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Circadian changes in neuronal networks

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The circadian clock generates circadian plasticity in some of the clock and non-clock neurons leading to the daily changes in their structure and in the number of synaptic contacts. This plasticity affects neuronal networks in the brain. The two best known examples of circadian changes in neuronal networks are those observed in the first optic neuropil (lamina) of the fly's visual system and between one group of clock neurons, the small ventral lateral neurons (s-LNvs), and their target cells in the dorsal part of the *Drosophila* brain. Both of these networks are remodeled in the course of the day by the circadian clock and they are further affected by external stimuli.

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Introduction

Neurons in the brain are organized in circuits devoted to specific functions and connected in networks that are established during development. They are, however, plastic since neurons are connected through synapses, which show plasticity in both physiology and structure. During development and later in life new synapses can also be formed and old ones retracted. This means that neurons can rewire to form new networks, especially after experience, learning and injury [1,2]. In addition to synapses, neurons also show plasticity in size and shape of dendrites and axons, which have been reported in insects and mammals [3,4]. In insects, plastic changes have been observed in synaptic neuropils of the brain of *Drosophila melanogaster* [5] in response to living conditions and in the fly's first optic neuropil (lamina) after light, visual and motor stimulations [6–8,9•]. In the fly's lamina, however, beside stimulus-dependent plasticity, a new type of plasticity, the circadian plasticity, has been detected [10,11]. The fact that the brain not only responds to external inputs [8] but is also remodeled during the day and night suggests that the distinct networks may be required for its diurnal and nocturnal functions. So far, the circadian remodeling of neurons' morphology and their synaptic contacts has been detected in the visual system of three fly species (*Musca domestica*, *Calliphora vicina*, *D. melanogaster*) [6,7,9•,12,13,14••,15•], and in the circadian system of the *Drosophila* brain [16•,17••] (Figure 1).

Circadian plasticity of clock neurons

In the brain of *Drosophila*, the network of clock neurons (described in detail in another chapter of this issue) is composed of about 150 cells (Figure 1a) that have been identified by the cyclic expression of the main clock genes, *period* (*per*) and *timeless* (*tim*). They are divided into seven groups, which seem to be specific in its properties and functions within the circadian network. Among the clock neurons, ventral lateral neurons (LNvs), especially those with small cell bodies (s-LNvs), have been proposed to be crucial for generating circadian rhythms in behaviour [18]. Large LNvs (l-LNvs) and s-LNvs, except for the 5th s-LNv, express the neuropeptide pigment-dispersing factor (PDF). The 5th s-LNv is immunoreactive to another neuropeptide — ion transport peptide (ITP) [19].

The organization of the circadian network is still under investigation but it has been suggested that clock neurons form a hierarchical system, in which s-LNvs are the most important, at least for maintaining the circadian rhythm in locomotor activity [20]. Generally clock neurons are functionally divided into morning and evening cells, generating morning and evening peaks of locomotor activity [21]. Recently it has also been suggested that clock cells form a network, in which groups of cells activate or inhibit each other [22,23], delivering the circadian (about 24 h) output to target cells of several brain circuits. Clock neurons, however, are also plastic and connections between them and their target neurons may change in response to external and internal stimuli. Light affects the pacemaker neuron branching in at least two insect species, in the cockroach [24] and in *Drosophila* [25]. In *Drosophila* larvae increased light stimulation reduces arborization and synaptic responses in LNvs, while decreased exposure has the opposite effect [25]. It indicates that the morphology of clock neurons and the network between photoreceptors of the Bolwig's organ, the larval photosensitive system, are plastic and they are shaped by external inputs. The larval LNvs do not show,

