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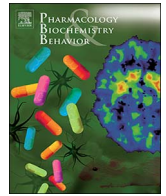
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Review

Contributions of the lateral habenula to circadian timekeeping



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ABSTRACT

Over the past 20 years, substantive research has firmly implicated the lateral habenula in myriad neural processes including addiction, depression, and sleep. More recently, evidence has emerged suggesting that the lateral habenula is a component of the brain's intrinsic daily or circadian timekeeping system. This system centers on the master circadian pacemaker in the suprachiasmatic nuclei of the hypothalamus that is synchronized to the external world through environmental light information received directly from the eye. Rhythmic clock gene expression in suprachiasmatic neurons drives variation in their electrical activity enabling communication of temporal information, and the organization of circadian rhythms in downstream targets. Here, we review the evidence implicating the lateral habenula as part of an extended neural circadian system. We consider findings suggesting that the lateral habenula is a recipient of circadian signals from the suprachiasmatic nuclei as well as light information from the eye. Further we examine the proposition that the lateral habenula itself expresses intrinsic clock gene and neuronal rhythms. We then speculate on how circadian information communicated from the lateral habenula could influence activity and function in downstream targets such as the ventral tegmental area and raphe nuclei.

As reviewed extensively elsewhere in this issue, the lateral habenula (LHb) is implicated in a wide range of brain and behavioral activities. One characteristic of the LHb that has been much neglected is its contribution to the brain's daily or circadian timekeeping system. The circadian system is responsible for coordinating internal physiology with the external world, including organizing day-night rhythms in rest and active brain states and behaviors. In this review, we provide an overview of the brain's circadian timekeeping system and evaluate the evidence that the LHb and to a lesser extent, the medial habenula (MHb), constitute important components of an extended neural circadian system. We then speculate on how circadian signals could influence the more established functions of this epithalamic structure.

1. The circadian timekeeping system

Intrinsic near 24 h or circadian rhythms pervade all aspects of physiology and behavior. In the brain, circadian rhythms influence neuronal activity, neurochemical synthesis and release as well as receptor availability and these all contribute to daily patterns in drug

sensitivity, cognitive performance, and rest-alert states (Perreau-Lenz and Spanagel, 2015; Silver and Kriegsfeld, 2014; Webb et al., 2015). These rhythms are generated by internal circadian timekeepers or clocks that have evolved to anticipate and prepare an organism for probable physiological and behavioral challenges across the 24 h temporal landscape (Mistlberger, 2011; Panda, 2016). Remarkably, the molecular basis for these clocks is largely conserved from flies to mice to humans and consists primarily of interlocking transcriptional-translational feedback loops (TTFL) that generate an internal molecular representation of the 24 h day. In mammals, the *Period* (*Per1-2*), *Cryptochrome* (*Cry1-2*) and *Bmal1* genes and their associated protein products are important components of the molecular circadian clock (Okamura, 2007; Partch et al., 2014; Reppert and Weaver, 2002) (Fig. 1). Substantial evidence indicates that the molecular clock is present in most cells and tissues in the brain and body (Gerber et al., 2015). However, in the brain, one structure, the suprachiasmatic nuclei (SCN) of the hypothalamus is imbued with particular properties, enabling it to function as the master circadian pacemaker, coordinating circadian rhythms throughout the body (Buijs et al., 2016). In rodents,

Abbreviations: AVP, arginine vasopressin; *Cry*, *Cryptochrome* gene; DA, dopamine; DBS, deep brain stimulation; DMH, dorsomedial hypothalamus; eGFP, destabilized green fluorescent protein; *fr*, *fasciculus retroflexus*; GRP, gastrin-releasing peptide; Hb, habenula; ipRGCs, intrinsically photosensitive retinal ganglion cells; LH, lateral hypothalamic area; LHb, lateral habenula; LHbL, lateral part of the lateral habenula; LHbM, medial part of the lateral habenula; Luc, luciferase; MHb, medial habenula; *Per*, *Period* gene; *Per1-luc*, *Per1*-luciferase transgene; PER2::LUC, *mPER2^{Luc}* fusion protein; PK2, prokineticin 2; PK2R, prokineticin 2 receptor; PVN, paraventricular nuclei of the hypothalamus; RMTg, Rostromedial Tegmentum; SCN, suprachiasmatic nuclei; 5HT, serotonin; SFR, spontaneous firing rate; TTFL, transcriptional-translational feedback loop; VIP, vasoactive intestinal polypeptide; VTA, ventral tegmental area; TTX, tetrodotoxin

