments were

equivalent (Muraro and Ceriani, unpublished results) and therefore the divergence cannot be attributable to differences in the extent of potassium channel expression. These controversies highlight the importance of using genetic manipulations with caution, not neglecting the effects that these might trigger during development or the functional consequences that homeostatic compensation might generate. Indeed, PDF function is important for the development of its own circuit (Gorostiza and Ceriani, 2013) and therefore silencing of these neurons (and preventing the release of PDF) throughout development may have effects on the establishment of a correct anatomy and physiology.

Circadian plasticity is necessary to adjust behavior in an adaptative way (e.g. during seasonal photoperiod changes) and even the top hierarchical clock pacemaker centers (sLNvs in the case of circadian locomotion in *Drosophila*) must be able to integrate information from the environment to modify behavior accordingly. This could be achieved by cell autonomous sensing of light (via Cryptochrome (CRY)-mediated photoreception) and/or through changes in the information coming from other brain centers, which could come anterogradely via neurotransmission or neuromodulation of subthreshold currents, or retrogradely, integrating also post-synaptic cues. Such network information, after being transduced to the cell nucleus, would impinge upon gene expression to adapt the circadian clock (and more relevantly, its outputs) to the new environmental condition. If this is true, then alteration of electrical activity should affect gene expression of pacemaker neurons, a hypothesis tested recently by Mizrak et al. (2012), who have analyzed the expression profile of isolated larval LNvs subjected to silencing (through the expression of KIR2.1) or hyperexcitation (through NachBac expression, a slow-inactivating bacterial sodium channel). As it has been described many times before, the expression of a large proportion of genes varies circadianly (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ceriani et al., 2002; Ueda et al., 2002; Wijnen et al., 2006; Keegan et al., 2007; Kula-Eversole et al., 2010; Nagoshi et al., 2010; Hughes et al., 2012; Rodriguez et al., 2013), the novelty of this study lies on the finding of a strong correlation between the membrane potential and the group of genes expressed at a given time of the day, with hyperexcitation producing a *morning-like* expression profile and hyperpolarization producing an *evening-like* expression profile (Mizrak et al., 2012). What does this mean? Is the membrane potential changing the clock and this, in turn, changing gene expression? Or is the membrane directly exerting an effect over gene expression? Interestingly, clock genes were only fine-tuned by these extreme treatments rather than suffering a complete activation or shutdown, suggesting that most changes in gene expression were not mediated by the circadian clock (Emery, 2012). An interesting turn of the page is that members of the cAMP-response element-binding protein (CREB) family offer a possible mechanism for translation of circadianly relevant membrane activity into alteration of gene expression in *Drosophila*, a molecule already thought to play this role in the mammalian clock (Gau et al., 2002).

Thus, asking whether electrical activity acts as a *zeitgeber* (i.e. a synchronizing cue) or a clock output might be as unhelpful as asking what was first, the chicken or the egg. More subtle manipulations than complete silencing or cracking up neuronal activity would be necessary to reveal which other cellular components, from membrane receptors and transduction pathways to gene expression changes and back to membrane activity, are necessary to assemble a plastic circadian clock.

INFORMATION PROCESSING BY SLNVS

Despite sLNvs importance as dominant pacemaker cells under both light–dark (LD) cycles and constant conditions (Grima et al., 2004; Stoleru et al., 2004; Rieger et al., 2006) we still lack a great deal of information on sLNvs physiology. Unlike lLNvs, which are larger and more superficial, and have been the subject of more thorough electrophysiological analysis (Park and Griffith, 2006; Cao and Nitabach, 2008; Sheeba et al., 2008a; Fogle et al., 2011; McCarthy et al., 2011), sLNvs lie deeper into the brain and have a smaller size, making them less accessible to electrophysiology. So far only a couple of papers have reported electrophysiological recordings of this cell type, revealing circadian variation on their resting membrane potential (Cao and Nitabach, 2008) and a depolarizing response to PDF (Choi et al., 2012). Their poor accessibility for electrophysiological recordings is not an obstacle for optical imaging. This experimental approach has recently revealed that nicotinic acetylcholine (ACh) receptors mediate increases of both intracellular calcium and cyclic AMP in sLNvs, and that they respond to GABA in an inhibitory manner (Lelito and Shafer, 2012). Optical imaging not only allows the experimenter to test responsiveness to a multiple array of molecules, such as neurotransmitters, but also one can assess connectivity among different clock neuron clusters by taking advantage of the use of binary systems of expression and *Drosophila* genetics (Yao et al., 2012).