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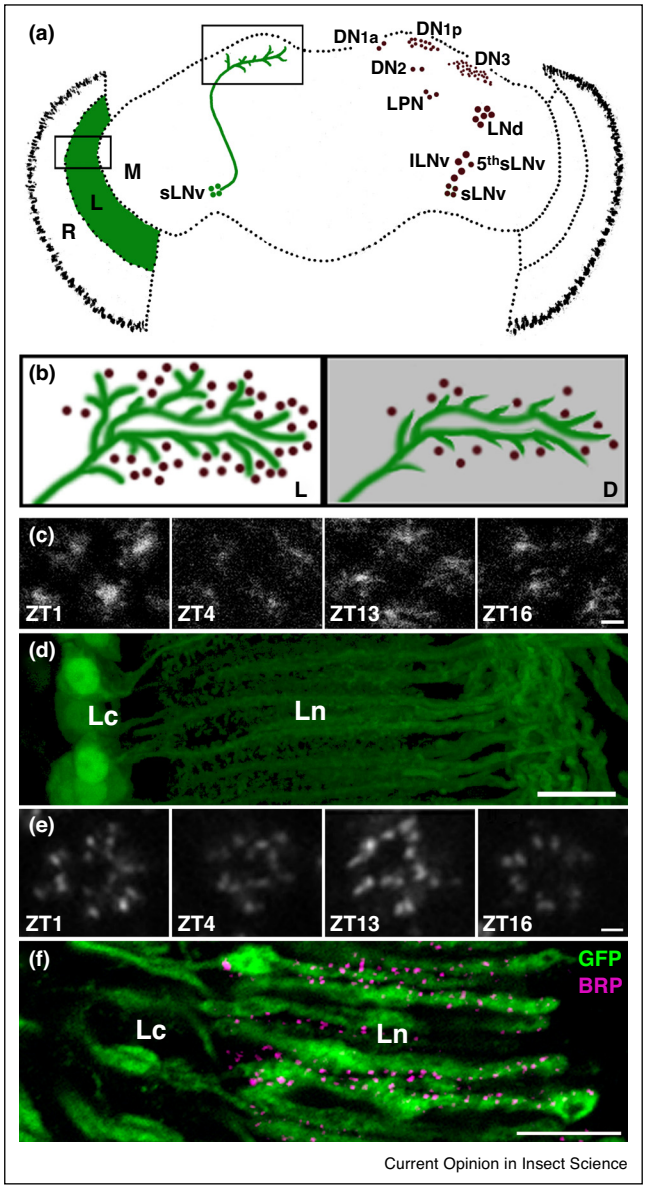
Figure 1



(a) Clusters of clock neurons that comprise the circadian network and sites of reported circadian plasticity (in green) in the brain of *Drosophila melanogaster*. The circadian plastic changes have been observed in the first visual neuropil or lamina (L) and terminals of the small ventral lateral neurons, s-LNvs (windows). R — retina, M — medulla, DN — dorsal neurons, LPN — lateral posterior neurons, LNd — dorsal lateral neurons, l-LNv — large ventral lateral neurons. **(b)** Remodeling of the s-LNv terminals under light-dark (LD) conditions. Differences in their branching pattern, which are observed between the day (L) and night (D), are accompanied by daily alterations in the number of synapses (dots) [17]. **(c)** Daily changes in the morphology of cross-sectioned dendritic tree of L2 (ZT1, ZT4, ZT13, ZT16) as revealed by the expression of Green Fluorescent Protein (GFP) in *21D-GAL4–UAS-S65T-GFP* transgenic line of *Drosophila melanogaster*. ZT — Zeitgeber Time, ZT1 — 1 h after lights-on, ZT4 — 4 h after lights-on, ZT13 — 1 h after lights-off, ZT16 — 4 h after lights-off (ZT0 and ZT12 denote the beginning of the day and night, respectively). Scale bar: 5 μm. **(d)** Cell bodies and axons of L2 monopolar cells in the lamina cortex (Lc) and lamina neuropil (Ln), respectively. The L2 axons posses short protruding dendrites forming a characteristic dendritic tree that continues throughout the depth of the lamina neuropil. Scale bar: 10 μm. **(e)** Daily changes in the abundance of BRP in the synaptic cartridge of the lamina neuropil in LD 12:12 (ZT1, ZT4, ZT13, ZT16). Scale bar: 1 μm. **(f)** The majority of BRP (magenta) is located in tetrad synapses of the terminals of R1–R6 photoreceptors, marked by the expression of GFP in *Rh1-GAL4–UAS-S65T-GFP* transgenic line of *Drosophila melanogaster*. Lc — lamina cortex, Ln — lamina neuropil, Scale bar: 10 μm.

however, daily oscillations in branching. In turn in adult flies, the s-LNvs display circadian plasticity that is modulated by light. In adult flies cyclic changes of the s-LNv terminal structure were more pronounced in constant darkness (DD) than in light/dark (LD) conditions [16], indicating that light have a similar inhibitory effect on the structure of s-LNvs as it has been observed in larvae [25].

