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Oscillating perceptions: the ups and downs of the CLOCK protein in the mouse circadian system

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Abstract

A functional mouse CLOCK protein has long been thought to be essential for mammalian circadian clockwork function, based mainly on studies of mice bearing a dominant negative, antimorphic mutation in the *Clock* gene. However, new discoveries using recently developed *Clock*-null mutant mice have shaken up this view. In this review, I discuss how this recent work impacts and alters the previous view of the role of CLOCK in the mouse circadian clockwork.

Keywords

Clock gene; suprachiasmatic nucleus (SCN); circadian rhythms; biological clocks; negative feedback loops; CLOCK; NPAS2

Introduction

Endogenous circadian clocks drive daily rhythms of physiology and behaviour in most organisms. In mammals, circadian clocks operate in nearly all cells and tissues, and are organized hierarchically (Reppert and Weaver 2002; Lowrey and Takahashi 2004). At the top of this hierarchy is a master clock that resides within the suprachiasmatic nuclei (SCN) of the anterior hypothalamus. The SCN clock is entrained to the 24-h period by the daily light–dark cycle acting through retina to SCN pathways, and, in turn, the entrained SCN drives a number of rhythmic outputs that synchronize the phase of circadian oscillators in peripheral tissues. Oscillators in nonSCN brain regions and peripheral tissues drive the rhythmic expression of genes involved in the physiological processes carried out by those tissues (see Duffield 2003; Lowrey and Takahashi 2004). SCN and peripheral tissues differ in that intracellular oscillators within the SCN appear to be coupled via SCN neural networking (Aton and Herzog 2005), while the oscillators within the cells of nonSCN tissues are not coupled; thus rhythms of SCN as a whole can be greater than the sum of its cellular oscillators, whereas rhythms in nonSCN tissues appear to be simply the sum of the intracellular rhythms, synchronized or not (Nagoshi *et al.* 2004;Welsh *et al.* 2004; Liu *et al.* 2007).

The intracellular molecular mechanism underlying the mammalian clockwork has been most extensively studied in the mouse, and is composed of transcriptional feedback loops that drive the selfsustaining clock mechanism in both the SCN and peripheral tissues (figure 1) (Reppert and Weaver 2002;Lowrey and Takahashi 2004). At the core of the molecular clock are a pair of PAS-containing bHLH transcription factors, CLOCK and BMAL1. CLOCK:BMAL1

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heterodimers drive the rhythmic expression of three *Period* genes (*mPer1–mPer3*) and two *Cryptochrome* genes (*mCry1* and *mCry2*) through E-box enhancer elements. The resultant proteins form PER/CRY complexes that translocate back into the nucleus to inhibit CLOCK:BMAL1-mediated transcription, completing the negative transcriptional feedback loop essential for clockwork function. Posttranslational processes, particularly PER protein phosphorylation by casein kinase I (CKI)δ and CKIϵ, appear to contribute to the time delays in the feedback mechanism needed for a 24-h clock (Lowrey *et al.* 2000;Lee *et al.* 2001, 2004). A modulatory, interlocking positive transcriptional feedback loop involves the rhythmic regulation of *Bmal1* transcription that is antiphasic to the *mPer* and *mCry* mRNA rhythms, via the coordinated actions of the *Ror* families of transcriptional activators and the transcriptional repressors *Rev-erb*α/β (Preitner *et al.* 2002;Ueda *et al.* 2002;Emery and Reppert 2004;Sato *et al.* 2004;Akashi and Takumi 2005;Liu *et al.* 2008). This model of mammalian clockwork function developed over 10 years (1997–2006) and was widely accepted before the role of CLOCK in this model became much less clear.

Identification and role of the Clock gene

The *Clock* gene was among the first genes to be identified as having a critical role within the mouse circadian clockwork. It was identified as a result of recovering a mutant mouse, named Circadian Locomoter Output Cycles Kaput (CLOCK for short), from a screen for chemically-induced dominant mutations that altered circadian behaviour (Vitaterna *et al.* 1994). Positional cloning and transgenic rescue approaches were used to identify the mutant gene, *Clock*, and the sequence analysis revealed that the mutant phenotype was due to a single point mutation within the intron between exons 18 and 19 of this gene (Antoch *et al.* 1997; King *et al.* 1997a). The mutation results in aberrant mRNA splicing, such that the portion of the mRNA encoded by exon 19 is spliced out without altering the subsequent translational frame (King *et al.* 1997b). The mutant CLOCK protein that results ($CLOCK^{\Delta 19}$) lacks the 51 amino acid residues encoded by exon 19, and this internal deletion renders $CLOCK^{\Delta 19}$:BMAL1 heterodimers functionally defective (Gekakis *et al.* 1998; Jin *et al.* 1999).

Heterozygous *Clock* mutant mice ($Clock^{\Delta 19/+}$) have long circadian periods, ~25–26 h (Vitaterna *et al.* 1994), and the mutant $CLOCK^{\Delta 19}$ appears to compete with wild-type CLOCK for binding with BMAL1 when both are present (King *et al.* 1997a); therefore this mutation in *Clock* is considered to be an antimorph (King *et al.* 1997a). Heterozygous mutant mice have exaggerated resetting responses to light that suggest a reduction in overall circadian oscillator amplitude (Vitaterna *et al.* 2006). Consistent with this idea, these mice also have slight reductions in the amplitudes of *mPer* gene expression in the SCN (Vitaterna *et al.* 2006).

Homozygous *Clock* mutant mice ($Clock^{\Delta 19/\Delta 19}$) have even longer periods, ~26–28 h, which can degenerate to arrhythmicity, depending on genetic background (Vitaterna *et al.* 1994; Oishi *et al.* 2002; Kennaway *et al.* 2003; Ochi *et al.* 2003). At the molecular level, $Clock^{\Delta 19/\Delta 19}$ mice have markedly blunted molecular rhythms in the SCN (Jin *et al.* 1999; Kume *et al.* 1999; Silver *et al.* 1999; Oishi *et al.* 2000; Ripperger *et al.* 2000; Cheng *et al.* 2002; Kennaway *et al.* 2006), due to impaired transcriptional activity of $CLOCK^{\Delta 19}$:BMAL1 heterodimers. The blunted gene expression rhythms in the SCN of $Clock^{\Delta 19/\Delta 19}$ mice are presumably the underlying cause of the behavioural rhythm defects. Further, the mice that are homozygous for a null allele of *Bmal1* have disrupted behavioural and molecular rhythms (Bunger *et al.* 2000), solidifying the notion that CLOCK and BMAL1 are essential circadian clock components that provide the positive transcriptional drive within the clock.