It has been already shown that PDF neuromodulation plays an important role both as a *zeitgeber* for other clock neurons of the circadian network and as a synchronizer for phase coherence among sLNvs (Peng et al., 2003; Lin et al., 2004b; Picot et al., 2007; Wulbeck et al., 2008; Yoshii et al., 2009b). But, which transduction pathways mediate circadian function? To shed light on the PDF signal transduction pathway, Duvall and Taghert (2012) have used a cAMP reporter together with RNAi-mediated downregulation of 12 different adenylate cyclases to find out the signal transducers of PDF receptor binding (Duvall and Taghert, 2012). They found out that, in sLNvs, PDF binding to PDF-R is coupled to Gsα60A and subsequently activates adenylate cyclase 3. They also report that activation of PDF-R in a different clock neuron cluster, the LNd *evening* cells, is not coupled to the same adenylate cyclase. Therefore, PDF differential function in the

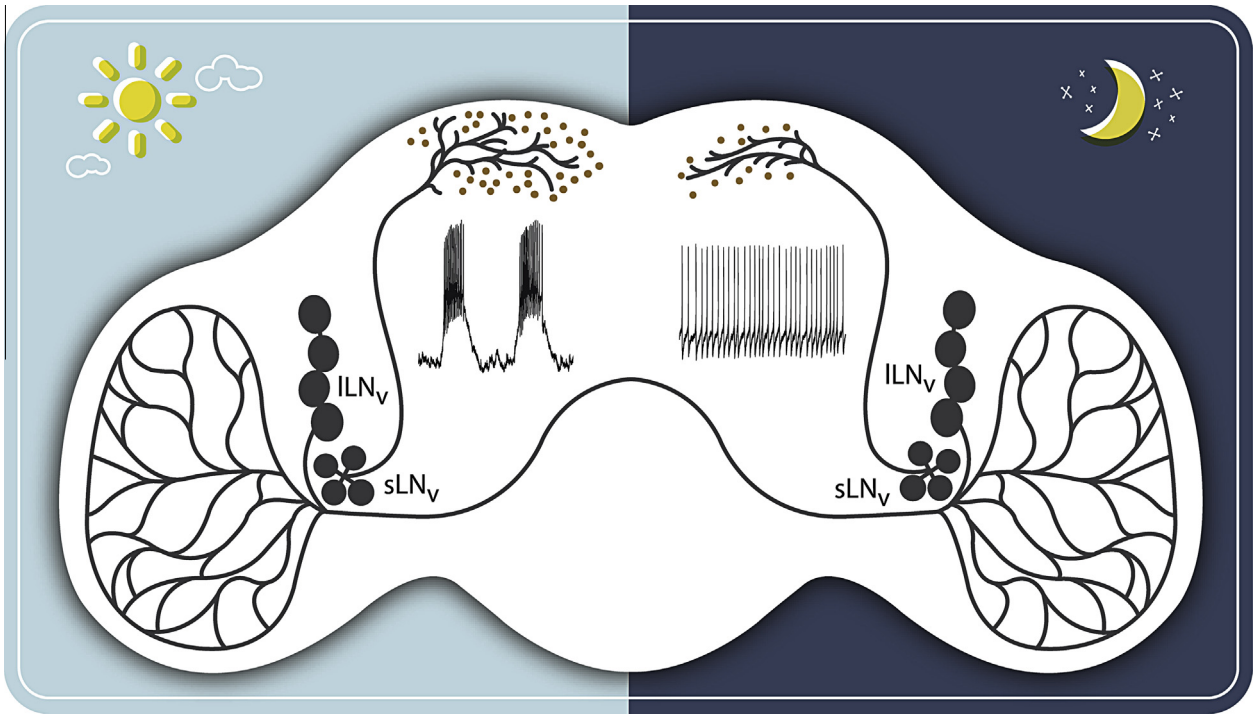

Fig. 2. Circadianly controlled outputs of the sLNvs: PDF, structural plasticity and electrical activity. During the day the projections of the sLNvs present higher levels of PDF immunoreactivity and adopt an *open* conformation (with the axonal terminals spreading throughout a larger area of the dorsal protocerebrum). On the contrary, at night-time PDF immunoreactivity decreases and the axonal terminals adopt a close conformation. Circadian variation of electrical activity has been a trademark of clock neurons in mammalian models. In *Drosophila*, where electrophysiological analysis of clock neurons is less developed, such variation has been already reported in the lLNvs, which exhibit a high activity bursting mode preponderant during the day and a lower activity tonic mode more prevalent during the night. The sLNvs have been considerably more difficult to record from, however, circadian variation in their resting membrane potential has been described (Cao and Nitabach, 2008). The recordings shown in the scheme are from lLNvs, although we have recorded from sLNvs in the bursting modality (NI Muraro & MF Ceriani, unpublished observations).

diverse clock neuron clusters might do so by means of specific signal transduction pathways. Fig. 3 provides a model of network information processing of sLNVs. Future research should further explore the mechanisms by which clock neurons are able to integrate both, network and environmental information, how this impacts the balance of the transcription–translation machinery of the molecular clock, and how this is able to modify neuronal outputs.

CIRCADIAN MODULATION OF STRUCTURAL PLASTICITY

There are multiple levels where circadian regulation can take place (Fig. 2). With that in mind, we believe that the regulation of the structure or morphology of the cells (e.g. neurons) involved in all the different circadianly regulated processes is an excellent substrate. Even though this subject has recently been reviewed (Pyza and Gorska-Andrzejak, 2008; Frenkel and Ceriani, 2011; Mehnert and Cantera, 2011) we believe the issue needs to be addressed here since several important questions remain unanswered. Henceforth we will refer to this remodeling phenomenon as structural circadian plasticity, or structural plasticity for short, as opposed to circadian plasticity (Mehnert and Cantera, 2011), since there are multiple levels that can undergo plasticity on a daily basis in addition to the structure of cells, as discussed along this review.

