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Circadian Clock Gene *Bmal1* Is Not Essential After All; Functional Replacement with its Paralog, *Bmal2*

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Summary

Most of the central circadian clock genes in the mouse exist as paralog pairs (*Per1/Per2*, *Cry1/Cry2*, *Clock/Npas2*) that must both be knocked out to confer arhythmicity [1,2,3]. The only exception to this pattern is *Bmal1/Mop3*, the single knockout of which confers arhythmicity despite the presence of its paralog *Bmal2/Mop9* [4]. The knockout of *Bmal1* also has significant effects on longevity, metabolism, *et al.* [5,6]. These results have led to the conclusion that *Bmal1* is a singularly essential clock gene and that *Bmal2* has a minimal role in the clock system. In contrast, we find that expression of *Bmal2* from a constitutively expressed promoter can rescue the clock and metabolic phenotypes of *Bmal1*-knockout mice, including rhythmic locomotor activity, rhythmic metabolism, low body weight, and enhanced fat deposition. Combined with the data of Bunger and coworkers who reported that knockout of *Bmal1* down-regulates *Bmal2* [4], we conclude that *Bmal1* and *Bmal2* form a circadian paralog pair that is functionally redundant, but that in the mouse, *Bmal2* is regulated by *Bmal1* such that knockout of *Bmal1* alone results in a functionally double *Bmal1/Bmal2* knockout. Therefore, the role(s) of *Bmal2* may be more important than has been appreciated heretofore.

Keywords

circadian; Bmal1; Bmal2; biological clock

Results and Discussion

For constitutive expression of *Bmal2*, we used the promoter region (2.5 kb) for *hEF1a* that is expressed in many rodent tissues, including the suprachiasmatic nuclei (SCN) of the hypothalamus [7]. The *hEF1a* promoter was fused to *mBmal2* cDNA with five tandem copies of the c-myc tag at the 5' end of the *mBmal2* cDNA (Fig. 1A). The construct included the SV40 intron and polyadenylation site at the 3' end. This construct is expressed after

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transient transfection into mouse 3T3 and human HEK293 cells; it is active in transactivation of E-box-containing promoters with both CLOCK and NPAS2 partners (Fig. S1; 2-fold with CLOCK and 7.4-fold with NPAS2 in 3T3 cells), as we have previously reported for transactivation of E-box-containing promoters with hBMAL2 (aka ARNTL2) in heterodimers with hCLOCK [8,9]. A transgenic mouse was made with this construct and c-myc-tagged BMAL2 was expressed strongly in spleen, thymus, pancreas and brain as well as weaker but significant expression in liver and lung (Fig. 1B). Expression of c-myc-tagged BMAL2 in mice driven by P_{hEF1a} (B2Tg) lengthens the behavioral locomotor activity by ~0.25 h as compared with wild-type (WT) mice (Figs. 1C, S2). Locomotor activity of *Bmal1*-knockout mice (B1ko) is arhythmic; however, P_{hEF1a}-driven expression of BMAL2 in B1ko mice rescues the rhythmicity (B1ko/B2Tg; Figs. 1C, S2). In most cases the rescue is apparently complete (as in B1ko/B2Tg-1 in Fig. 1C, n = 4), while in other cases the rescue is clear but not absolutely complete (as in B1ko/B2Tg-2 in Fig. 1C, n = 3). There is less total locomotor activity in the B1ko mouse, but P_{hEF1a}-driven expression of BMAL2 also partially rescues this loss of activity (Fig. S2D and S2E). Some mice tested in the locomotor activity assay harbored the P_{mPer2}::mPER2::LUC knockin genetic construct so that their tissues could also be tested for luminescence rhythms *in vitro* (see below).

Ectopic expression of *Bmal2* also rescues metabolic phenotypes that are associated with the knockout of *Bmal1*. For example, WT mice exhibit circadian rhythms of oxygen consumption that are disrupted in B1ko mice (Figs. 2A; B1ko mice exhibit either arhythmicity or ultradian rhythms of oxygen consumption, Fig. S3). Constitutive expression of *Bmal2* in the WT background (B2Tg) does not significantly affect these metabolic rhythms, but it will rescue robust rhythmicity in the B1ko background (B1ko/B2Tg, Figs. 2A, S3). Other metabolic phenotypes that are associated with the knockout of *Bmal1* are significantly (i) reduced body weight (Fig. 2B), (ii) enhanced fat index (Fig. 2C), and (iii) reduced daily food intake (Fig. 2D) in 8-week old mice. All three of these metabolic phenotypes exhibited by B1ko are rescued by the constitutive expression of *Bmal2* (B1ko vs. B1ko/B2Tg), but are not affected by *Bmal2* expression in the WT background (WT vs. B2Tg, Fig. 2B, 2C, 2D).