Surprises from CLOCK-deficient mice

The view that a functional CLOCK protein was required for circadian oscillator function was shaken up by our recent studies of mice homozygous for a null mutation in *Clock* (*Clock*$^{-/-}$) (DeBruyne *et al.* 2006). We originally sought to determine the relationship between SCN and peripheral circadian oscillators by generating a null allele of the *Clock* gene that would allow tissue-specific gene disruption, hypothesizing that if we could disrupt circadian oscillator function in a specific peripheral tissue, we would be able to ascertain the specific role of the circadian oscillator in that peripheral tissue. The *Clock* gene appeared to be ideal for this as all of the data generated using *Clock*$^{\Delta 19/\Delta 19}$ mutant mice strongly suggested that the CLOCK protein was a vital component of the circadian clockwork (see above). However, since a null-mutation of the *Clock* gene had not been reported, we first conducted studies to confirm the widely-held belief that CLOCK was indeed a required component of the circadian clock.

To determine the effect of CLOCK deficiency on behavioural rhythmicity, we monitored wheel-running activity of wild-type, heterozygous and homozygous *Clock* null-mutant mice held in constant darkness (DD). Consistent with a previous report that monitored behavioural rhythms in a strain heterozygous for a chromosomal deletion containing the *Clock* gene (King *et al.* 1997a), heterozygous *Clock* null-mutant mice displayed normal and robust circadian patterns of behaviour, with rhythms similar to those of wild-type animals. Much to our surprise, all of the homozygous *Clock* null mutant mice we tested also displayed robust behavioural rhythmicity comparable to that of their wild-type siblings (figure 2,A). These CLOCK-deficient mice are not without some behavioural rhythm defects: their circadian periods were on average about 20 min shorter than their wild-type siblings, and their circadian responses to light pulses were altered (DeBruyne *et al.* 2006). Nonetheless, these findings demonstrated that CLOCK is not required for the generation of robust circadian rhythms in locomotor activity, contrary to our expectations.

The role of CLOCK in the circadian clockwork (figure 1) was proposed based largely on the analyses of mice homozygous for the antimorphic *Clock*$^{\Delta 19}$ mutation, and posits that the CLOCK protein, dimerized with BMAL1, provides the positive transcriptional drive of rhythmically expressed genes harbouring E-box elements within their promoters. Since CLOCK-deficient mice maintain robust behavioural rhythms, we examined whether or not several putative direct CLOCK:BMAL1 target genes were still rhythmically expressed without CLOCK. In the SCN, we found that expression of most genes still oscillates without CLOCK, although most had a ~50% reduction in amplitude compared to their wild-type siblings (figure 2,B) (DeBruyne *et al.* 2006). The mRNA abundance rhythms of the *mPer2* and *Dbp* genes were the exceptions: the *mPer2* rhythm was essentially normal in the SCN of CLOCK-deficient mice, and the *Dbp* mRNA rhythm was nearly abolished (figure 2,B). Examination of nuclear protein abundance rhythms for some of these genes mirrored a similar consequence—the mPER2 and mCRY1 proteins cycled in nuclear abundance in the SCN of CLOCK-deficient mice with amplitudes reduced by ~60% compared to wild-type (DeBruyne *et al.* 2006). Intriguingly, although peak *mPer1* mRNA levels were reduced to ~50% in the CLOCK-deficient SCN (figure 2,B), peak nuclear mPER1 accumulation was detectable in only ~10% of SCN nuclei. Importantly, BMAL1 still accumulated in the nuclei of CLOCK-deficient SCN, but was detectable in only ~10% of SCN neurons despite elevated mRNA expression levels within the SCN (DeBruyne *et al.* 2006), suggesting that CLOCK has a partial role in regulating nuclear accumulation of BMAL1 at the posttranscriptional level. Taken together, these results suggested that the positive E-box based transcriptional drive within the circadian clockwork is still active in the SCN, albeit to a somewhat lesser extent, and the molecular oscillator as a whole still functions without CLOCK.

Enigmatic NPAS2

The finding that CLOCK-deficient animals maintain circadian rhythmicity at both the behavioural and molecular levels within the SCN suggested the existence of another clock gene expressing a protein whose function partially overlaps with the function of CLOCK. Further, the existence a *Clock*-like gene is suggested by the observation that BMAL1 homodimers do not act as transcriptional enhancers, at least *in vitro* (Rutter *et al.* 2001). In fact, the mouse circadian clockwork generally seems very resistant to gene knockout approaches: there appears to be genetic redundancy shared between *mPer1* and *mPer2*, and *mCry1* and *mCry2* (double knockout mice of either pair of genes are required to completely abolish circadian rhythmicity). Only in a single case, *Bmal1*, has a single-gene knockout completely abolished rhythms (Bunger *et al.* 2000). Therefore, it was likely that another BMAL1 dimerization partner existed in the SCN clockwork, but its function within the clockwork is yet to be determined.

NPAS2 (also called MOP4) is a paralogue of CLOCK (Hogenesch *et al.* 1997; Zhou *et al.* 1997), and thus appeared to be the best candidate gene that could have a similar function as CLOCK. Further, NPAS2 can dimerize with BMAL1 in both the brain and in cell lines (Kume *et al.* 1999; DeBruyne *et al.* 2006), and appears to function in a clockwork mechanism in mouse forebrain (Reick *et al.* 2001). Its function in the SCN, however, had been questioned because initial attempts were unable to detect *Npas2* expression within the SCN (Shearman *et al.* 1999; Reick *et al.* 2001). A more sensitive technique, real-time PCR, has now demonstrated that *Npas2* is, in fact expressed within the SCN (Ueda *et al.* 2005; Kennaway *et al.* 2006; DeBruyne *et al.* 2007a), and one group has speculated that NPAS2 might maintain the blunted molecular rhythms in the SCN and the long-period behavioural rhythmicity of $Clock^{\Delta 19/\Delta 19}$ mice (Kennaway *et al.* 2006).