So far synaptic contacts between clock neurons have not been described and the paracrine transmission of circadian information has been postulated [15•,26,27•], yet silencing the presynaptic scaffolding protein Bruchpilot (BRP) in *per* expressing cells changes locomotor activity level in *Drosophila* [Pyza and Damulewicz, unpublished results]. This strongly implies that synaptic contacts within the clock network and/or with its output neurons affect behaviour. Some of the clock neurons possess PDF receptors [28] so they may receive information from s-LNvs and l-LNvs by the volume transmission of PDF. In turn, the clock neurons form a network with target cells and the s-LNvs are especially interesting owing to the fact that their terminals show the circadian rhythm in morphology. They cyclically release PDF [29] and their terminals show pronounced circadian changes in branching [16•]. Their primary branches oscillate in fasciculation and defasciculation and new processes can be added or retracted in the secondary or tertiary order branches [17••]. The shortest primary processes were observed at the end of subjective night. In addition, minor terminals change their position, probably to contact different target cells of the dorsal protocerebrum in the course of the day [17••] (Figure 1b). Indeed, circadian changes in the number of synaptic contacts between the s-LNv terminals and postsynaptic cells have been detected using immunohistochemistry with the antibody against BRP and the synaptic vesicle protein Synaptotagmin (SYN) [17••]. The SYN immunoreactivity changes revealed that synaptic vesicles start to accumulate in the s-LNv terminals at the end of the day and BRP level increases at the beginning of the day. In turn, GFP reconstitution across synaptic partners (GRASP method) detected different clusters of neurons receiving synaptic inputs from the s-LNvs along the day. The structural remodeling of s-LNvs depends on the transcription factor MEF2 [30], involved in activity dependent neuronal plasticity in mammals [31]. In *Drosophila* the expression of *Mef2* is controlled by the circadian clock and this gene has been

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detected in both l-LNvs and s-LNvs [32,33]. MEF2 targets axon fasciculation and cell adhesion genes, including *Fas2* that encodes a NCAM homolog [30].

Although the main role of the s-LNvs in generating the circadian rhythm has been established by studying locomotor activity, they can provide circadian inputs not only to motor but also to other brain circuits [17••]. The s-LNvs may promote and inhibit processes at the same time not only by modulating the connectivity with both other clock neurons and output neurons, but also by establishing different circadian networks during the day. The circadian plasticity of s-LNv terminals do not exclude, however, a possibility that other clock cells provide circadian inputs to other regions of the brain, for example to the optic lobe. Large LNvs, according to present opinion, synchronize morning and evening peaks of locomotor activity in light/dark regime (LD 12:12) to the beginning of day and night, respectively [34]. These cells are important for arousal [35] but cannot maintain the rhythm in locomotor activity in constant darkness (DD). They have dense arborization with numerous varicosities, the sites of peptide release, in the medulla, the second optic neuropil of the visual system. These varicosities display daily changes in size in the housefly [36]. The similar oscillations of varicosities and cyclic release of PDF have not been found in *Drosophila*. It is possible, however, that in the smaller brain of *Drosophila* these cyclic changes were below the detection level. Another possibility is that other mechanisms are involved in transmission of circadian information to the visual system in this species. Because the lack of PDF affects the network of neurons processing photic information in the lamina [37••], it may indeed be one of the transmitters of circadian information from the clock neurons (the pacemaker) to the visual system.

Clock-controlled visual network

The first example of circadian plasticity in the brain was found in the lamina [6,7] (Figures 1a and 2a) of the optic lobe. In this neuropil, tetrad synapses, which are formed between the retina photoreceptor terminals and the lamina cells (Figure 2), change in number during the day and night. Among eight photoreceptors in each ommatidium of the compound eye of flies, only six (R1–R6) form tetrad synapses in the lamina (Figure 2b–d) while the two others, R7 and R8, pass the lamina to terminate in the medulla. Since tetrad synapses show daily changes in the frequency, the circuit consisted of photoreceptors and their four postsynaptic cells: monopolar cells L1 and L2 (in *Drosophila* also L3), amacrine and glial cells, changes in the course of the day. In *Drosophila* tetrad synapses peak in number at the beginning of the day (morning peak) and at the beginning of the night (evening peak) [Pyza and Woznicka, unpublished results] resembling the pattern of locomotor activity in this species. In the housefly, the increase of the tetrad synapse number also correlates with the peak of locomotor activity [6]. Additionally, oscillations in the number of their presynaptic elements are paralleled with changes of sizes of postsynaptic cells L1 and L2 [12]. Their axons are thicker and dendrites longer when the number of tetrad presynaptic profiles increases [12,38] (Figure 1c and d). The L2 cell morphology changes, however, with lamina depth. In the proximal lamina the L2 dendrites are largest at the beginning of the night [Pyza and Kijak, unpublished results], whereas in the distal lamina at the beginning of the day [38]. In the lamina, not only tetrad synapses but also feedback synapses (Figure 2e) cycle during the 24 h period. They are formed between L2 and two postsynaptic cells; one of the six photoreceptor terminals and the so-called medulla T1 cell. The feedback synapses increase in the number during the night in the housefly and in *Drosophila* ([6]; Pyza and Woznicka, unpublished results). Unfortunately, circadian plasticity of other synaptic contacts in the lamina have not been studied. Nevertheless the cycling of just these two types of synapses (tetrads and feedbacks) provides an evidence for daily remodeling of the network between the retina and lamina, to secure the efficient transmission of visual information during the high motor activity of flies.