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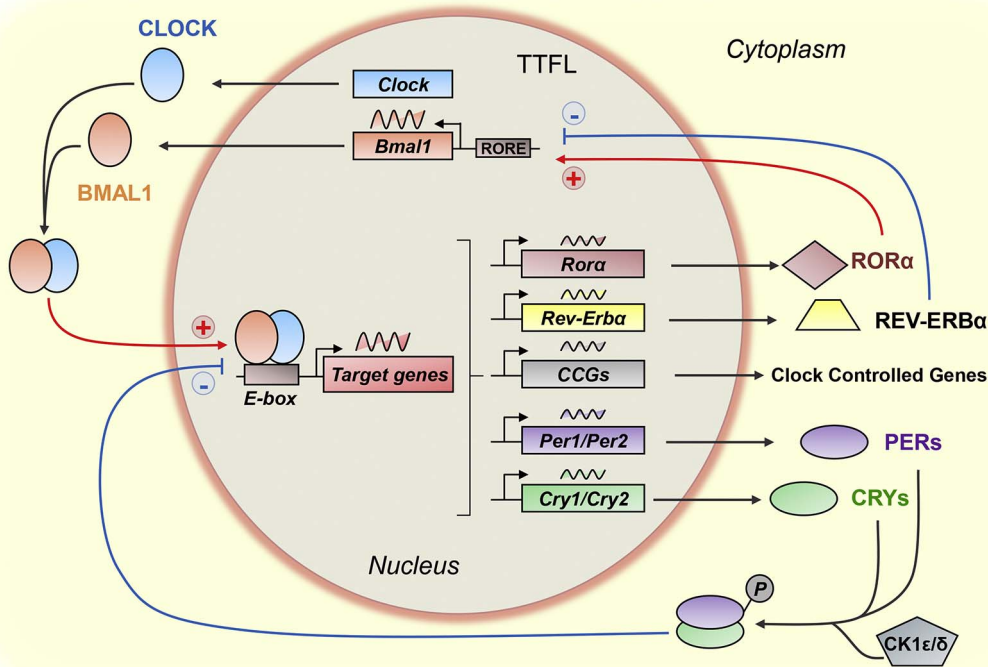


Fig. 1. Simplified model of the mammalian molecular circadian clock. The core molecular clock machinery is composed of an autoregulatory genetic oscillator, integrating negative and positive transcription/translation feedback loops (TTFL). CLOCK and BMAL1 drive the positive arm. They form heterodimers that activate transcription of target genes by binding to E-box sequences. These include *Per*, *Cry*, *Rev-erba*, *Rora* as well as other *clock-controlled genes* (CCGs). Negative feedback is mediated by PER and CRY proteins which inhibit the activity of CLOCK:BMAL1, and therefore, repress their own transcription. A secondary stabilizing loop involves inhibition of *Bmal1* transcription by REV-ERB α and activation by ROR α through binding to ROR response elements (ROREs). Functioning of the clock is modulated by post-translational modifications including phosphorylation by proteins such as Casein kinase 1 epsilon (CK1 ϵ) and delta (CK1 δ) which control the stability and translocation of proteins to the nucleus. The activity of this molecular clock gives rise to rhythmic patterns of gene expression which in turn drive circadian rhythms in physiology and behavior.

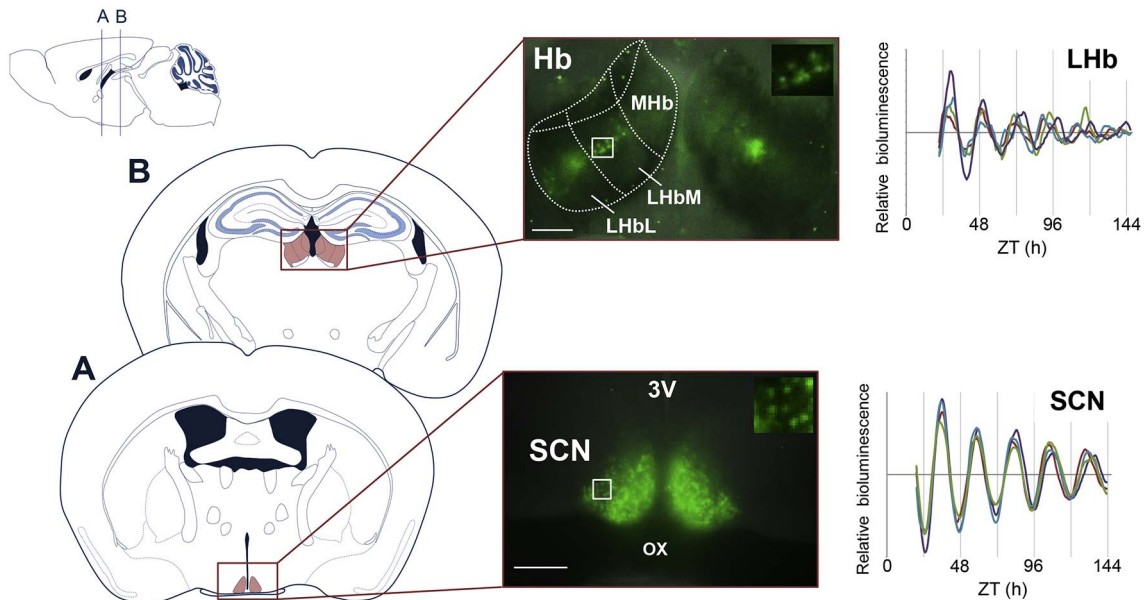


Fig. 2. Endogenous circadian rhythms in PER2::LUC expression in the mouse suprachiasmatic nuclei (SCN) and the habenula (Hb). Schematic coronal brain sections containing either the SCN (A) or the Hb (B) colored in red. Insets are EM-CCD images overlaid on bright-field photomicrographs of SCN or Hb cultured explants showing PER2::LUC bioluminescence (green). The dotted white lines delineate the anatomical regions of the medial habenula (MHb) and the medial and lateral subdivisions of the lateral habenula (LHbM and LHbL, respectively). Calibration bar 250 μ m. Plots on the right show relative bioluminescence of individual representative cells from single slices of the SCN (bottom right) and LHbM (top right). Schematic sagittal and coronal brain sections are adapted from the mouse brain atlas (Paxinos and Franklin, 2001). 3V, third ventricle; OX, optic chiasm; ZT, Zeitgeber Time. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

experimental lesion of the SCN or impairment of its molecular clock abolishes many daily rhythms in consummatory behavior, locomotor activity, rest-wake states, and endocrine function (Silver and Kriegsfeld, 2014; Welsh et al., 2010).