Homozygous *Npas2*-mutant mice ($Npas2^{-/-}$), which do not express functional NPAS2 (Garcia *et al.* 2000), display robust circadian rhythms in locomotor behaviour (Dudley *et al.* 2003) (figure 2,A). Like CLOCK-deficient mice, $Npas2^{-/-}$ mice also have subtle circadian defects: a slightly shortened circadian period and an altered response to perturbations in the light–dark cycle (Dudley *et al.* 2003). However, these circadian phenotypes were initially thought to be due to disrupted crosstalk between forebrain and SCN clocks, and not due to NPAS2 deficiency within the SCN (Dudley *et al.* 2003; Green and Menaker 2003).

To unequivocally determine whether NPAS2 has overlapping function with CLOCK, we generated $Clock^{-/-}$; $Npas2^{-/-}$ double knockout mice, as well as their siblings containing null mutations in three out of four possible *Clock* and *Npas2* alleles, and examined their circadian patterns of behaviour. CLOCK-deficient animals with only one wild-type allele of *Npas2* ($Clock^{-/-}$; $Npas2^{+/-}$) had marked defects in circadian behaviour: in constant darkness, these mice displayed unusually short circadian periods that often degenerated into complete arrhythmicity after a few weeks (DeBruyne *et al.* 2007a). The free-running periods displayed by these mice were shorter than those of single mutants ($Clock^{-/-}$ or $Npas2^{-/-}$) or NPAS2-deficient animals with only one functional allele of *Clock* ($Clock^{+/-}$; $Npas2^{-/-}$), suggesting that NPAS2 and CLOCK may have partially overlapping roles in determining behavioural rhythmicity, with CLOCK having a more prominent role than NPAS2. Mice lacking functional alleles of both *Clock* and *Npas2* ($Clock^{-/-}$; $Npas2^{-/-}$) displayed completely arrhythmic patterns of locomotor behaviour immediately upon placement in constant darkness, confirming that CLOCK and NPAS2 have overlapping roles in maintaining circadian behaviour (figure 2,A) (DeBruyne *et al.* 2007a). Further, E-box driven clock genes in the SCN of $Clock^{-/-}$; $Npas2^{-/-}$ mice were not rhythmically expressed but instead were expressed at constitutively low levels (DeBruyne *et al.* 2007a). This further implicates NPAS2 as a dimerizing partner of BMAL1 to generate rhythmic behaviour in the absence of CLOCK.

To confirm that NPAS2 does indeed function within the SCN, we generated CLOCK-deficient mice that express firefly luciferase (LUC) fused to the endogenous mPER2 protein (mPER2::LUC) (Yoo *et al.* 2004). The mPER2::LUC fusion protein is expressed from a knocked-in $mPer2^{Luc}$ allele, and allows for real-time monitoring of circadian dynamics from isolated tissue explants in culture (Welsh *et al.* 2004; Yoo *et al.* 2004). Our rationale was that if CLOCK-deficient SCN are still rhythmic in culture, then NPAS2 must be functioning within the SCN, not another brain region, to maintain SCN-level rhythmicity. Using real-time reporting of bioluminescence from SCN explants, we found that isolated SCN from CLOCK-deficient mice expressing the mPER2::LUC fusion protein ($Clock^{-/-}$; $mPer2^{Luc}$) still maintained selfsustained molecular oscillations in culture that were similar to those from wild-type or $Npas2^{-/-}$ SCN expressing mPER2::LUC ($Npas2^{-/-}$; $mPer2^{Luc}$; figure 3) (DeBruyne *et al.* 2007a). In addition, SCN explants from $Clock^{-/-}$; $Npas2^{-/-}$; $mPer2^{Luc}$ mice were not rhythmic, consistent with observed behavioural patterns. Finally, we found that *Npas2* mRNA is expressed in the SCN of both wild-type and CLOCK-deficient mice at comparable levels (DeBruyne *et al.* 2007a). These data indicate that without CLOCK, NPAS2 does indeed maintain the SCN clockwork, independent of a major influence from other brain regions.

Whether or not NPAS2 functions in the SCN clockwork of wild-type mice, in the presence of *CLOCK*, remains to be resolved. As mentioned above, single $Npas2^{-/-}$ mice do display some subtle defects in circadian behaviour. These mice also have some very subtle differences in rhythmic expression of the *mPer*2 and *Bmal*1 genes in SCN (DeBruyne *et al.* 2007a). Since *Npas2* mRNA has now been found in the SCN of wild-type mice (Ueda *et al.* 2005; Kennaway *et al.* 2006; DeBruyne *et al.* 2007a), it seems that the most likely cause of the $Npas2^{-/-}$ circadian phenotypes is actually the loss of NPAS2 function within the SCN itself. Clearly, the importance of CLOCK or NPAS2 function in the SCN clockwork is much better appreciated when one or the other is missing. Whether this is a reflection of a genetic compensatory response mechanism or simply partially redundant function shared between two proteins is unknown; however, this and other evidence seem to suggest that CLOCK and NPAS2 function in a partially redundant fashion to maintain mouse clockwork function in the SCN.

CLOCK and NPAS2 in peripheral clocks

The circadian clockwork mechanism in peripheral oscillators such as those in liver or lung tissues and fibroblast cell lines is thought to be very similar to that within SCN neurons (Cuninkova and Brown 2008). As such, oscillators in peripheral tissues have been instrumental for understanding the biochemical and transcriptional mechanisms underlying circadian gene expression (e.g., Lee *et al.* 2001; Preitner *et al.* 2002; Etchegaray *et al.* 2003; Ripperger and Schibler 2006). We therefore examined the effects of the loss of CLOCK in mRNA and protein accumulation rhythms in the liver *in vivo*.

In vivo sampling of mRNA abundance in liver tissues collected around the clock on the first day in constant darkness suggested that the loss of CLOCK had roughly the same impact on the liver clockwork as in the SCN. The E-box driven genes *mPer1, mPer2, Rev-erb*α and *Dbp* were rhythmically expressed in the livers of CLOCK-deficient mice, however these rhythms were of lower amplitude than those in wild-type livers (DeBruyne *et al.* 2006). Also, circadian rhythms in accumulation of the mPER2 and mCRY1 proteins in liver nuclei were rhythmic, with amplitudes indistinguishable from those of wild-type. The levels of nuclear mPER1 accumulation in the liver of CLOCK-deficient mice were also comparable to mPER1 levels in the livers of wild-type mice, and unlike the phenotypic difference seen with nuclear mPER1 in the SCN. The cause for this apparent difference in the regulation of nuclear mPER1 in different tissues of CLOCK-deficient mice is unknown, but this difference is the only substantial phenotype-specific and tissue-specific difference that we observed. *Bmal1* mRNA levels were elevated without CLOCK, but nuclear BMAL1 accumulation was reduced

compared to wild-type, suggesting that, as in the SCN, CLOCK has a role in regulating BMAL1 nuclear accumulation. In sum, these results suggested that, as with the SCN, NPAS2 may be maintaining the liver clockwork in the absence of CLOCK. Indeed, *Npas2* mRNA and nuclear protein levels are substantially upregulated in CLOCK-deficient livers *in vivo*, compared to wild-type (DeBruyne *et al.* 2006).