The number of tetrad synapses, examined by counting presynaptic profiles, correlates with the expression of the presynaptic scaffolding protein BRP [14••] (Figure 1e and f). Therefore any changes in synaptic contacts can be evaluated by measuring the level of BRP in particular synapse type. In tetrad synapses (Figure 2b–d) this protein oscillates in abundance in both LD 12:12 (12 h of light and 12 h of darkness) and DD. In LD 12:12, BRP in tetrad synapses cycles with two, morning and evening, peaks (Figure 1e) but in DD shows a peak at the beginning of the subjective night. A similar pattern of BRP oscillations has also been found in the whole brain homogenate of *Drosophila*, suggesting that the majority of synapses in the brain peaks at the beginning of the day and night [14••]. The circadian rhythm of BRP depends on clock genes, because it is abolished in the arrhythmic per^0 null mutant, however, the two peaks are regulated in different ways. Our studies on BRP level in the lamina cartridges (Figure 1e) [14••] revealed that the morning peak is predominantly regulated by light, since it is present neither in wild type Canton S flies in DD, nor in the *norpA* phototransduction mutant. In turn, the second (evening) peak is regulated by the circadian clock because it is not present in per^{01} and tim^{01} mutants. Considering that *per* is expressed not only in the clock neurons but also in the retina photoreceptors and glial cells, silencing *per* in those cells has shown that circadian changes in BRP level in tetrad synapses are regulated by three circadian inputs, from the retina, glial cells and the pacemaker. The decreased level of PER in the retina and in glial circadian oscillators changes the pattern of BRP rhythm in tetrad synapses [14••]. Therefore the

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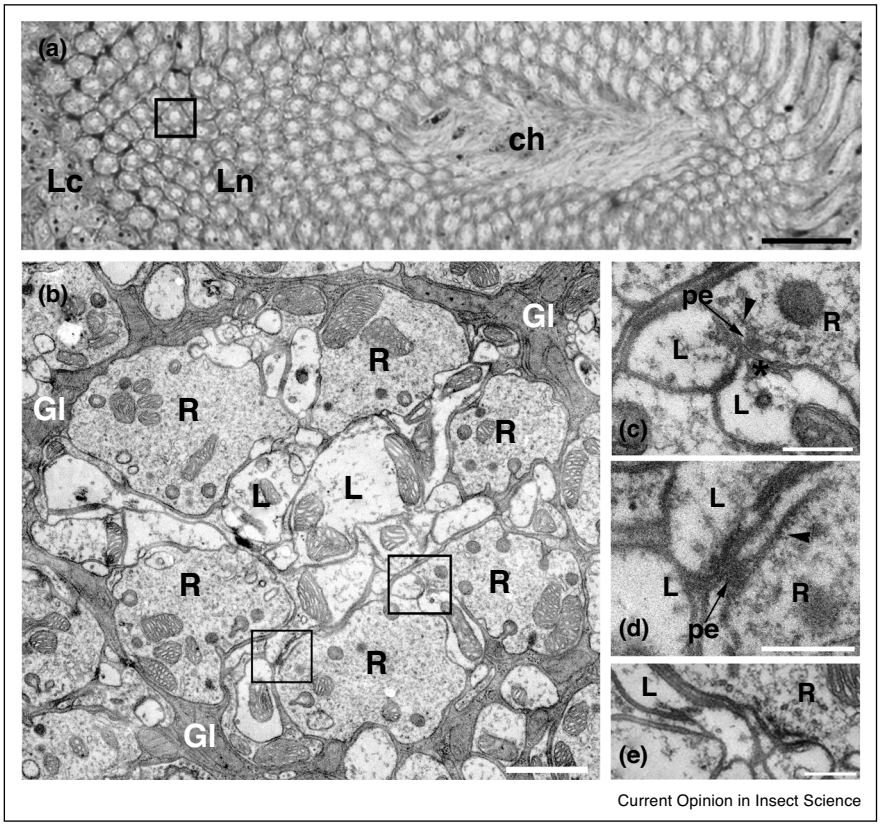
Figure 2