The SCN pacemaker is synchronized to the external world by retinal efferents that convey information on environmental light levels directly to the SCN. This glutamatergic pathway originates from the intrinsically photosensitive retinal ganglion cells (ipRGCs). The photopigment melanopsin is contained in ipRGCs rendering them sensitive to blue-green wavelengths of light as well as intensities of light associated with dawn and dusk (Hughes et al., 2015; Lucas et al., 2014; Schmidt et al., 2011). They function to integrate and signal environmental lighting information to the SCN as well as other structures in the brain including the intergeniculate leaflet, olivary pretectal nucleus, lateral habenula and medial amygdala (Brown, 2016; Hattar et al., 2006; Martersteck et al., 2017). Thus, ipRGCs not only participate in circadian photoentrainment, but can drive changes in neuronal activity of different brain regions leading to widespread effects in adaptive behavior (Milosavljevic et al., 2016).

The SCN circadian pacemaker is also responsive to stimuli that elevate internal arousal. These so-called non-photic cues include scheduled meals in the rest period, physical exercise, and social interactions (Bass, 2012; Challet, 2013; Hughes and Piggins, 2012; Mistlberger and Antle, 2011). Non-photic information is conveyed to the SCN *via* neural pathways distinct from that of the direct light-input, and originate in forebrain, thalamic, and brainstem structures (Morin, 2013).

The development of rodent models with fluorescent (*e.g.* destabilized green fluorescent protein or eGFP; (Kuhlman et al., 2000) or bioluminescent reporters [*e.g.* luciferase or luc; (Yamaguchi et al., 2003)] of the molecular clock has enabled visualization of the SCN circadian clock in living tissue explants (Fig. 2A). With suitable imaging, rhythms can be tracked at single cell level and from such recordings, it is now recognized that there is variation in the rhythm generating capabilities of different SCN subregions (Evans et al., 2013; Inagaki et al., 2007; Yamaguchi et al., 2003). Moreover, with the use tetrodotoxin (TTX) to block action-potential dependent synaptic communication, the amplitude of and coordination between single cell rhythms decrease, indicating that intercellular communication is critical for both rhythm generation and maintaining synchrony among the cell autonomous oscillators of the SCN (Aton et al., 2005; Yamaguchi et al., 2003). Current evidence indicates that the neurotransmitter GABA, and the neuropeptides arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) play key roles promoting cell-cell synchrony in the SCN (Aton et al., 2005; DeWoskin et al., 2015; Maywood et al., 2011; Myung et al., 2015).

The molecular clock drives SCN neurons to exhibit a marked 24 h rhythm in the spontaneous firing rate (SFR) of action potentials (Colwell, 2011). This can be recorded as multiunit activity *in vivo* and remarkably as single neuronal discharge in brain slices *in vitro* as well as in dissociated SCN neurons in culture (Brown and Piggins, 2007). This rhythm in electrical activity facilitates the communication of time of day/circadian cycle information to the rest of brain and body *via* paracrine and neural signals (Buijs et al., 2016). The principal target of SCN efferents is the hypothalamus, particularly the subparaventricular zone and its adjacent structures (Moore, 2013; Morin, 2013; Vujovic et al., 2015). In addition, extrahypothalamic structures receive direct SCN projections (Kalsbeek et al., 2011), including potentially the habenula (Hb) (Buijs et al., 1978; Zhang et al., 2009). The neurochemical nature of these output signals is not well-understood, but is believed to include the neurotransmitter GABA which is synthesized by the majority of SCN cells as well as the neuropeptides AVP, VIP, gastrin-releasing peptide (GRP), and prokineticin 2 (PK2) which are differentially expressed in the SCN (Antle and Silver, 2005; Moore, 2013). Indeed AVP-containing terminals were initially identified in the Hb and this raised the possibility that they originated from neurons in the SCN

(Buijs et al., 1978). Trans-synaptic pathways also link the SCN with the pineal, pituitary, and adrenal glands, with SCN signals shaping the daily patterns of release of hormones such as melatonin and corticosterone to communicate circadian information throughout the body (Kalsbeek and Fliers, 2013; Oster et al., 2016).

2. The habenula as a component of the extended neural circadian system

Intriguingly, rhythmic clock gene expression is not restricted to the SCN and there is now considerable evidence that oscillators of varying robustness are found in a number of brain structures including the Hb (Abe et al., 2002; Abraham et al., 2005; Granados-Fuentes et al., 2004; Guilding et al., 2009; Guilding et al., 2010; Guilding and Piggins, 2007). This suggests that the neural circadian timekeeping system is more extensive than previously believed (Guilding and Piggins, 2007), and determining how extra-SCN oscillators regulate local tissue physiology and function has emerged as an important task in neuroscience.