However, it is very difficult to discern if a peripheral oscillator still functions normally using *in vivo* sampling, as intact clockwork function within the SCN can drive apparent rhythms in expression of some clock genes in peripheral tissues even if the autonomous oscillator endogenous to the peripheral tissue is abolished (Pando *et al.* 2002; Kornmann *et al.* 2007; Liu *et al.* 2007). Therefore, we determined if the autonomous oscillators in peripheral tissues depended on CLOCK and/or NPAS2 by measuring bioluminescence rhythms produced by cultured liver and lung explants obtained from wild-type, $Clock^{-/-}$, $Npas2^{-/-}$, and double knockout ($Clock^{-/-}$; $Npas2^{-/-}$) mice that also express the $mPer2^{Luc}$ reporter (DeBruyne *et al.* 2007b).

Like the SCN, bioluminescence rhythms produced by liver and lung explants from $Npas2^{-/-}$ mice were very comparable to those of wild-type mice (figure 4). Surprisingly, bioluminescence profiles of both liver and lung tissue explants from $Clock^{-/-}$ mice were arrhythmic (figure 4). In fact, bioluminescence profiles and rhythm amplitudes produced by CLOCK-deficient liver and lung explants were indistinguishable from those of arrhythmic $Clock^{-/-}$; $Npas2^{-/-}$ mice (figure 4). Importantly, arrhythmicity was not due to low luciferase activity or rapid desynchronization: media change acutely induced PER2::LUC activity but failed to restore rhythmicity in CLOCK-deficient liver or lung explants (DeBruyne *et al.* 2007b). Thus, while NPAS2 maintains rhythmic SCN function in the absence of CLOCK, NPAS2 alone is unable to maintain rhythmicity in peripheral tissues, despite a dramatic upregulation in its expression in the livers of $Clock^{-/-}$ mice. This result further supports the argument that NPAS2 function in the SCN oscillator is not the product of a genetic compensatory mechanism. Further, these results highlight a newly emerging distinction between SCN and peripheral oscillator function when measured at the tissue-level (Liu *et al.* 2007): rhythmicity in the SCN tissue can be maintained via coupling between SCN cellular oscillators through neuronal interactions, whereas rhythmicity in peripheral tissues cannot be maintained because they lack coupling.

CLOCK and NPAS2 in the mouse circadian system

CLOCK and NPAS2 have partially overlapping functions within the SCN clock, but not in the peripheral tissues tested, as summarized in figure 5. But what is the relationship between the two proteins in the SCN, and what is different about NPAS2 that it cannot maintain peripheral oscillator function in the absence of CLOCK?

As suggested above, NPAS2 probably contributes to SCN clock function in wild-type animals. However, the relationship between CLOCK and NPAS2 in the SCN does not appear to be equal, as suggested by the behavioural genetics on mice carrying only a single functional allele of either gene (figure 2). Further, in CLOCK-deficient SCN, nuclear BMAL1 staining is reduced to only ~10% of the nuclei, despite overall elevated *Bmal1* mRNA expression (DeBruyne *et al.* 2006). This finding suggests that nuclear accumulation of BMAL1 is dependent on CLOCK in the vast majority of, but not all, SCN neurons. Further, since *Npas2* is present in CLOCK-deficient SCN, it is likely that the residual BMAL1 nuclear localization in CLOCK-deficient SCN is probably dependent on NPAS2. The reciprocal relationship appears to be true CLOCK and NPAS2 nuclear localization depends on BMAL1 (Kondratov *et al.* 2003,2006). Therefore, we predict that the remaining BMAL1 positive cells in the SCN of CLOCK-deficient mice also coexpress nuclear NPAS2. Further, if we assume

that these SCN neurons with detectable nuclear BMAL1 are the only neurons that contain functioning intracellular oscillators within the CLOCK-deficient SCN, it would follow that the wild-type SCN may contain two populations of oscillators, ~90% that are CLOCK-dependent, and ~10% that are NPAS2 dependent (figure 5).

It is not known, however, if CLOCK and NPAS2 are expressed in the same or distinct neurons within the SCN. Therefore, CLOCK and NPAS2 could have a variety of relationships, depending on whether NPAS2 is also present in the nuclei of the same neurons as CLOCK (figure 5). If CLOCK and NPAS2 are expressed in different, nonoverlapping populations of SCN neurons, it would suggest that there are distinct CLOCK-dependent or NPAS2-dependent oscillators within the SCN, with both contributing to SCN function (figure 5,A). Alternatively, CLOCK may be colocalized with NPAS2 in some neurons (i.e. ~10%), perhaps acting interchangeably or in an E-box/promoter-specific manner in this subpopulation of SCN neurons (figure 5,B). Further experiments will be required to discern these possibilities.

In either case, it is likely that NPAS2-dependent oscillators present in ~10% of CLOCK-deficient SCN are driving rhythms in at least some of the remaining ~90% SCN neurons that do not have endogenous oscillator function. This is suggested by the apparent discrepancy between the number of BMAL1 (~10%) and mPER2 (~60% of wild-type) positive nuclei of CLOCK-deficient animals (DeBruyne *et al.* 2006). Also, the amplitudes of the mPER2::LUC rhythms produced by CLOCK-deficient SCN are ~60% compared with wild-type, not 10% (DeBruyne *et al.* 2007a, data not shown). Presumably, some of these 'driven' oscillations occur in SCN neurons that normally contain CLOCK-dependent oscillators, and the 'driving' mechanism is presumably interneuronal coupling within the SCN (Liu *et al.* 2007). The intriguing result that mPER1 only accumulates in the nuclei of 10% of CLOCK-deficient SCN neurons (DeBruyne *et al.* 2006) suggests that perhaps nuclear mPER1 accumulation in the SCN may be dependent on its interactions with CLOCK:BMAL1 or NPAS2:BMAL1 heterodimers, and that detectable nuclear mPER1 in the SCN may mark 'true' oscillator cells in the SCN.