As of today, the role that structural plasticity has on the regulation of the rest–activity cycles has not been addressed in *Drosophila*. Nevertheless, multiple examples of circadian structural plasticity have been found in the last few years in cells from very different systems, which range from the insect visual neuropil to VIP-releasing neurons in the mammalian SCN (see references across this section).

There are multiple aspects that need to be addressed to understand the role that structural circadian plasticity plays on the regulation of clock activity. One could first ask how this plasticity is regulated in a circadian fashion, and which pacemakers are in charge of controlling this phenomenon. One of the earliest reports to show circadian changes in the morphology of neurons was contributed by Pyza and Meinertzhagen (1995). In this pioneering work they showed that axons of the L2 monopolar interneuron in the optic neuropil of the housefly *Musca domestica* undergo daily changes in diameter. These structures are largest during the beginning of the day and smallest during the middle of the night, when flies are kept in a normal LD condition, suggesting a circadian effect on the morphology of

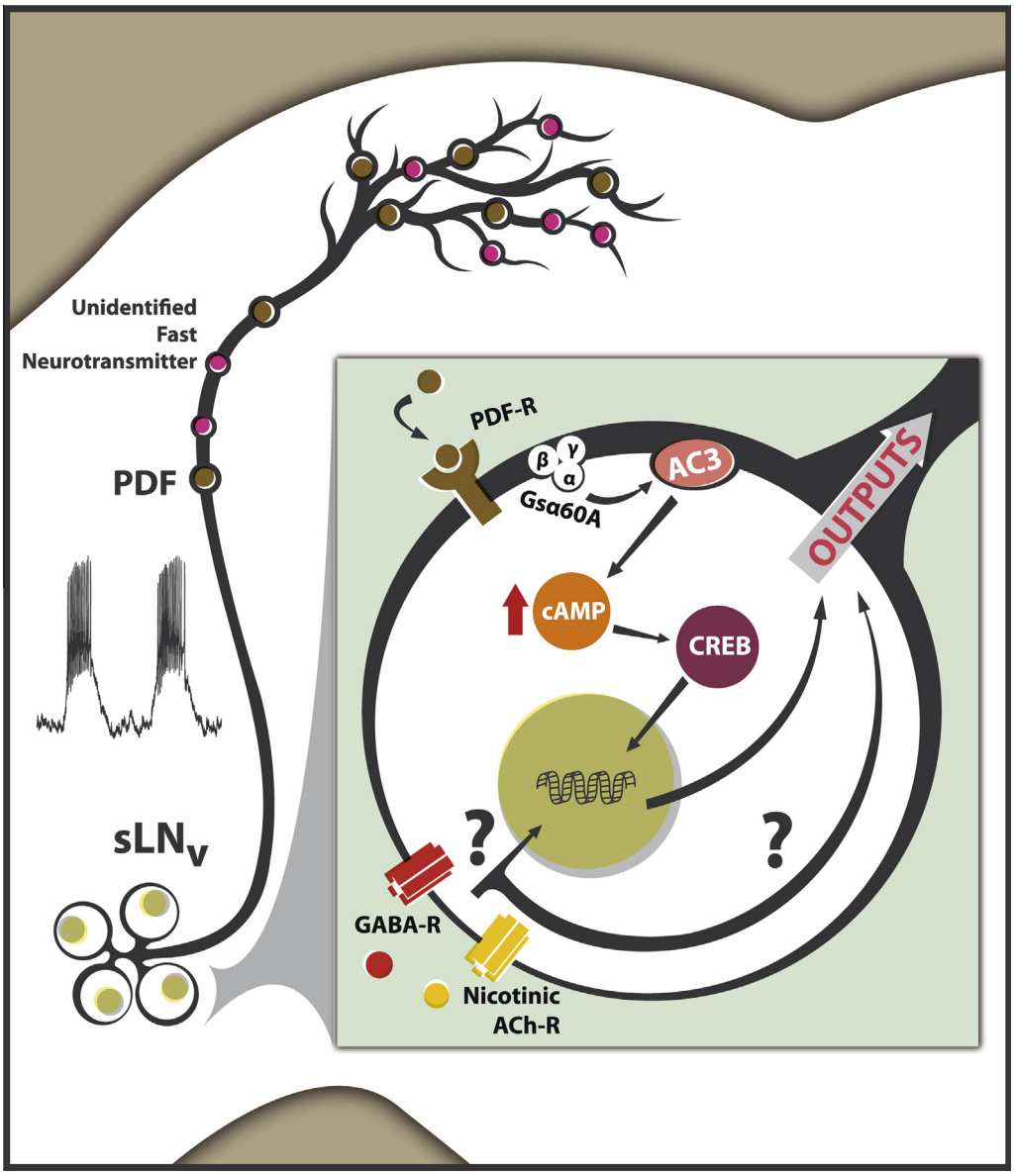

Fig. 3. sLNvs, the main pacemaker cells under free running conditions (DD), are able to process network information to fine tune their outputs. Although their importance in the hierarchy of circadian neurons is well known, how sLNvs process information from the network and how this affects their outputs is only starting to be unraveled. This model summarizes the proven chemical inputs to sLNvs: PDF, GABA and ACh. In all cases the source of these neurotransmitters is still unknown. PDF binds to its specific receptor which is coupled to a Gsα60A G-protein and activates adenylate cyclase AC3. This increases cAMP levels and activates CREB which subsequently modifies gene expression. A lot less is known about the roles of GABA and ACh. They do produce inhibition and excitation, respectively, which could directly modify the electrical output of the neuron, and therefore neurotransmitter release. Additionally, this effect on membrane hyper or depolarization could be affecting transduction cascades that ultimately modify gene expression, but this has not been demonstrated. All these sLNvs inputs could impact on their outputs: electrical activity, release of PDF and that of an unidentified fast neurotransmitter, and structural plasticity of axonal projections. Cell-autonomous sensing of light via Cryptochrome is a well-known input to sLNvs that certainly affects its function, but has been left out of the model since its activity does not depend on the neuronal network.