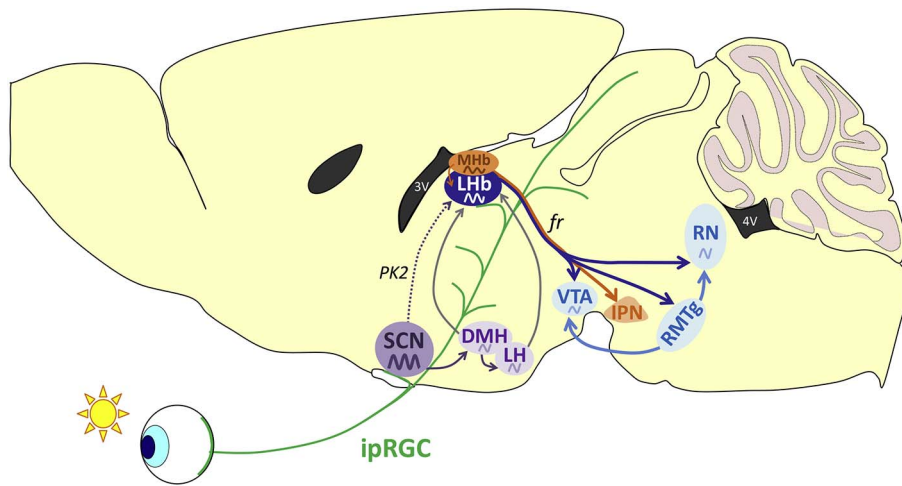
The Hb is a bilateral structure located in the epithalamus, adjacent to the dorsal third ventricle and above the posterior-dorsal edge of the thalamus. It was named based on its elongated shape (from the Latin word *habena* meaning, “little rein”) and is highly conserved across vertebrates (Bianco and Wilson, 2009). Anatomically, the Hb is strategically positioned to act as a relay station, receiving, integrating, and conveying information from forebrain to midbrain structures. Its main input pathway is the *stria medullaris* and it sends efferent projections through the *fasciculus retroflexus* (*fr*). Together with its afferents and efferents, the Hb forms the dorsal diencephalic conduction system (Herkenham and Nauta, 1979; Sutherland, 1982).

The Hb is composed of two major subregions, the medial (MHb) and the lateral habenula (LHb) (Ramon y Cajal, 1894) that differ in their anatomical connections, neurochemistry and gene expression profiles as well as function (Lecourtier and Kelly, 2007; Namboodiri et al., 2016; Wagner et al., 2016a). The LHb is further broadly partitioned into medial (LHbM) and lateral compartments (LHbL), although cytoarchitecture and immunohistochemical studies have revealed a more complex and heterogeneous organization with up to 10 subnuclei in mouse and rat LHb (Aizawa et al., 2012; Wagner et al., 2016b; Wagner et al., 2014).

2.1. Extrinsic circadian regulation of LHb activity

As noted above, the idea that the Hb has a role in circadian rhythmicity initially stemmed from immunohistochemical evidence that SCN neurons contain AVP and that AVP-ir fibers were present in the LHb (Buijs et al., 1978; Sofroniew and Weindl, 1978; Vandesande et al., 1975), leading to speculation that the SCN could signal timekeeping information directly to the LHb *via* AVP. More recently, an investigation of a mouse model in which the projections of PK2 cells in the SCN are indicated by a fluorescent reporter construct also suggested direct connections from the SCN to the LHb (Zhang et al., 2009). However, this finding has not been independently replicated, while a new study reports that AVP-ir axons in the LHb originate from magnocellular neurons of the paraventricular nuclei (PVN) of the hypothalamus (Hernandez et al., 2015). Thus it remains unclear as to whether the SCN directly projects to the LHb.

Anatomical studies suggest that the SCN innervates the dorsomedial hypothalamus (DMH), and *via* the DMH, the lateral hypothalamic area (LH) (Morin, 2013; Saper, 2013). Interestingly, these extra-SCN hypothalamic areas show circadian rhythms in neuronal activity (Guilding et al., 2009; Ono et al., 1981) and tract-tracing experiments in rodents have revealed that such areas send projections to the LHb (Poller et al., 2013; Stamatakis et al., 2016; ter Horst and Luiten, 1986). Therefore, these hypothalamic structures form an indirect pathway by which circadian timekeeping signals from the SCN could be conveyed to the LHb (Fig. 3).



they are not intrinsically driven. The functional role of the LHb oscillator in providing circadian timekeeping information to its target areas remains unknown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Another possible conduit of indirect communication between the SCN and the LHb is via the hormone melatonin. The circadian pattern of synthesis and release of this hormone from the pineal gland is tightly controlled by the SCN (Pevet and Challet, 2011). Using autoradiography, melatonin binding sites have been described in the LHbM (Weaver et al., 1989). The presence of functional melatonin receptors within the LHb is further demonstrated in a recent electrophysiological study showing that melatonin increases glutamatergic synaptic transmission onto LHbM neurons (Evely et al., 2016).

Cyclical fluctuations in temperature within a physiological relevant range can provide an important resetting cue for mammalian extra-SCN oscillators *in vitro* (Brown et al., 2002; Buhr et al., 2010; Saini et al., 2012). Thus, by controlling circadian rhythms in body temperature, the SCN can relay temporal information to entrain peripheral oscillators. However, the influence of body temperature rhythms on the phase or presence of circadian rhythmicity in the LHb remains unexplored.