In contrast to the SCN, the oscillator mechanisms in the liver and lung require CLOCK in order to function (figure 5). One possible explanation for this difference could be that while the intracellular SCN oscillators are coupled via neuronal networking (discussed above), the liver and lung lack functional coupling between the oscillators. If this is the case, then there may be a small population of cells in which NPAS2 alone can maintain oscillator function in the liver and lung, but these oscillations are not propagated throughout the tissue due to the lack of intercellular coupling. In our experiments (DeBruyne *et al.* 2007b), rhythmicity by this small population of cells could have been masked by a substantially larger population of cells expressing constitutive levels of the reporter. Single cell bioluminescence recordings from the liver and lung are needed to examine this possibility.

Another more intriguing explanation for the difference between SCN and liver/lung oscillators may be that NPAS2 function within the oscillator requires another unknown factor that is present in the SCN, but absent in the liver or lung. Conversely, NPAS2 function could be blocked by an unknown factor present in liver and lung, but absent in SCN. If either were the case, NPAS2 function in the oscillator would be determined by the presence and/or regulation by this unknown factor. This unknown factor could be another protein, cofactor, or even posttranslational modification dependent on an enzyme that has a limited tissue distribution (figure 5). We saw very few differences in the molecular clockwork between the SCN and liver, but one difference was in the regulation of nuclear mPER1. Perhaps the differential regulation of nuclear mPER1 in the SCN and the liver of CLOCK-deficient mice holds the key to identifying this unknown factor.

Concluding remarks

Although the role of CLOCK in the circadian clock was initially questioned by the persistence of nearly normal rhythms in CLOCK-deficient mice, the role of the CLOCK protein in the circadian clockwork is indeed quite important. The analysis of CLOCK-deficient mice has further refined CLOCK's role in the circadian system, in addition to demonstrating that NPAS2 is also a clock component.

Analysis of null mutations can determine if a protein is required for a certain process but null mutations can often have very subtle effects due to overlapping function of a related paralogue. The *Clock* gene's involvement in the circadian clockwork was discovered by recovery of a dominant-negative mutant allele. This discovery subsequently led to the identification of BMAL1 as an essential clock component (Gekakis *et al.* 1998; Bunger *et al.* 2000) and the development of tools such as transfection-based transcriptional assays that have allowed the identification of the primary negative feedback loop as a key mechanism underlying clock function. Further, studies using $Clock^{\Delta 19/\Delta 19}$ mutant mice led to the discovery of numerous clock-controlled genes that may lead to rhythms in physiological processes. The antimorphic nature of the mutant $Clock^{\Delta 19}$ allele was the key to its discovery; a null *Clock* allele never would have been identified via mutagenesis screening for mutants with behavioural rhythm period defects. Therefore, if it was not for the initial discovery and recovery of '*Clock* mutant mice' more than a decade ago (Vitaterna *et al.* 1994), mammalian circadian biologists may still be operating in the dark when it comes to understanding the importance of circadian rhythmicity and the timekeeping mechanism driving circadian rhythms.

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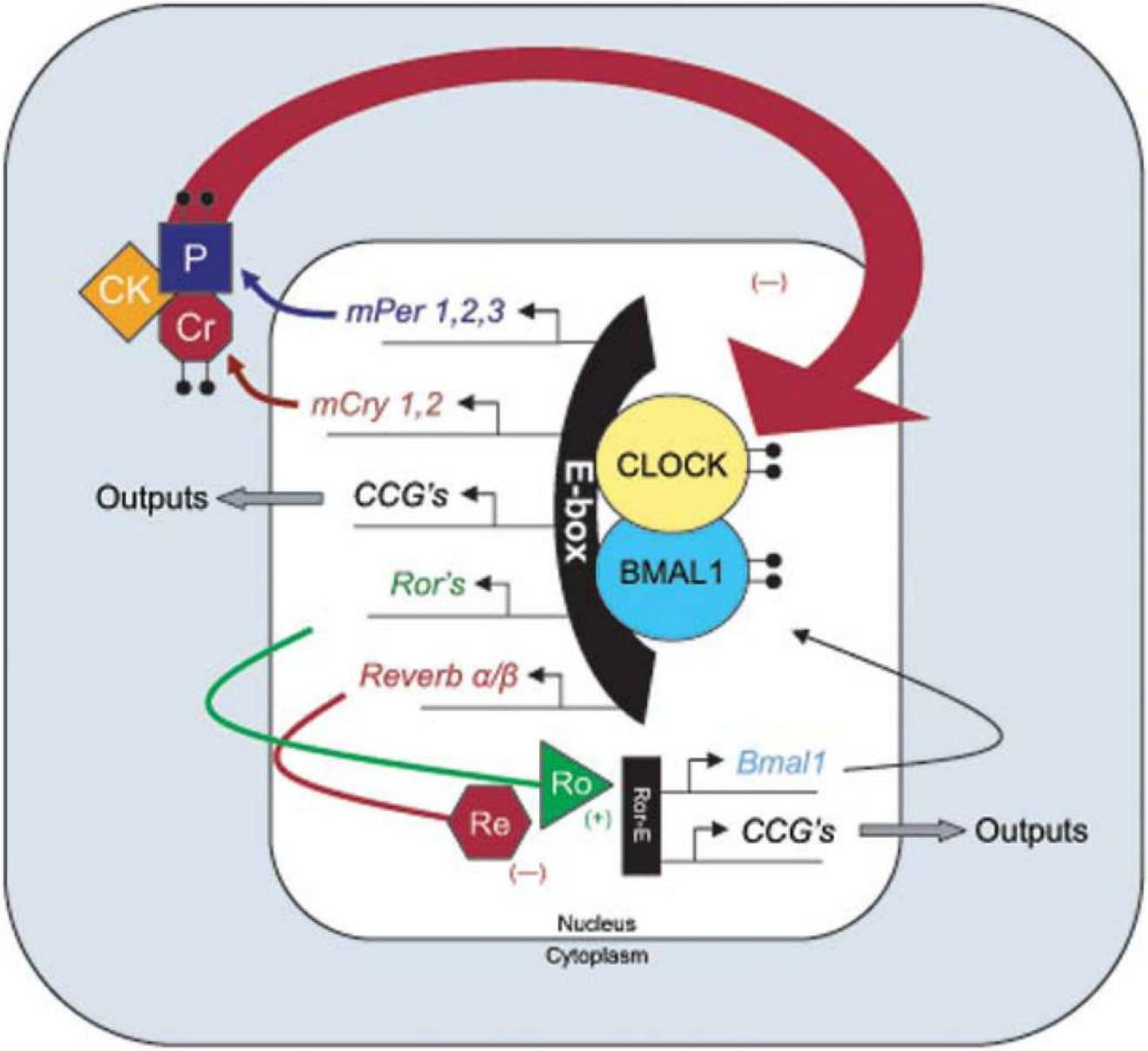

Figure 1.

