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CLOCK and NPAS2 have overlapping roles in the suprachiasmatic circadian clock

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Abstract

Heterodimers of CLOCK and BMAL1, bHLH-PAS transcription factors, are believed to be the major transcriptional regulators of the circadian clock mechanism in mammals. However, a recent study shows that CLOCK-deficient mice continue to exhibit robust behavioral and molecular rhythms. Here we report that the transcription factor NPAS2 (MOP4) is able to functionally substitute for CLOCK in the master brain clock in mice to regulate circadian rhythmicity.

Circadian rhythms regulate biological events like the timing of the sleep-wake cycle and are generated by a hierarchy of circadian clocks^{1,2}. Atop this hierarchy is a master clock that resides in the hypothalamic suprachiasmatic nuclei (SCN). The SCN clock is entrained by the daily light-dark cycle to the 24-h period by retina to SCN pathways, and it synchronizes the phase of circadian clocks in peripheral tissues that drive rhythmic changes in local physiology.

The intracellular circadian clock mechanism in the mouse is regulated by transcriptional feedback loops that drive the self-sustaining clock mechanism in both the SCN and peripheral tissues^{1,2}. The critical molecular mechanism is thought to involve CLOCK:BMAL1 heterodimers that drive the rhythmic expression of three *Period* genes (*mPer1–3*) and two *Cryptochrome* genes (*mCry1* and *mCry2*). The resulting proteins form PER:CRY complexes that translocate back into the nucleus to inhibit their own transcription, creating a negative feedback loop. A modulatory, interlocking positive transcriptional feedback loop involves the rhythmic regulation of *Bmal1* transcription, through the coordinated actions of *Rev-erba* (repressor) and *Rora* (activator), whose mRNA oscillations are antiphase to the *mPer* and *mCry* mRNA rhythms.

Recent genetic evidence has shown that CLOCK is not essential for the circadian rhythm in locomotor activity in mice (Fig. 1a, middle panel)³. However, compared with wild-type controls, CLOCK-deficient mice do have a slightly shortened circadian period in constant darkness and show altered circadian responses to light³. Without CLOCK, molecular and biochemical rhythms are also altered, but most persist³. Because BMAL1 is essential for the expression of circadian behavioral rhythms⁴ and homodimers are not transcriptionally active⁵, we sought an alternative dimerization partner for BMAL1.

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NPAS2 (also called MOP4) is a paralog of CLOCK^{6,7} that can dimerize with BMAL1 and appears to function in a clockwork mechanism in mouse forebrain⁸. Its function in the SCN has been questioned, however, as previous studies were unable to detect *Npas2* expression in the SCN^{8,9}. Homozygous *Npas2*-mutant mice (*Npas2*^{-/-}), which do not express functional NPAS2¹⁰, display robust circadian rhythms in locomotor behavior¹¹ (Fig. 1a, right panel). Like CLOCK-deficient mice, *Npas2*^{-/-} mice also have a slightly shortened circadian period and an altered response to perturbations in the light-dark cycle¹¹. These circadian phenotypes have been proposed to be a result of disrupted crosstalk between forebrain and SCN clocks, and not a result of NPAS2 deficiency in the SCN^{11,12}.

To examine whether NPAS2 is the missing BMAL1 partner, we generated CLOCK-deficient mice carrying either one or no functional *Npas2* alleles by interbreeding CLOCK-deficient mice with a previously generated null allele of *Npas2* (ref. ¹⁰, Fig. 1b–d and Supplementary Methods online). Our animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the UMass Medical School. CLOCK-deficient animals carrying only one normal allele of *Npas2* (*Clock*^{-/-}; *Npas2*^{+/-}) had substantially shorter circadian periods in constant darkness (22.7 ± 0.2 h) compared with wild-type mice (23.8 ± 0.1 h), with progressive rhythm instability (Fig. 1b,e,f). CLOCK-deficient mice with no functional NPAS2 (*Clock*^{-/-}; *Npas2*^{-/-}) exhibited arrhythmic locomotor behavior immediately on placement in constant darkness (Fig. 1c,e,f). These findings suggest that NPAS2 is the missing BMAL1 partner and that NPAS2 has a direct function in the SCN, the generator of rhythmic locomotor behavior. Notably, NPAS2 mutant mice carrying only one allele of *Clock* (*Clock*^{+/-}; *Npas2*^{-/-}) displayed behavioral rhythmicity in constant darkness that was similar to wild-type animals (Fig. 1d,e,f), suggesting that CLOCK may have a more prominent role than NPAS2 in the SCN clock.

We used *in situ* hybridization to evaluate the status of the molecular clock in the SCN of the double mutant (*Clock*^{-/-}; *Npas2*^{-/-}) mice. We found that the circadian rhythms of *mPer1*, *mPer2*, *Rev-erba* and *Bmal1* mRNA expressed in the SCN of wild-type mice were abolished in the double mutants (Fig. 2). Consistent with the idea that the transcriptional drive of both the negative and positive feedback loops are disrupted in the double mutants, *mPer1*, *mPer2* and *Rev-erba* mRNA levels were at constant low levels over the circadian cycle, whereas *Bmal1* mRNA levels were at constant high values (Fig. 2), consistent with the idea that *Bmal1* is repressed by Rev-erba^{1,2}.

Single knockout *Npas2*^{-/-} mice displayed subtle alterations in the rhythmic expression of some genes in the SCN. For example, the *mPer2* mRNA rhythm of *Npas2*^{-/-} mice appeared to be slightly damped compared with wild-type mice (Fig. 2). In addition, *Bmal1* levels, although rhythmic, were increased throughout the circadian day in *Npas2*^{-/-} mice, compared with wild types (Fig. 2). The molecular defects in *Npas2*^{-/-} SCN are more subtle than those observed in CLOCK-deficient mice³, suggesting that CLOCK normally has a more prominent role than NPAS2 in controlling circadian gene expression.