these cells. These changes were circadian in origin since control groups (kept either in DD or LL) showed plasticity as well.

When thinking about how time cues are transferred among different members of the circadian network, one possible mechanism entails altering the release of the molecules involved in transmitting this information. The fly visual system provides another example of such circadian plasticity. Using confocal microscopy Pyza and Meinertzhagen (1997) showed that the size and spacing of putative pigment dispersing hormone (PDH) peptide (the ortholog of PDF) release sites exhibit circadian plasticity, having fewer and larger varicosities during subjective day than during subjective night. Keeping the flies in DD did not eliminate the changes; thus as with the previous L2 interneuron plasticity we can conclude that this plasticity is of endogenous origin. This form of plasticity deserves attention because it suggests that

PER-expressing PDH cells could be a *release site* for circadian information. As the authors pointed out, these changes in the size of the varicosities could be associated with rhythm in the synthesis and or release of the peptide in the neurites of PDH cells. Pyza and colleagues also showed that *Drosophila* also displays circadian variability in the size and shape of neurons in the first optic neuropil, the lamina (Pyza and Meinertzhagen, 1999; Gorska-Andrzejak et al., 2005), as well as on the dendrites of the L2 cells, where these structures were longer at the beginning of the day and shorter toward the end of the day and at night (Weber et al., 2009).

It is also worth pointing out that the role of glial cells in this plasticity is not totally understood, but there is evidence suggesting that they might be involved in controlling the rhythms found in the optical neuropil. Glial cells also show circadian plasticity but in the opposite direction as the change observed for the L1 and L2 cells (Pyza and Gorska-Andrzejak, 2004b). By blocking gap junctions, Pyza and Gorska-Andrzejak (2004a) were able to disrupt the coupling between lamina cells, thus preventing the rhythmic change in morphology observed both in neurons and glial cells. This result strongly supports the hypothesis that glial cells are involved in the generation of the circadian plasticity observed in the fly's visual system neuropil.

As already discussed, several studies have shown circadian changes in the shape of optic lobe interneurons both in *Musca* and *Drosophila* (Meinertzhagen and Pyza, 1996; Pyza and Meinertzhagen, 1999; Pyza and Gorska-Andrzejak, 2004b; Gorska-Andrzejak et al., 2005). In addition, Cantera and colleagues identified an example of circadian plasticity on the morphology of the neuromuscular junctions on flight muscles, which change rhythmically between day and night (Mehnert et al., 2007). The neuronal branches are thicker and have larger boutons during the day. In order to test for light-driven effects, flies were placed in DD and the results are consistent with a true circadian phenomenon, a claim further supported by the observation that mutations on the circadian genes *per* and *tim* disrupt this form of plasticity. Interestingly, differences in bouton size were reduced during DD, suggesting that light stimulates bouton growth (Mehnert et al., 2007). In a study attempting to dissect the origin of these rhythmic changes, Mehnert and Cantera (2008) found that suppressing synaptic activity in the mornings, by using the *shibire* mutation in glutamatergic motorneurons, did not prevent the observed growth in bouton size, which was indistinguishable from that of control flies. Surprisingly, this rhythm persists even in decapitated flies, suggesting that it might be independent of movement, synaptic activity and the central pacemaker as well. The authors suggest that the observed plasticity must be driven by a peripheral pacemaker, an interesting suggestion, since thus far all of the examples of circadian plasticity we are aware of are under tight control of the central nervous system. What is the link between these circadianly controlled morphological changes in neurons with changes in synaptic activity by these same cells, or their counterparts is a very important question still open.

In search for a mechanism that could be responsible for this alternate state, Gorska-Andrzejak et al. (2013) analyzed the expression pattern of bruchpilot (BRP), a marker of active zones (Kittel et al., 2006; Wagh et al., 2006). They analyzed the expression of this protein at different time points and light conditions, as well as in different mutant backgrounds, such as per^{01}, tim^{01} and cry^{01}. They found that both light and circadian inputs play a role in controlling this plasticity. BRP expression shows a bimodal distribution, showing a morning (ZT1) and an evening (ZT13) peak. This bimodal expression of BRP correlates very nicely with the behavior of these animals, and also with previous results from the same group, showing morphological changes in the L1 and L2 axons (Pyza and Meinertzhagen, 1999). When wild-type flies were kept in DD, BRP expression pattern changed and showed only a single peak. This study suggests that the rhythm in BRP expression pattern is regulated by a circadian oscillator in photoreceptors and glial cells, as well as input from the central pacemaker in the brain (Gorska-Andrzejak et al., 2013).