An early anatomical tracing study described a retinal projection to the LHb (Qu et al., 1996), raising the possibility that the eye directly conveys photic information to LHb neurons. However, subsequent mapping using a mouse model in which the efferents of ipRGCs are visualized with a lacZ reporter found that ipRGCs preferentially innervate the region immediately supra-adjacent to the LHb [the parabehabula; (Morin and Studholme, 2014) and not the LHb itself (Hattar et al., 2006; Sakhi et al., 2014b)] (Fig. 3). Through *in vivo* recordings, Zhao and Rusak (2005) found that neurons in the LHb responded to retinal illumination, mostly by increasing firing activity. Further, using multielectrode probes that enable simultaneous recording of activity throughout many levels of the Hb, Sakhi et al. (2014b) confirmed that LHb neurons respond to retinal illumination. However, by using a much finer temporal resolution than the 5–10s recording epochs utilized by Zhao and Rusak (2005), they determined that these light-evoked effects were too sluggish to represent direct responses (occurring some 350–400 ms following the onset of the stimulus). Instead, these effects of retinal illumination were most likely attributable to integrated responses of other visual centers that then conveyed this information to the LHb. Importantly, the mapping of responses illustrated that the ‘hotspot’ was in the very edge of LHbL and extended into the parabehabula region. Further these responses were found to be of low sensitivity (*i.e.* requiring higher levels of illumination than other brain structures receiving ipRGC input). Therefore, while the LHb has access to retinal information, the input route is more likely polysynaptic than monosynaptic and it is the LHbL/para-habenula region that is most responsive and not the main body of the LHb.

Fig. 3. Lateral habenula (LHb) as a component of the extended circadian system. Intrinsically photosensitive retinal ganglion cells (ipRGCs, green) innervate the master pacemaker (suprachiasmatic nuclei, SCN), the LHb, and other brain areas contributing to photic synchronization of circadian rhythms. Putative projections from the SCN to the LHb may convey extrinsic timekeeping signals by releasing Prokineticin 2 (PK2). However, temporal information from the SCN could also reach the LHb through polysynaptic pathways formed from direct and indirect projections to the dorsomedial hypothalamus (DMH) and the lateral hypothalamic area (LH). The LHb possesses intrinsic circadian timekeeping capabilities manifest as daily changes in neuronal activity and clock gene expression in both the LHb and medial habenula (MHb). The LHb modulates the activity of monoaminergic centers in the midbrain via direct and indirect efferents to the ventral tegmental area (VTA) and the raphe nuclei (RN). Although MHb neurons preferentially innervate the interpeduncular nucleus (IPN), unidirectional projections to the LHb also exist. VTA and RN show daily changes in electrical activity although current data suggest

2.2. Lateral Habenula (LHb) intrinsic timekeeping capabilities

The expression of the immediate early gene, *c-fos*, is typically used as a proxy for neuronal activity, (Krukoff, 1999) and immunohistochemical detection of its protein product, c-Fos, has enabled the assessment of LHb neuronal activity *in vivo*. Rhythmic changes in c-Fos-ir associated with active behavioral state are seen in the rodent LHbM (Schwartz article this issue). Thus when a rodent is resting during the day, c-Fos-ir in the LHbM is low, whereas in the behaviorally active night, c-Fos-ir in the LHbM is significantly elevated (Paul et al., 2011). This suggests a number of possibilities including: 1) neuronal activity in the LHbM is responsive to feedback from active behavioral states; 2) output from the SCN drives rhythms in the LHbM or 3) the LHbM possesses a degree of intrinsic daily rhythmicity. A pioneering electrophysiological study by Zhao and Rusak (2005) directly addressed these possibilities. They found that neuronal activity in rat LHb brain slices measured by single unit extracellular recording, varied across the 24 h cycle with peak spontaneous firing rate occurring around the middle of the day, with lower rates during the night. Thus, when isolated from feedback from behavioral state or SCN input, the LHb can sustain daily variation in neuronal activity *in vitro*.

Subsequent studies investigated whether the Hb expresses intrinsic molecular clock gene rhythms. Assessment of rhythmicity in Hb brain slices with luminometry indicated that the PER2::luciferase (LUC) fusion protein expression was rhythmic, albeit weakly, for up to 48–72 h *in vitro* (Guilding et al., 2010; Guilding et al., 2013). Visualization of this signal at single cell resolution with an EM-CCD camera-equipped microscopy system showed that rhythmic PER2::LUC expression was most prominent in the LHbM, with less distinctive signal detected in the lateral division of the LHb (Fig. 2B). Indeed, assessment of multiunit neuronal activity showed that rhythms in spontaneous discharge rate also changed across 24 h *in vitro*.