To further assess NPAS2 function in the SCN, we generated CLOCK-deficient mice carrying the *mPer2*^{Luciferase} (*mPer2*^{Luc}) allele. Mice carrying this allele at the *mPer2* locus express a mPER2::LUC fusion protein, which allows real-time monitoring of circadian dynamics from tissue explants in culture (see Supplementary Methods)¹³. Using real-time reporting of bioluminescence from SCN explants, we found that isolated SCN from CLOCK-deficient mice expressing the fusion protein (*Clock*^{-/-}; *mPer2*^{Luc}) still maintained self-sustained molecular oscillations in culture that were similar to those from wild-type SCN expressing the fusion protein (*Clock*^{+/-}; *mPer2*^{Luc}, Fig. 3 and Supplementary Fig. 1 online). These data suggest that NPAS2 maintains the SCN clockwork without CLOCK, independent of a major influence from other brain regions.

To verify that *Npas2* is actually expressed in the SCN of wild-type and CLOCK-deficient mice, we used a quantitative real-time PCR approach. Our experiment clearly shows *Npas2* expression in the SCN of both wild-type and *Clock*^{-/-} mice (Supplementary Fig. 2 online).

We conclude that NPAS2 has a newly found, unexpected role in the SCN clock mechanism that controls circadian behavior. CLOCK and NPAS2 can independently heterodimerize with BMAL1 in the SCN to maintain molecular and behavioral rhythmicity. We cannot distinguish whether NPAS2 normally functions to regulate circadian gene expression in the SCN of wild-type mice, or whether NPAS2 only has functionally relevant effects on gene expression in the absence of CLOCK. Nevertheless, the results show that NPAS2 maintains circadian function in the absence of CLOCK. The differences in gene expression profiles between the *Clock*^{-/-} and *Npas2*^{-/-} single-knockouts suggests that different circadian promoters may have different affinities or requirements for CLOCK:BMAL1 versus NPAS2:BMAL1 heterodimers. Thus, NPAS2 may function coordinately with CLOCK in the SCN. These findings show a new level of transcriptional control in the SCN clockwork.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Locomotor activity rhythms are abolished in double knockout mice. **(a–d)** Representative activity rhythms of **(a)** wild-type (left), *CLOCK*-deficient (*Clock*^{-/-}; middle) and *NPAS2*-deficient mice (*Npas2*^{-/-}; right), **(b)** *CLOCK*-deficient mice having one functional allele of *Npas2* (*Clock*^{-/-}; *Npas2*^{+/-}), **(c)** double knockout mice (*Clock*^{-/-}; *Npas2*^{-/-}), and **(d)** *NPAS2*-deficient mice having one functional allele of *Clock* (*Clock*^{+/-}; *Npas2*^{-/-}). Each horizontal line represents 48 h; the second 24-h period is plotted both to the right and below the first. Vertical bars represent periods of wheel-running activity. Animals were initially housed in a 12-h light, 12-h dark cycle (LD) and were then transferred to constant darkness (DD). The timing of the LD cycle is indicated by the bar above the top records, and gray shading in the records indicates darkness. Numbers on the left indicate days of study. The yellow lines delineate the phase of activity onset in constant darkness. **(e)** Periodogram estimates of period for each genotype. Each bar is the mean $\pm$ s.e.m.; the number of animals is indicated in each bar. * $P < 0.05$, ANOVA compared with wild type (Tukey HSD for unequal n), AR indicates arrhythmicity. **(f)** Circadian amplitude (power from periodogram analyses) from days 6–15 in DD. The four rhythmic genotypes did not differ (ANOVA, $P > 0.05$).

Figure 2.

Clock gene mRNAs are arrhythmic in the SCN of double knockout mice. **(a)** Brains from wild-type mice (black lines), NPAS2-deficient mice ($Npas2^{-/-}$, green lines) and double-knockout mice ($Clock^{-/-};Npas2^{-/-}$, brown lines) were collected at 4-h intervals on the first day in constant darkness. mRNA levels for *mPer1*, *mPer2*, *Bmal1* and *Rev-erba* were determined by *in situ* hybridization. Each point represents the mean $\pm$ s.e.m. ($n = 3-4$ animals for each time point except for circadian time (CT) 2 in which $n = 2$ for *mPer1* and *Bmal1* in the double knockout mice). The horizontal bar at the bottom of each panel represents the light-dark cycle (gray = light, black = dark) the animals were housed in before placement in constant dark. For each gene, the mRNA levels were rhythmic in wild-type and NPAS2-deficient SCN (ANOVA, $P < 0.05$), whereas the mRNA levels were not rhythmic in the double knockout SCN (ANOVA, $P > 0.1$). **(b)** Representative *in situ* hybridization autoradiographs showing *mPer1* and *Bmal1* mRNA expression levels in wild-type and double-knockout ($Clock^{-/-};Npas2^{-/-}$) brains collected at CT 6. Consecutive coronal sections, at the level of the SCN, are shown for each genotype.

Figure 3.

SCN molecular rhythms persist in culture without CLOCK. Bioluminescence rhythms from cultures of the SCN of wild-type mice expressing *mPeriod2::Luciferase* (*Clock*^{+/+};*mPer2*^{Luc}, top panel) and CLOCK-deficient mice expressing the fusion protein (*Clock*^{-/-};*mPer2*^{Luc}, bottom panel). The data shown (*n* = 2, wild-type SCN; *n* = 2, CLOCK-deficient SCN) were normalized by the average luciferase activity over the duration of the experiment and 24-h trends were removed. The raw data from these and additional experiments are shown in Supplementary Figure 1.