Another way to study how circadian plasticity affects the activity of the system is by studying synaptic vesicles, their morphology and their location on the synapse. Employing this approach in the *Drosophila* neuromuscular junction, Cantera and colleagues were able to show an important daily reorganization of the size and distribution (i.e. to be part of the active zone or the reserve pool) of synaptic vesicles, both in LD and DD. Active zone vesicles were smaller at ZT1 and ZT13 showing a strong correlation with the two daily peaks of locomotion observed in *Drosophila*, a result that suggest that synaptic vesicles are smaller in size in periods of high activity. Results from these experiments implied that an important feature of neural activity, such as size of synaptic vesicles, changes along the day (Ruiz et al., 2010).

So far we had only discussed examples of circadian plasticity in insects, although such examples are also present in vertebrates. In mammals, it has been shown that the central pacemaker lies within the SCN and that this nucleus synchronizes the activity to the LD cycles following a circadian cycle (Reppert and Weaver, 2002). The clock is synchronized to the environment primarily by signals arriving from the retino-hypothalamic tract terminating in a region of the SCN called the SCN core, which contains vasoactive intestinal peptide (VIP)-producing neurons (Morin and Allen, 2006). To enquire whether neurons or astrocytes undergo structural remodeling along the day, Becquet et al. (2008) looked for ultrastructural rearrangements in the axon terminal and somato-dendritic coverage of neurons expressing VIP or arginine vasopressin (AVP), the main two effectors released by the SCN to synchronize its clock neurons. They showed for the first time that the SCN undergoes circadian plasticity of its neuronal–glial network along the day, and that these changes are dependent on the identity of the neuronal population, whether they are AVP or VIP neurons. This observation supports the idea that

the mammalian SCN undertakes circadian structural plasticity and that this plasticity may be necessary for the synchronization of the clock to the LD cycle. In follow-up experiments Girardet et al. (2010) studied the contribution of glutamatergic synapses, known to play a crucial role on the functioning of the SCN. Although the levels of expression of glutamate transporters or the number of glutamatergic terminals did not show any circadian oscillation, the density of the synapses on neurons expressing VIP increased during the day, and this increase was present in glutamatergic and non-glutamatergic synapses (Girardet et al., 2010). These results show that the SCN undergoes circadian plasticity and that these changes take place on the VIP neurons (i.e. the photic input region) and not on the AVP neurons.

In zebrafish, hypocretin/orexin (HCRT) neurons have been postulated to regulate sleep–activity cycles and project to several areas of the brain, including the wake-regulating hindbrain and sleep-regulating pineal gland. By using two-photon imaging of living animals, Appelbaum and colleagues showed that these neurons exhibit circadian plasticity across the day (Appelbaum et al., 2010). Most of the previous examples of circadian structural plasticity discussed so far are based on fixed tissue experiments, thus we would like to emphasize the important change in the methodology embodied in the zebrafish experiments. Using this technique, Appelbaum et al. (2010) showed circadian regulation of synaptic plasticity in single axons of living vertebrates, both in LD and DD conditions. The number of active synapses projecting to the hindbrain and pineal gland is increased during the day. The phase of this increase is different in these two regions, suggesting that even in the presence of a similar input, the postsynaptic regulation of this circadian plasticity can differ. In order to test the role that homeostasis plays in this plasticity, the authors performed a sleep-deprivation experiment. They found that only a long period (6 h) of sleep deprivation has an effect on increasing the number of active synapses near the pineal gland during subjective night, showing that the observed synaptic plasticity in the hypocretin neurons is regulated by sleep homeostasis, in addition to having circadian control.

It has been shown in the last few years that one of the most important players in the regulation of circadian function in *Drosophila* is the PDF neuropeptide (Renn et al., 1999; Lin et al., 2004a; Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005; Shafer and Taghert, 2009; Yoshii et al., 2009b). This neuropeptide has been proposed to be the molecule that transfers time information across cells, and its rhythmic release the mechanism for transferring this information (Park et al., 2000). To close this section on circadian structural plasticity we would like to discuss recent studies that address a novel mechanism in which the morphology of the neurons in charge of releasing PDF undergo daily remodeling of their axonal terminals (Fernandez et al., 2008). In order to compare the complexity of the circuit (a measure of the number of axonal processes), two different time points were chosen, one early in the day (ZT2) and a second one at the early night (ZT14) (Fig. 4), corresponding to the peak and trough of PDF intensity, respectively (Park et al., 2000). Interestingly the axonal projections of these core pacemaker neurons are more complex at a timepoint in which PDF levels are high, and less so at night, when PDF levels are low. In contrast to the motorneuron terminals, no light-dependent effect was observed on these arborizations, i.e. structural changes remained unaltered under DD, underscoring its circadian nature. Consistent with this possibility, no circadian plasticity was observed in the PDF circuit in per^{01} and tim^{01} flies, supporting the role for the endogenous circadian clock as the source of the observed plasticity (Fernandez et al., 2008). Following up on this issue, Depetris-Chauvin et al. (2011) analyzed the role of electrical activity on the complexity of the PDF neurons axonal arborization at different times of day. To *silence* the electrical activity of these neurons, a modified GAL4 driver (GeneSwitch, Osterwalder et al., 2001) was employed, allowing for temporal and spatial