Recently, a more detailed investigation of LHb neuronal activity using whole-cell patch clamp recording, consolidated the findings of Zhao and Rusak (2005) and reported a prominent rhythm in spontaneous discharge activity. Mouse LHb neurons increase SFR from a late night/early day nadir to peak firing around the late day/early night (Sakhi et al., 2014b; Zhao and Rusak, 2005). Indeed, the early-late day variation in neuronal activity was sustained in LHb brain slices prepared from animals housed in constant dark conditions, indicating that in the absence of daily change in environmental lighting levels, circadian timekeeping can drive rhythmic changes in LHb cellular discharge rate. Consistent with this, the early to late day increase in neuronal LHb activity was absent in LHb brain slices from mice lacking a functional molecular circadian clock (*Cry1*^{-/-} *Cry2*^{-/-} mice). Interestingly, in

these recordings of *Cry1^{-/-}Cry2^{-/-}* Lhb activity, SFR was constitutively low at both early and late day time points sampled, suggesting that the molecular clock acts to increase SFR around the late day (Sakhi et al., 2014b). As noted above, weak PER2::LUC rhythms are measurable in LHbM and assessment of *Per1*-luc expression with an EM-CCD camera system revealed rhythmic expression of this TTFL reporter/gene in the LHbM which was absent in LHb brain slices prepared from *Cry1^{-/-}Cry2^{-/-}* mice. Therefore, molecular and neurophysiological evidence points to the LHb as possessing a degree of intrinsic circadian timekeeping. Interestingly, a recent study has reported that presynaptic release probability of LHb afferents varies according to the time of day. This increases across the day such that there is a higher release probability during the afternoon. Therefore, the peak in LHb SFR is coincident with maximal evoked presynaptic efficacy (Park et al., 2017). This suggests that extrinsic and intrinsic mechanisms co-operate to drive daily variation in LHb neuronal activity.

The occurrence of peak firing rate in the LHb brain slices during the mid to late day (Sakhi et al., 2014b; Zhao and Rusak, 2005) further suggests that rhythmic neuronal activity in the LHb is coincident with or phase-delayed with respect to the firing rate rhythms of SCN neurons which peak around mid-day (Cutler et al., 2003; Green and Gillette, 1982; Groos and Hendriks, 1982). As noted above, there is anatomical evidence for a putative projection from the SCN to the LHb, but there is no physiological or functional support for this conjecture. One potential candidate of this pathway is PK2 since PK2-containing neurons may innervate the Hb and mRNA for prokineticin 2 receptor (PK2R) is expressed in the LHb (Cheng et al., 2002; Zhang et al., 2009). To test if LHb neurons are responsive to PK2, Sakhi et al. (2014b) made current- and voltage-clamp recordings and found that PK2 acts on presynaptic terminals in the LHb to elevate GABA release which then signals through post-synaptic GABA_A receptors to suppress LHb neurons. Therefore, the slight delay in the firing rate rhythm noted in the mouse LHb may arise in part from the suppressive actions of an SCN-derived PK2 signal, although considerably more research is required to scrutinize this possibility.

One difficulty in determining the extent of intrinsic molecular timekeeping capability in the Hb complex *in vivo* is the variability in the description of clock gene expression in this structure. An early study (Shieh, 2003) reported expression of *Per1* and *Per2* in the rat MHb, with no expression in the LHb. In that investigation, clock genes were evaluated using a radioactive isotope and *in situ* hybridization and while the MHb appears to be clearly delineated in the autoradiograms presented, expression in the adjacent LHbM cannot be discounted due to scattering of the radioactive signal. Similarly, a recent study using a rat model of depression in which molecular clock activity was assessed using radioisotopic *in situ* hybridization, reported daily changes in *Per1*, *Per2*, and *Bmal1* in the rat LHb (Christiansen et al., 2016). However, visual inspection of the published autoradiograms suggests that it is very difficult to accurately discriminate the MHb from the LHb. Another study using qPCR to assess gene expression in microdissected brain sections reported that the molecular clock genes *Per1*, *Per2*, and *Bmal1* were expressed in the rat LHb (Zhang et al., 2016). Unfortunately, the authors do not provide precise description of tissue preparation or histological evidence to discount the possibility that their microdissected samples also contain MHb tissue. Thus there remain several procedural challenges to overcome to decisively determine if clock gene expression is widespread in the Hb or whether it occurs in particular divisions or subnuclei. This is presumably best addressed through non-isotopic *in situ* hybridization and/or immunohistochemical visualization of clock gene protein expression in fixed Hb brain sections. Indeed, in the *in vitro* assessment of *Per1*-luc and PER2::LUC in Hb tissue slices, rhythmic signal is readily monitored in the (presumed) ependymal cell layer along the medial border of the MHb, suggesting non-neuronal contributions to circadian timekeeping in the Hb at large. Further, since neuronal activity in the mouse MHb varies across the day, a degree of circadian influence on the cells of this structure is apparent (Sakhi et al.,

2014a). Interestingly, unidirectional projections from the MHb neurons to the LHb are reported (Kim and Chang, 2005). This suggests the existence of an intrinsic circuit within the Hb complex in which the MHb could modulate the electrical circadian output of the LHb; however this possibility requires further experimental scrutiny.

Collectively, these investigations suggest that the LHb is the recipient of circadian-relevant information and that the LHb possesses a degree of intrinsic circadian timing capability. An important experimental challenge is to determine how these sources of circadian regulation are integrated in the expression of molecular clock and neuronal activity rhythms.

2.3. Potential roles for the Habenula circadian oscillator

As comprehensively reviewed elsewhere in this volume, the LHb is implicated in a range of neural processes, including cognition, reward, aversion, motivated behavior and sleep-wake cycles. Interestingly, many of these show daily and/or circadian variation, but if and how rhythmicity in the LHb contributes to this is unknown.