(a) The fragment of cross-sectioned lamina showing the cylindrical units of its neuropil, so called cartridges (small window). Lc — lamina cortex, Ln — lamina neuropil, ch — optic chiasm. Scale bar: 50 μm. **(b)** EM micrograph of a single cartridge with L1, L2 monopolar cell profiles (L) at its axis and the surrounding terminals of R1–R6 photoreceptors (R), as well as the epithelial glial cells (Gl). The presynaptic elements (T-bars) of typical tetrad synapses can be observed inside two photoreceptor terminals (windows). Scale bar: 1 μm. **(c)** and **(d)** The magnification of tetrad synapses from panel b showing the platforms (arrowheads) and the pedestals (pe) of presynaptic ribbons in photoreceptor terminals (R), opposite which lie the profiles of L1 and L2 (L) with postsynaptic cisternae (asterisk). Scale bar: 250 nm. **(e)** Profiles of tetrad and feedback synapses localized in the axon of photoreceptor (R) and L2 monopolar cell (L), respectively. Scale bar: 250 nm.

generation of circadian changes in the lamina network is complex and it is not only regulated locally but also by the input from the central clock. In contrast to BRP protein, the level of BRP mRNA does not oscillate in head homogenates [9•]. However, daily oscillations in *brp* expression have been recently detected in photoreceptors and the lamina L2 cells [Pyza and Damulewicz, unpublished results].

We have found that apart from BRP, other synaptic proteins, such as Synapsin, a protein involved in regulating the reserved pool of synaptic vesicles [39], and the presynaptic and postsynaptic Disk-Large (DLG) protein oscillate in DD, in the pattern specific for each protein [9•].

The network between clock cells and the visual system

In the visual system not only synaptic proteins change their level during the day and night but also the α subunit of the Na^+/K^+-ATPase, the main ion pump in all cells, including neurons and glia [37••,40]. The rhythm of the α subunit in the lamina is regulated, as the rhythm of BRP, by circadian inputs from the retina, glial cells and the pacemaker [37••]. By changing the level of catalytic subunit (α) of the sodium pump during the day, the pacemaker and peripheral clocks in the retina and glial cells may regulate neuron excitability and/or adhesion between cells. Such properties of the sodium pump in all neurons of the brain may affect their network in a circadian fashion. The α subunit of the sodium pump in the lamina seems to receive circadian modulatory inputs from the pacemaker by PDF and ITP. The lack of just one of these neuropeptides affects the pattern of the daily rhythm of the α subunit of Na^+/K^+-ATPase [37••]. PDF is probably released from varicosities of PDF neuronal processes in the medulla and diffuses to the lamina. The ITP-immunoreactive processes are in the distal lamina. They seem to originate from the 5th s-LNv [15•].

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Conclusions and perspectives

The two examples of the circadian control of networks in the brain, between s-LNvs and their target cells in the dorsal protocerebrum and within the visual system, indicate that circadian clocks control both presynaptic and postsynaptic cells in the networks by increasing or decreasing strength and/or number of contacts between neurons. Moreover, networks could be formed between different sets of neurons if presynaptic and/or postsynaptic neurons change morphology and size of their processes. Therefore, the networks are remodeled during the day and night to accomplish different functions of the brain in the course of the day.

Although this review describes circadian neuronal networks and their plasticity in the brain, a similar structural circadian plasticity has also been observed in the peripheral nervous system. The synaptic bouton structure and synapse number in motor terminals of *Drosophila* also show circadian rhythms [41,42]. All changes in synaptic contacts are generated by the central and/or peripheral clocks, and modulated by external stimuli.

Networks in the brain are devoted to different functions but they interact with each other and various brain functions are clock-controlled in time. For example, the visual circuit in flies receives and processes sensory information in response to light but its activity is regulated in time by the circadian network of clock cells — the pacemaker and by local circadian oscillators. It is tempting to formulate a hypothesis that the circadian system controls different networks in the brain by the cyclic reorganization of synapses and the remodeling of dendrite and axon morphology. This can be obtained by two mechanisms, a direct synaptic transmission and the paracrine signaling of neuropeptides. The latter may control, for example, the sodium pump activity in neurons and glial cells. Future studies should concentrate on the mechanisms of circadian control of various brain functions.

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