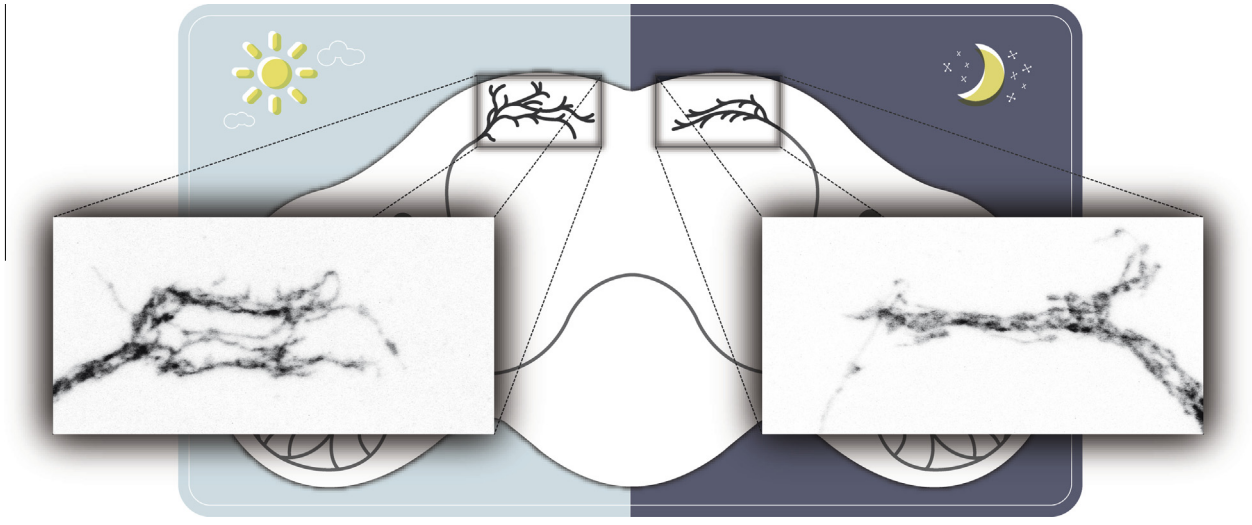

Fig. 4. Structural plasticity of sLNvs dorsal projections. Confocal images of sLNvs dorsal projections showing the *open* conformation during the day (on the right, ZT2) and a *close* conformation at night-time (on the left, ZT14).

control of gene expression. This was used to drive the expression of KIR2.1. When the complexity of the circuits was analyzed, the total number of crosses was reduced in the silenced brains. Interestingly, even though there was a reduction on the degree of plasticity, circadian remodeling was still taking place, supporting the notion that the molecular oscillator is functional (Depetris-Chauvin et al., 2011). As a final remark we can state that circadian plasticity appears to be a much extended phenomenon across different phyla, and that one of the multiple functions of this form of plasticity is to encode time-of-day information; in those examples within pacemaker neurons it may provide the substrate for differential connectivity along the day.

CIRCADIAN PLASTICITY AT ITS BEST: WHEN ENVIRONMENT ALTERS HIERARCHY BETWEEN OSCILLATORS

Circadian control of rhythmic behavior has recently been reviewed (e.g. (Klemm et al., 1986; Dubruille and Emery, 2008; Nitabach and Taghert, 2008; Yoshii et al., 2012). Under natural conditions, organisms use different cues from the environment to synchronize their biological clocks, and they might do so slightly differently from what we have learnt from analysis under laboratory conditions (Menegazzi et al., 2012, 2013; Vanin et al., 2012). In *Drosophila*, light and temperature changes are directly perceived by clock cells (Emery et al., 2000b; Ivanchenko et al., 2001; Glaser and Stanewsky, 2005), photoperceptive organs (Helfrich-Forster et al., 2001), and specific structures involved in mechanoperception (Sehadova et al., 2009); and these input signals are integrated by pacemaker neurons, for example at the transcriptional level (Boothroyd et al., 2007; Boothroyd and Young, 2008), for optimal timing of physiology and behavior (Yoshii et al., 2009a). In mammals, photoperiodic (changes in the duration of day length across the year) responses depend on the SCN, and are thought to be a property of the SCN cells working as an ensemble, as opposed to be encoded within individual neurons: available data supports the notion that an heterogeneous neuronal population within the SCN ensures a proper photoperiodic response (Vansteensel et al., 2008). In *Drosophila*, similar analyses have only recently begun to be explored. With the goal of revisiting what is known about how plasticity is achieved within the fly circadian network, this section will focus on two distinct aspects, whose understanding is rapidly evolving, namely, the interaction between light and temperature as *zeitgebers*, and the role of PDF.

Pioneer work from the Rouyer and Rosbash's laboratories established that relatively specific neuronal clusters define the pattern of locomotor activity throughout the day–night cycle, where the sLNvs (Fig. 1) control the rise and fall of activity around dawn (as well as in DD), while a subset of the LNds together with the PDF-negative 5th sLNv, determine the peak of activity around dusk (Grima et al., 2004; Stoleru et al., 2004). However, in recent years the notion that environmental cues strengthen the output of specific clusters, and in doing so they tinker with this hierarchical organization, has begun taking shape (e.g. Zhang et al., 2010).