Therefore, P_{hEF1a}-driven expression of BMAL2 can rescue circadian and metabolic phenotypes associated with the knockout of *Bmal1* in intact mice. Is this rescue also true for isolated tissues that can express circadian rhythms *in vitro*, as can be assayed by light emission of tissues from mice with transgenic reporters [10]? Isolated SCN, lung, and liver slices exhibit circadian rhythms of luminescence *in vitro* from WT and B2Tg animals (Fig. 3; note that the period is significantly longer in lung of B2Tg mice). In contrast, isolated tissues–including the SCN–of neither B1ko nor B1ko/B2Tg mice exhibit circadian rhythms *in vitro*. To be confident that B1ko/B2Tg mice in which locomotor activity was rescued corresponded exactly with animals whose SCN activity rhythms *in vitro* were not rescued, we tested four separate B1ko/B2Tg mice (with the P_{mPer2}::mPER2::LUC knockin construct) for locomotor activity and then sacrificed them for SCN luminescence activity. Figure S4 shows that all four mice exhibited a rescue of the locomotor activity rhythm, but their SCN luminescence activity *in vitro* was not rhythmic. Glucocorticoids have been reported to synchronize peripheral clocks both *in vitro* and *in vivo* [11,12,13]. After treating liver explants with dexamethasone (DEX, a synthetic glucocorticoid), we observed persistent circadian rhythms of PER2::LUC luminescence in B1ko/B2Tg slices as compared with highly damped oscillations from in B1ko slices (Fig. 3). Therefore, even though expression of *Bmal2* is able to rescue behavioral rhythmicity and metabolic phenotypes of intact B1ko mice (Figs. 1 & 2), it appears to be less effective in rescuing B1ko phenotypes in isolated tissues (Fig. 3).

Since the endogenous *Bmal2* gene is intact in *Bmal1*-knockout mice, why does it not sustain rhythmicity in B1ko mice in the same way that it does in the B1ko/B2Tg mice? A clue to an explanation is found in the original *Bmal1*-knockout data [4]; when *Bmal1* is knocked out, *Bmal2* mRNA levels are knocked down to basal levels. We repeated these findings (Fig. 4A, 4B). Therefore, it is possible that *Bmal2* expression in WT mice is regulated by BMAL1 so that knockout of *Bmal1* leads to a condition that is essentially a double *Bmal1/Bmal2* knock-out/down. Indeed, examination of the upstream promoter/enhancer region of *Bmal2* indicates the presence of 8 E-boxes (2 canonical E-boxes {CACGTG} and 6 non-canonical E-boxes {CA-GC/TG-TG}) that could be regulated by BMAL1/CLOCK and BMAL1/NPAS2 heterodimers [14,15,16]. To test this hypothesis, we made a reporter construct with the putative promoter/enhancer region of *mBmal2* fused to luciferase (P_{Bmal2}::luc). In transient transfection assays that introduced this reporter into either mouse 3T3 cells or human HEK293 cells, we found that BMAL1/NPAS2 or BMAL2/NPAS2 heterodimers could activate *Bmal2* expression in both cell types and the BMAL1/NPAS2 transactivation of P_{Bmal2} could be inhibited by expression of mCRY2 in HEK293 cells (Figs. 4C and S1E) [14–17]. Additionally, in mouse 3T3 cells, BMAL1/CLOCK and BMAL2/CLOCK heterodimers could also activate the *Bmal2* promoter/enhancer (Fig. 4C).

We conclude that BMAL1 is not absolutely necessary for circadian rhythms of locomotor activity or metabolic phenotypes, but that it may functionally replaced by expression of its paralog BMAL2 from a constitutively expressed promoter. Combined with the data of Bunger and coworkers who reported that knockout of *Bmal1* down-regulated *Bmal2* [4], we conclude that *Bmal1* and *Bmal2* form a circadian paralog pair that is functionally redundant, but that in the mouse, *Bmal2* is regulated by *Bmal1* such that knockout of *Bmal1* alone results in a functionally double *Bmal1/Bmal2* knockout. This conclusion is consistent with our observation that the promoter of *mBmal2* is regulated by BMAL1/NPAS2 and BMAL1/CLOCK transactivation. Some siRNA analyses have led to the conclusion that *Bmal2* knockdown has no effect on the circadian rhythmicity of human osteosarcoma (U2OS) cell cultures [18]. On the other hand, other siRNA studies in 3T3 fibroblasts have supported the opposite conclusion, namely that knocking *Bmal2* mRNA levels down disrupts the circadian system in cell cultures [19], which is more consistent with the implications of our study, and perhaps indicates that there are tissue/species specific variations in the role of *Bmal2* in the clock mechanism.