Model of the mammalian circadian clockwork. The bHLH-PAS transcription factors CLOCK and BMAL1 heterodimerize, and bind E-box elements to drive expression of the *mPer1, mPer2* and *mCry1* and *mCry2* clock genes. The PER (P) and CRY (Cr) proteins form a complex along with CKI δ/ε (CK) and translocate into the nucleus where they bind CLOCK:BMAL1 to inhibit transcription (−), completing the essential negative feedback loop. Posttranslational modification (black lollipops) of several of these components appears to help maintain ~24 h rhythmicity. CLOCK:BMAL1 heterodimers also drive expression of Ror's (Ro) and *Rev-erb* α/β (Re), which are transcriptional activators (+) or repressors (−), respectively, that drive cyclical *Bmal1* expression via Ror elements within the *Bmal1* promoter, in a secondary feedback loop that appears to stabilize rhythms. Clock controlled genes (CCG's) are output genes directly regulated by this central clockwork (adapted from Reppert and Weaver (2002), and Emery and Reppert (2004)).

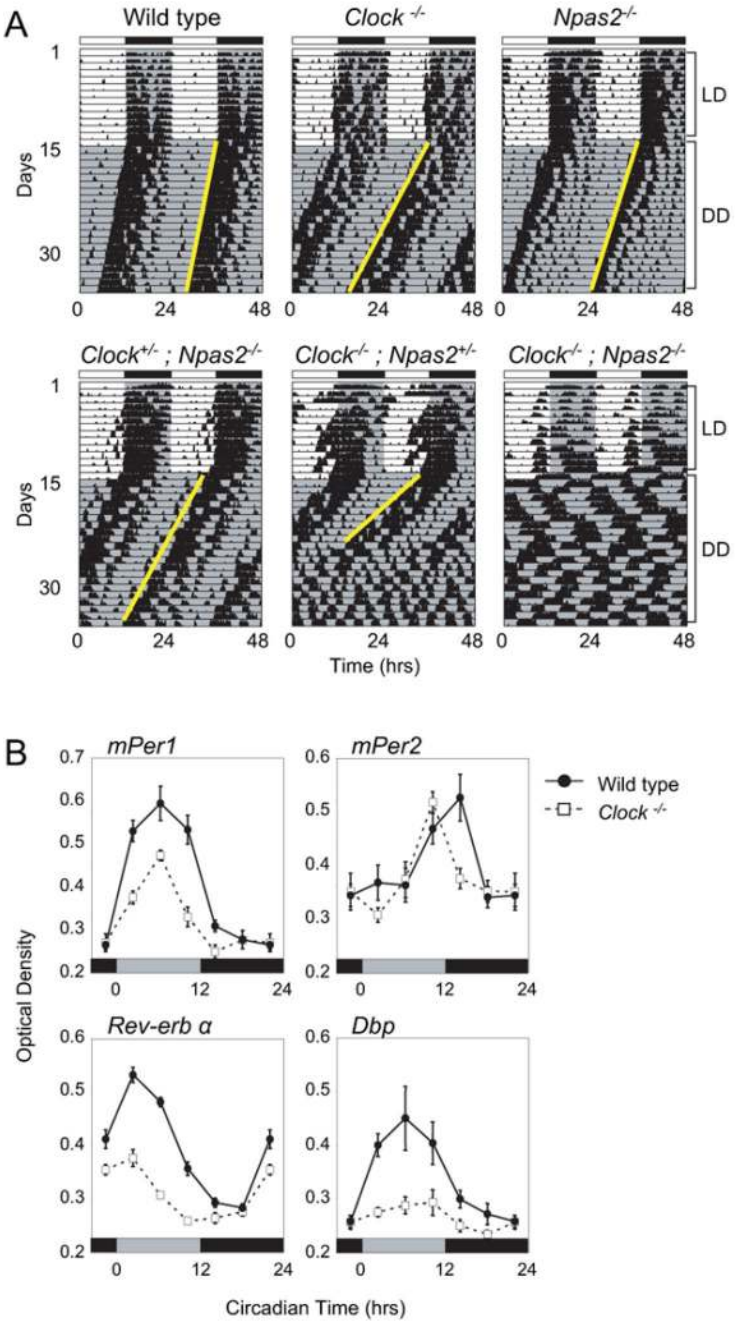

Figure 2.

Behavioural rhythms persist in *Clock*$^{-/-}$ mice, but not *Clock*$^{-/-}$; *Npas2*$^{-/-}$ mice. (A) Representative double-plotted actograms depicting behavioural rhythms obtained from mice of the genotypes indicated. Each horizontal line represents two days of recording, and data from each day are plotted twice: on the upper line from 24 to 48 h and the subsequent lower line from 0–24 h. Activity levels are depicted by black marks above each horizontal line. Alternating white and black bars at the top of each plot represents the light cycle, the animals were maintained on, and the white and gray within the records indicates time when the animals were in the light or dark, respectively (data shown were adapted from DeBruyne *et al.* (2007a) with permission from *Nature Neuroscience*). (B) Representative mRNA abundance

profiles in the SCN of wild-type and *Clock*$^{-/-}$ mice (the data shown were adapted from DeBruyne *et al.* (2006), with permission from *Neuron*).