Light is a key *zeitgeber* in most organisms which efficiently resets the fly clock (Suri et al., 1998; Yang et al., 1998; Ozkaya and Rosato, 2012). In the early days it was assumed that all clocks were made equal, and thus, recreating a clock in a dish would teach us much of its molecular underpinnings; however, pretty soon this notion proved to be incorrect, and for example, CRY, a dedicated circadian photoreceptor, was found to play a role as part of core machinery in peripheral oscillators (Krishnan et al., 2001), which is reminiscent of CRY's role within the mammalian clock. Along the same line, it is becoming increasingly clear that environmental conditions affect unevenly the properties of subsets of circadian neurons, thus impinging upon their relative hierarchy within the network. Although we still require a thorough understanding mostly due to the lack of appropriate genetic tools, a picture is emerging. An interesting example of plasticity at the circuit level arose when animals were exposed to LL, a condition that drives behavioral arrhythmicity in wild-type flies (Emery et al., 2000a). In 2007, Stoleru et al. reported behavioral rhythmicity in LL in flies overexpressing the clock component SHAGGY in all clock cells, presumably through modulation of CRY function; they also showed that under these conditions rhythmic behavior depended on the action of certain dorsal clusters, namely the DN1s, a conclusion shared by Emery and colleagues (Murad et al., 2007). Interestingly, Stoleru et al. (2005) proposed that only in the absence of light (i.e. at night on a regular LD cycle) the sLNvs function as the master clock and dominate the network, while the DN1s process light information and control rhythmicity during daylight (Stoleru et al., 2007). In parallel, Rouyer and colleagues also analyzed behavioral rhythmicity in LL. However, and likely due to the use of different genetic strategies, they found that the PDF-negative LNs (including the 5th sLNv as well as a subset of the LNds) were the relevant clusters driving rhythmic locomotor activity in LL (Picot et al., 2007). Interestingly, they concluded that light differentially affects the output of specific clusters, inhibiting the output of the sLNvs and activating that of the LNds, in line with the possibility raised by Stoleru and colleagues. Finally, recent work from the Emery lab also supports that the environmental condition defines the contribution of a subgroup of DN1s to locomotor rhythmicity. By means of a specific Gal4 driver that allows expression in a very restricted circadian pattern, the authors showed that these so-called E-neurons promote activity around dawn or dusk depending on the temperature and lighting conditions (Zhang et al., 2010). In line with these observations in the fly model, nocturnal rodents kept under constant light uncovered an unexpected property of the SCN; under these extreme conditions animals showed split behavior, which correlated with antiphasic oscillations of core clock as well as output genes in specific regions, such as left/right SCN in the rat, and even in the ventrolateral/dorsomedial areas within the same

hemisphere in the hamster SCN (de la Iglesia et al., 2000; Yan et al., 2005; Vansteensel et al., 2008), reminiscent of the picture emerging on the fly model.

Temperature cycles are a major cue and synchronize the biological clock both in DD as well as in LL (Matsumoto et al., 1998; Glaser and Stanewsky, 2005; Yoshii et al., 2005). Temperature-dependent entrainment of the clock takes place in a cell-autonomous fashion and, perhaps surprisingly, does not require the antenna (Glaser and Stanewsky, 2005). In fact, Stanewsky and colleagues elegantly demonstrated that entrainment to temperature cycles depends on the action of peripheral sensory structures known as chordotonal organs, which signal to the central brain and contribute to the synchronization of the circadian clock (Sehadova et al., 2009). Employing genetic manipulations that allowed reconstitution of a functional clock only in specific subsets of circadian neurons, Gentile et al. (2013) defined that the dorsal clusters would mediate synchronization to higher temperature cycles (i.e. 20:29 °C) while the ventral clusters appear to play a more central role at lower temperature cycles (16:25 °C). Interestingly, their work highlighted another layer of complexity whereby CRY, a key player in cell-autonomous light entrainment (Ceriani et al., 1999; Busza et al., 2004), counteracts synchronization through temperature cues by yet undefined mechanisms (Gentile et al., 2013). In agreement with this possibility, when CRY levels are depleted (through exposure to constant light conditions or in the absence of a functional CRY – such as in cry^b) a larger proportion of neurons within the circadian network become synchronized to temperature cycles (Glaser and Stanewsky, 2005, 2007). These results suggest a clear connection between these two important *zeitgebers* to ensure coherence in the output of the network.

MANY ROLES FOR A TINY NEUROPEPTIDE

The relevance of PDF in the control of fly rest-activity cycles was discovered serendipitously (Renn et al., 1999), and its function is potentially conserved (Beckwith et al., 2011); moreover, and quite surprisingly for a molecule that embodies rhythmic behavior, it does not oscillate either at the mRNA or peptide levels (Park and Hall, 1998), with the notable exception of the terminals of the neuronal processes where it is expressed (Park et al., 2000) (Fig. 2). Remarkably, PDF is expressed in a very restricted subset of neurons, namely the small and large LNvs in the adult brain (Fig. 1, Helfrich-Forster and Homberg, 1993; Helfrich-Forster, 1997).