Lhb neurons are mainly glutamatergic and their activity is tightly linked with monoaminergic modulatory centers, including the dopaminergic (DA) ventral tegmental area (VTA) in the midbrain (Fig. 3) (Lecourtier and Kelly, 2007). This LHb-VTA pathway plays a critical role in signaling negative reward prediction errors and motivation (Hikosaka, 2010). LHb neurons are activated by aversive stimuli (Stamatakis and Stuber, 2012) or negative reward prediction error (lower outcome in comparison with expectation or predicted outcome) (Matsumoto and Hikosaka, 2007), while dopaminergic VTA neurons are inhibited by such stimuli/outcomes. Since LHb neurons exhibit intrinsic daily changes in their firing rate (Sakhi et al., 2014b; Zhao and Rusak, 2005) (higher middle to late day/early night), this raises the possibility that the LHb provides rhythmic neural input to its midbrain targets. Indeed, rhythms in the firing activity of VTA DA neurons as well as in the number of spontaneously active DA neurons are reported (Dominguez-Lopez et al., 2014; Luo et al., 2008). Since rhythms in VTA activity are not detectable when the VTA is isolated in brain slices, this suggests that they are driven by signals arising from other parts of the brain (Abe et al., 2002; Mendoza and Challet, 2014; Salaberry and Mendoza, 2015; Webb et al., 2009). Further studies are needed to specifically address the potential contribution/influence of intrinsic LHb oscillations to rhythms in DAergic activity. For example, one of the main targets of LHb efferents is the GABAergic neurons of the RMTg (Herkenham and Nauta, 1979; Jhou et al., 2009) which then innervate VTA DAergic neurons. The higher firing rate of LHb neurons during the late day could then indirectly reduce VTA neuronal activity at this time (Barrot et al., 2012; Brinschwitz et al., 2010; Jhou et al., 2009). In turn this could influence the time of day at which stimuli are perceived as more or less rewarding.

Lhb neurons also send dense projections to the serotonergic (5HT) dorsal and median raphe nuclei (Pollak Dorocic et al., 2014; Quina et al., 2015), and stimulation of the LHb suppresses 5HT neurons in the raphe (Stern et al., 1979; Varga et al., 2003). This inhibitory effect appears to be mediated by activation of local GABAergic interneurons and indirectly through activation of GABAergic neurons in the RMTg area (Fig. 3). Since the dorsal and median raphe nuclei project widely to many brain regions (Vertes and Linley, 2008), including the hippocampus, SCN and lateral preoptic area, the modulation of 5HT-neuronal activity by the LHb could indirectly influence functions of their efferent targets including cognition and sleep-wake cycles [for review see (Hikosaka, 2010; Zhao et al., 2015)]. Indeed, ablation of the *fr* or specific lesion of the LHb leads to disruption of REM sleep (Aizawa et al., 2013b; Goldstein, 1983; Valjakka et al., 1998) indicating that the LHb plays an important role in sleep regulation (Aizawa et al., 2013a).

In addition, pharmacological inhibition of LHb neuronal activity (Goutagny et al., 2013) or Hb lesion (Lecourtier et al., 2004) disrupts performance in hippocampus-dependent spatial cognition tasks. As the

hippocampus does not appear to receive direct projections from the Lhb, it is believed the functional connectivity between both regions occurs *via* the serotonergic median raphe (Aizawa et al., 2013b). Theta oscillations in hippocampal neurons play a key role in memory formation (Buzsáki, 2002) and are reported to display circadian modulation (Munn et al., 2015). Intriguingly, Lhb neurons generate spontaneous theta oscillatory activity that is phase locked to hippocampal theta oscillations (Aizawa et al., 2013b; Goutagny et al., 2013). Moreover, these phase locked neurons are mainly found in the LHbM region which is also the Lhb region with the most prominent rhythmic *PER2::LUC* and *Per1-luc* expression (Aizawa et al., 2013b; Guilding et al., 2010; Sakhi et al., 2014b). However, to the date, it is unclear if theta oscillatory activity in the Lhb is under circadian influence.

Since the Lhb modulates the monoaminergic systems, it is not surprising that Lhb dysfunction is associated with the pathophysiology of psychiatric disorders, including mood alterations, schizophrenia, and drug addiction [for review see (Lecca et al., 2014; Proulx et al., 2014; Velasquez et al., 2014)]. (For detailed literature review, please see other articles published in this special Lhb issue). Hyper-activation of the Lhb is reported in both humans and animals models of depression, while lesion or inhibition of the Lhb (eg. Deep brain stimulation (DBS), pharmacology, genetically) in animal models alleviates depression-like symptoms [reviewed in (Boulos et al., 2017; Fakhoury, 2017)]. Interestingly, circadian rhythm disruption often accompanies mood alteration (Kronfeld-Schor and Einat, 2012; McCarthy and Welsh, 2012; McClung, 2013; Parekh and McClung, 2015; Wulff et al., 2010). For example, post-mortem examination of the brains of patients who had a history of major depressive disorder reveals abnormal patterns of circadian clock gene levels in extra-SCN brain regions including large shifts in the timing of peak expression, and potential dysregulation in the temporal relationship between individual genes (Li et al., 2013). Similarly, the induction of depression-like state in animals through exposure to chronic stress also elicits circadian dysregulation at behavioral and molecular level (Christiansen et al., 2016; Logan et al., 2015). This relationship appears reciprocal since conditions forcing misalignment in the internal temporal organization of the organism also promote depression-like phenotypes (Ben-Hamo et al., 2016). This raises the possibility that psychiatric conditions and Lhb timekeeping reciprocally interact such that alterations in Lhb rhythmicity impact on psychiatry diseases and that such disorders trigger circadian disruption in the Lhb. Further studies are needed to investigate whether and how circadian processes in the Lhb could help to implement DBS and pharmacological therapies by considering time-of-day.