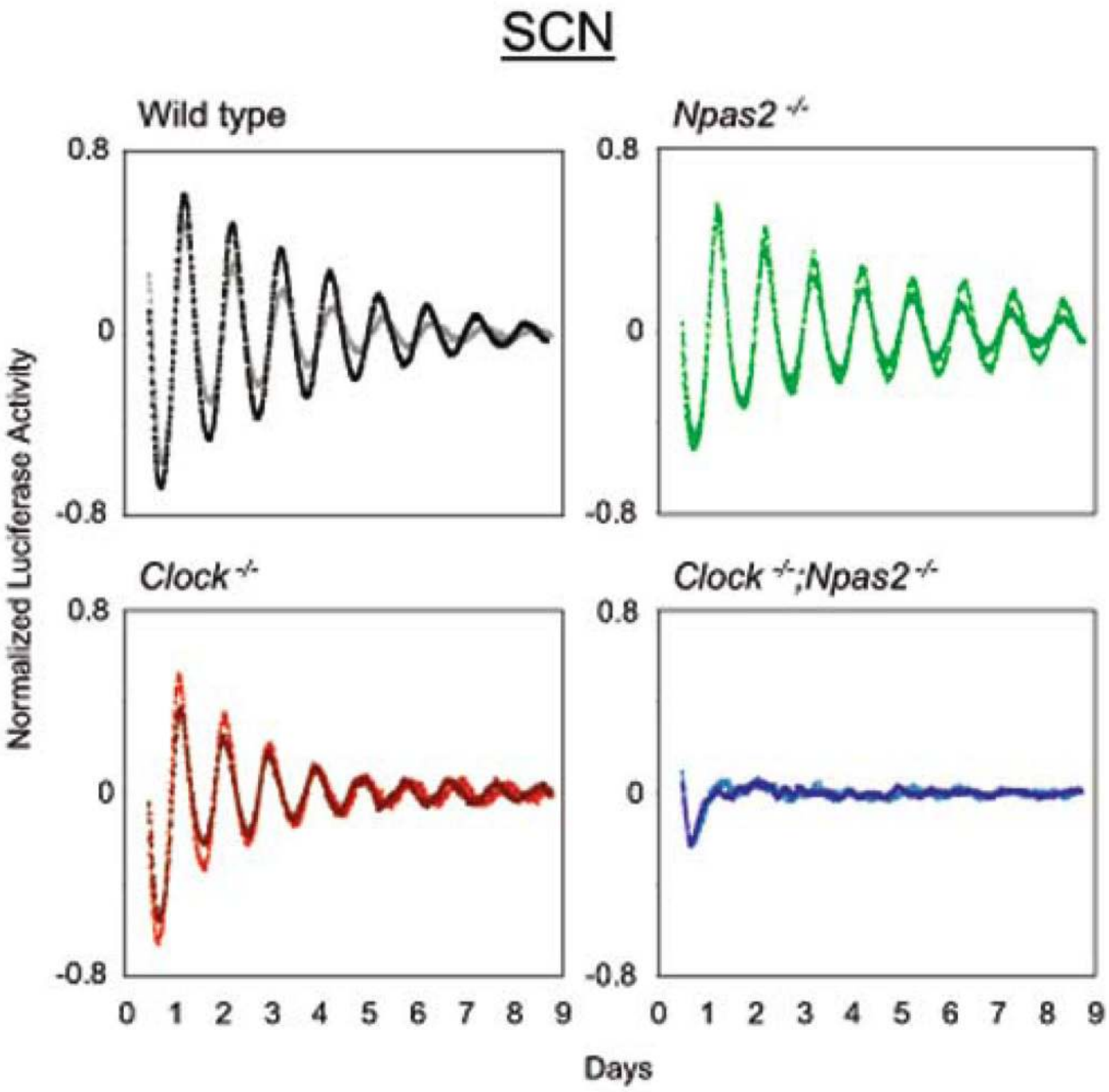

Figure 3.
SCN mPER2::LUC rhythms are abolished in *Clock*$^{-/-}$; *Npas2*$^{-/-}$ double-knockout mice. Representative normalized bioluminescence rhythms obtained from SCN isolated from the indicated genotypes. Two individual records are shown in each graph (data are replotted from DeBruyne *et al.* (2007b), with permission from *Current Biology*).