Although this neuropeptide has recently been shown to play different functions throughout the life of the fly (Talsma et al., 2012; Gorostiza and Ceriani, 2013), the focus is now to review its role in synchronization within the circadian network. *pdf* null mutants (as well as mutants in its receptor PDFR (Hyun et al., 2005; Lear et al., 2005; Mertens et al., 2005)) become gradually arrhythmic in the absence of environmental cues, although a proportion displays low amplitude and short period rhythms, observations that led Taghert, Hall and colleagues to suggest that PDF is the circadian output signal responsible for coupling molecular oscillations in the circadian network to the behavioral output (Renn et al., 1999). Later on, this hypothesis was refined and PDF was proposed to contribute to the functional integration, i.e. synchronization, of independent circadian clusters under free-running conditions (Peng et al., 2003). In support of this possibility, in the absence of PDF molecular oscillations become out of sync in the sLNvs, while the LNds stay synchronized and expose a lower amplitude, slightly faster molecular clock (Lin et al., 2004b), which parallels the phenotype of the fraction of *pdf* null mutants that stay rhythmic (Renn et al., 1999). Moreover, downregulation of *pdf* levels in specific neuronal subsets led Shafer and colleagues to suggest that PDF from the small LNvs and not the large LNvs is responsible for the maintenance of free-running activity rhythms (Shafer and Taghert, 2009).

In recent years several laboratories have attempted to define the role of PDF in synchronization of molecular oscillations, and hence rhythmic behavior, through various approaches. A comparison between the activity profiles of *pdf* null flies and those lacking small and large LNvs (through expression of pro-apoptotic genes in the PDF pattern) entrained to LD or temperature cycles suggested that PDF-positive light-entrainable cells regulate the phase of the temperature-entrainable ones (that is, the DNs and LPNs) to be synchronized to their own phase using PDF as the coupling mediator (Tomioka et al., 2008); however, loss of PDF neurons would also deprive the system from a still elusive fast neurotransmitter (Yasuyama and Meinertzhagen, 2010), thus compromising the analysis. In parallel, Sheeba et al. (2008b) generated long-term alterations on LNvs electrical excitability (through constitutive expression of NaChBac) to inquire about their effects on molecular oscillations in specific clusters, as well their impact on locomotor rhythmicity. Not surprisingly, upon transfer to constant conditions chronic alteration of LNvs excitability initially led to desynchronization of molecular rhythms in different circadian subpopulations, as indicated by loss of rhythmicity at the behavioral level; however, after 5–6 days in DD roughly half of the population displayed complex behavioral rhythms (i.e. one short and one long rhythmic component), suggesting that resynchronization of the molecular oscillations within specific clusters had indeed taken place; specifically, they reported PER oscillations in DN1s and DN2s coinciding with the two peaks of activity, while the remaining clusters displayed single peaks coinciding with either one of those. Interestingly, only the long-period component was PDF-dependent (Sheeba et al., 2008b).

As mentioned earlier (see also Fig. 1), with the notable exception of the large LNvs, almost every circadian neuron responds to PDF (Shafer et al., 2008). To inquiry about the role of PDF in sustaining rhythmicity and synchronization within each independent cluster, Yoshii et al. (2009b) followed TIM oscillations through immunohistochemistry for 5 consecutive days under free-running conditions. In wild-type flies they observed

a sustained cycling pattern in CRY-positive groups (PDF-positive and negative sLNvs, CRY-positive LNds, anterior and posterior DN1s); surprisingly, the CRY-negative cells behaved differently: while no oscillations could be detected in the DN3s, the LNds (and to some extent the posterior DN1s and DN2s) changed after the first couple of days in DD and supported an almost anti-phasic molecular oscillation. As an explanation for this behavior the authors proposed that different clusters run with a different free-running period, as it appears to be the case in the mammalian SCN (Quintero et al., 2003; Yamaguchi et al., 2003). Next they investigated whether TIM immunoreactivity was affected in the *pdf* null mutant and found that in the absence of PDF both CRY-positive and -negative LNds display in-phase, high-amplitude cycling, with little to no effect on the remaining CRY-negative clusters. These and other observations led the authors to propose that PDF acts on circadian neurons in a rather complex manner, mediating synchronization of certain subsets (sLNvs, anterior DN1s) but not in other clusters (such as the CRY-positive LNds and PDF-negative sLNv); remarkably, in all these clusters PDF would promote period lengthening, in contrast to the CRY-negative LNds, in which it promotes period shortening. Additionally, certain dorsal clusters (CRY-negative posterior DN1s, DN2 and DN1s) do not require PDF signals for synchronization, but PDF affects the speed of their clocks (Yoshii et al., 2009b). Their conclusions were corroborated in part by Zhang et al. (2009), who also investigated the interaction between PDF and CRY in mediating entrainment of molecular clocks; employing different genetic backgrounds they also concluded that PDF and CRY cooperate to set the phase and amplitude of the molecular clocks of the evening oscillators.

CONCLUDING REMARKS

Although we are still on the early days, and the connectivity within the network is far from being understood, it is important to keep in mind that neural clocks, although built upon very similar building blocks, are not a unique entity, and these differences in the molecular underpinnings of independent clocks are required to confer the organism the ability to cope with a changing environment. In other words, a robust clock within a plastic network ensures coherence in circadian outputs.

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