The Hb is strongly associated with addiction [for review, see (Lecca et al., 2014)], with the Lhb implicated in cocaine and alcohol dependence and the Mhb in nicotine and opioid/morphine dependence (Velasquez et al., 2014). Interestingly, many facets of substance abuse vary with time of day including patterns of drug self-administration, responsiveness to drugs of abuse, and withdrawal severity symptoms [see (Webb et al., 2015) for review]. For example, the rewarding properties of cocaine (measured in the conditional place preference paradigm) are greater when drug was administered during early day compared to late afternoon (Abarca et al., 2002). In rats, alcohol intake follows a daily rhythm with higher consumption at night (Perreau-Lenz et al., 2012). In addition, clinical studies report a time of day effect in emergency room admission of drug overdose patients (Morris, 1987; Raymond et al., 1992). Whether the rhythmicity in Lhb neurophysiology (eg. daily patterns of receptor expression, changes in excitability, electrical activity patterns) is responsible or contributes to the daily rhythms seen in drug reward/aversion and motivation behaviors remains unknown. Further studies exploring daily responses of Lhb neuronal activity to drugs of abuse, as well as the extent to which circadian signals are conveyed from the Lhb to the RMTg-VTA, are needed to determine their influence on daily variations in reward-related neurophysiology and behavior. Such endeavors will illuminate the potential role of the Lhb as an interface between the reward and circadian

systems. Notably, this could provide insight into the optimal time-of-day at which to titrate and target pharmacotherapeutic interventions to reduce drug addiction (Salaberry and Mendoza, 2015).

As discussed above, activation in Lhb neurons is associated with active behavioral states. Under certain environmental conditions, the normally consolidated running-wheel rhythms of nocturnal rodents can disassociate into two main bouts whose onsets stabilize some 12 h out of phase with one another. Examination of *c-Fos-ir* in the SCN and LHbM of hamsters exhibiting so-called ‘split’ rhythms suggests that *c-Fos-ir* is coincidentally elevated in one lobe of the SCN and its ipsilateral LHbM, whereas in the contralateral SCN and LHbM, it is reduced (Tavakoli-Nezhad and Schwartz, 2005). This has led to speculation that the Lhb acts to promote a temporal window permissive of the expression of locomotor activity (Tavakoli-Nezhad and Schwartz, 2006). Thus, anatomical evidence has pointed to the Lhb as potential regulator of SCN output signals. Further investigations indicate voluntary exercise in wheels induces *c-Fos* expression in the LHbM (Paul et al., 2011), while sleep deprivation can change molecular clock gene expression in the Lhb (Zhang et al., 2016). This indicates that the Lhb is responsive to so-called ‘non-photoc’ stimuli. Experimental lesioning of the main output projection of the Hb, the *fasciculus retroflexus* reduces the amplitude of night-time locomotor activity, indicating that the Lhb promotes intense nocturnal activity. However, since output signals from the Mhb are also contained in the *fr*, it is unclear if this diminution in nocturnal activity is exclusively attributable to Lhb output. Indeed, an investigation in which efferent signals originating from the dorsal aspect of the Mhb were impaired reports suppression in night-time active behaviors, suggesting that the Mhb and not the Lhb contributes to promoting active behaviors at night (Hsu et al., 2014).

3. Summary & future perspectives

The Lhb acts as a relay station conveying information from fore-brain to midbrain structures. Cytoarchitecture, immunohistochemical and genetic studies have revealed a complex and heterogeneous organization within the Lhb with a variety of cell types and circuits. Here, we reviewed cumulative evidence supporting the Lhb as a part of an extended circadian system. Circadian rhythms pervade many aspects of our lives allowing organisms to coordinate their internal physiology and behavior to increase their chances of survival in a cyclical/re-current environment. This system is an organized hierarchy of multiple circadian oscillators, with the SCN as the main circadian pacemaker, synchronizing oscillators residing in other regions of the nervous system and peripheral tissues. These extra-SCN oscillators, including the Lhb, are believed to be important for regulating tissue specific physiology. Lhb not only responds to extrinsic temporal cues but importantly, expresses intrinsic circadian properties. Circadian processes taking place in the Lhb add an important layer of complexity in understanding Lhb neurophysiology and functionality, and raise important fundamental questions: (i) What is the functional role of the local Lhb clockwork? What are the consequences of its disruption? (ii) How does the Lhb integrate extrinsic and intrinsic circadian cues (e.g. light, SCN cues, rhythmic behavioral feedback, and neuromodulators) *in vivo* to regulate Hb outputs and functions?

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