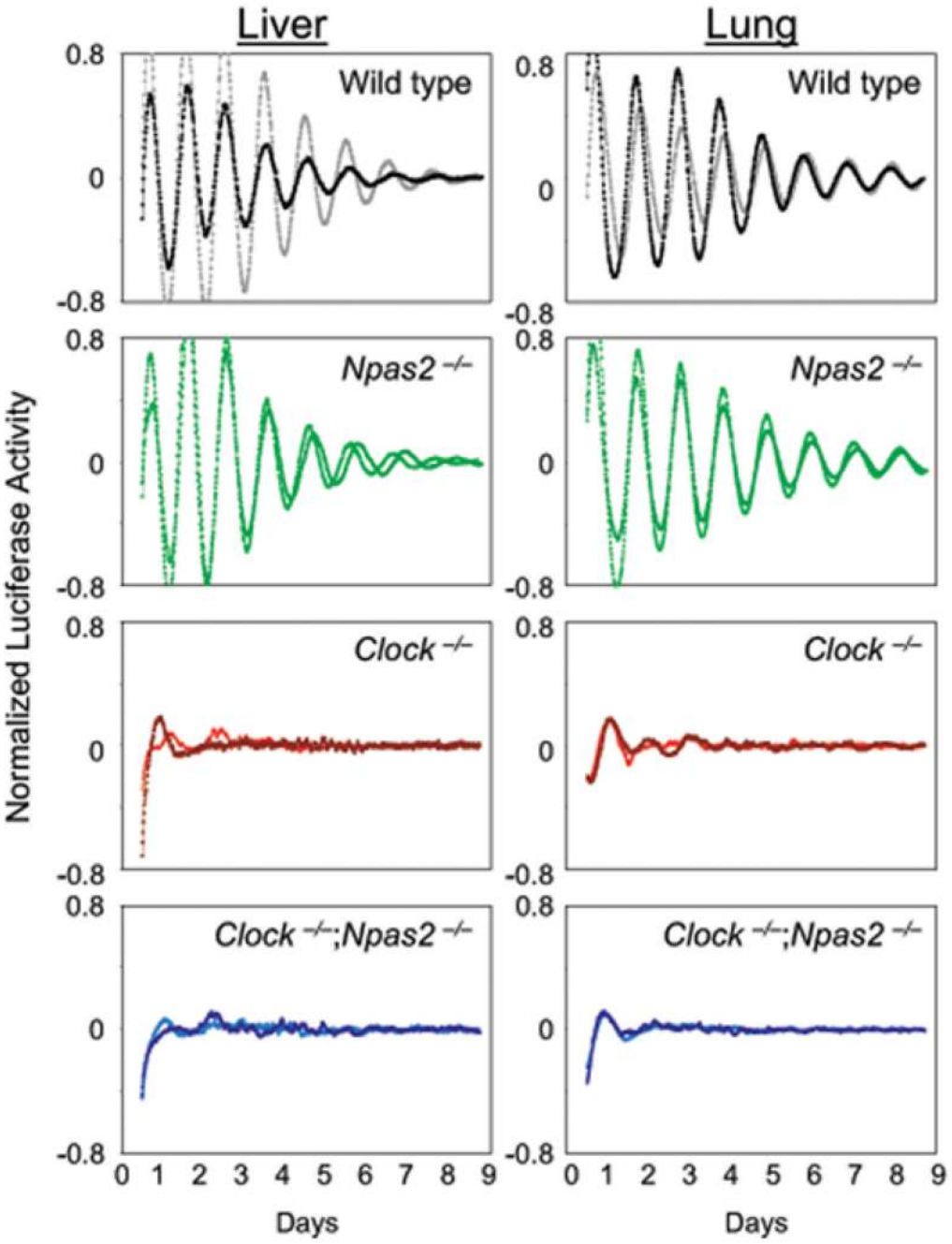

Figure 4.
Circadian oscillator function in isolated liver and lung explants is abolished in CLOCK-deficient animals. Representative normalized bioluminescence profiles obtained from isolated liver (left side) and lung (right side) explants taken from the indicated genotypes. Each panel contains data from two independent animals (data are replotted from DeBruyne *et al.* (2007b), with permission from *Current Biology*).

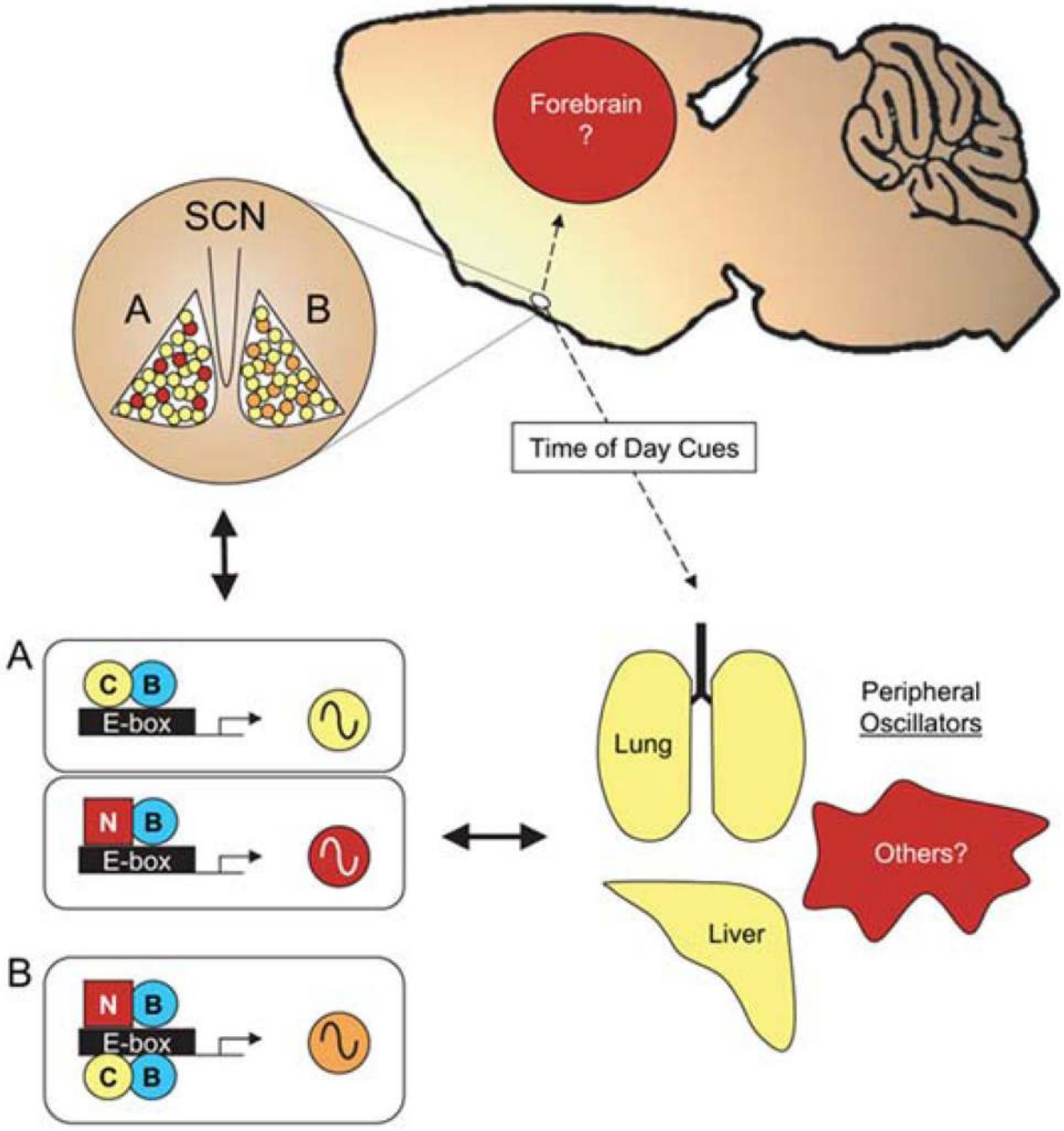

Figure 5.

Schematic depiction of the spatial relationship between CLOCK and NPAS2 dependent oscillators. Two possible spatial relationships of CLOCK and NPAS2 within the SCN are depicted in the SCN diagram, labelled A and B, and correspond to the simplified clockwork models labelled A and B in the bottom left. The simplified clockwork models illustrate either CLOCK (C) and NPAS2 (N) heterodimerizing with BMAL1 (B) and binding E-box elements to drive circadian gene expression and ultimately oscillator function (oscillator symbol). In A, different SCN neurons contain either CLOCK-dependent (yellow) or NPAS2 dependent (red) oscillators, with no overlap. In B, most cells contain CLOCK-dependent oscillators, but some cells also have oscillators that can use CLOCK or NPAS2 more or less interchangeably

(orange). The nonSCN and peripheral oscillators shown are drawn assuming the model shown in A extends to these tissues. The liver and lung contain CLOCK dependent circadian oscillators (yellow), whereas there may be some nonSCN and peripheral tissues that contain NPAS2-dependent oscillators (red). The forebrain may contain a strictly NPAS2-dependent oscillator (Dudley *et al.* 2003), but it is not known if rhythms in the forebrain are driven by cell-autonomous oscillators. The model shown in B is also a possibility with non-SCN and peripheral tissues (not shown).