Despite the rescue of behavioral/metabolic phenotypes by expression of *Bmal2* from the *hEF1a* promoter, constitutive expression of *Bmal2* does not rescue robust and/or sustained rhythmicity of isolated SCN, liver, or lung tissue *in vitro*. Because the SCN is thought to be the central pacemaker underlying the behavioral locomotor rhythm, it is perplexing that the B1ko/B2Tg mice in which the behavioral rhythm is rescued does not have a concomitant rescue of the *in vitro* rhythm of the SCN. This result is not without precedent, however. For example, another study using *Per1*−/− mice found robust circadian rhythms of locomotion but the luminescence activity of SCN slices *in vitro* was essentially arhythmic [20]. That report concluded that either (i) a small population of rhythmic neurons in the *Per1*−/− SCN is sufficient to drive locomotor rhythms, or (ii) *in vivo* physiological factors can compensate for the atypical activity of *Per1*−/− SCN. Our results with B1ko/B2Tg mice may be another example of this phenomenon. In summary, our data support a key role for *Bmal2/Arntl2* in the clock system that has been overlooked by previous studies. *Bmal2/Arntl2* may be responsible for many of the important clock and non-clock functions that have been attributed to *Bmal1* in the mouse. This is noteworthy because polymorphisms of *Arntl2* in humans may have important consequences such as susceptibility to diabetes [21]. Therefore, as we extend our understanding of mammalian circadian rhythms from the mouse model to humans, it will be important to appreciate the potential significance of *Arntl2*.

Experimental Procedures

Complete Experimental Procedures are described in Supplemental Data.

Generation of *Bmal2* transgenic mice (B2Tg)

To generate a transgenic mouse strain expressing *mBmal2* constitutively, we selected a promoter for elongation factor-1α ($P_{hEF1\alpha}$) that can drive constitutive expression in many rodent tissues, including the SCN [7]. $P_{hEF1\alpha}$ was ligated to five tandem repeats of the c-myc tag ("5myc"), followed by the *mBmal2* ORF ([22], Fig. 1A). Then, the SV40 small-T intron and the SV40 early gene transcriptional termination/polyadenylation signal sequence [23] was fused to the 5' end of the construct. The final construct is called "$P_{EF1\alpha}$-Myc-mB2-SV40intronPA." The transgenic mouse was made by the Vanderbilt Transgenic Mouse/Embryonic Stem Cell Resource and founder mice were screened by PCR for *Bmal2* and for the SV40 poly(A) signal. We obtained three founders of $P_{EF1\alpha}$-*mBmal2* transgenic mice (B2Tg) and all of them were fertile. Two lines of the B2Tg mice (designated #1 and #3) showed free-running periods significantly longer than those of wild-type mice in DD. The other line (#2) showed no significant difference in the free-running period. In this study, we used line #3 to analyze molecular and physiological circadian rhythms. All the B2Tg mice used in this study were heterozygous for the $P_{EF1\alpha}$-Myc-mB2-SV40intronPA transgene.

Promoter/enhancer assays

Mouse 3T3 and human HEK293 cells were transfected using Lipofectamine™ 2000 (Invitrogen) with luminescence reporter constructs: PK2.8-Luc (reporter for the PK promoter [24]) or pGL4-PmB2 (reporter for *mBmal2* promoter activity), and the pCMV-Rluc as a transfection control. To make pGL4-PmB2, a fragment extending from the mouse *Bmal2* upstream region (−4706 from transcription start site) to the first intron (+339 from transcription start site) was amplified by PCR from mouse genomic DNA (C57BL/6 strain) and inserted into XhoI and BglII sites of the pGL4.11 luciferase vector (Promega).

Locomotor behavior assay

Animal care and use procedures were approved by Vanderbilt University institutional guidelines. Mice were singly housed in cages equipped with running wheels on a LD 12:12 cycle for at least 10 d before being released into constant darkness. ClockLab software (Actimetrics, Evanston, IL) was used to collect data and produce double-plotted actograms as well as the periodogram and activity analyses. χ^2 periodogram analyses were performed to obtain periods using the first 20 days of data in DD. To measure circadian phase angle at the termination of the LD cycle, linear regressions were fit through the activity onsets for the 7 days before and 6–20 days after release into DD (the first 5 days in DD were excluded to avoid "transients" [25]).

Oxygen consumption, body weight/fat composition, and food intake assays

Sex and age matched mice were used for all metabolic experiments. The mice were singly housed in indirect calorimetry cages ("OxyMax," Columbus Instruments). Mice were monitored for 2 d in LD, 3 d in DD, and followed by 1–2 d in dim red light (red light intensity inside mouse cages was ~1 μmol/m^2s), during which time oxygen consumption (VO_2) was measured every 15 min. Energy expenditure was calculated as described previously [26]. LumiCycle software (Actimetrics Inc., Evanston, IL) was used to subtract the 24-h moving average from the raw oxygen consumption (VO_2) results for the 3 d in DD and to smooth the data by 2-h adjacent averaging. The period of the VO_2 rhythm in DD was determined by periodogram analyses of the smoothed data. Body fat composition was determined with an mq10 nuclear magnetic resonance analyzer (Bruker Optics). For food

intake assessment, mice were placed in individual cages and the amount of food that was consumed in 24 h was measured.

Tissue culture and *in vitro* luminescence recording

P_{mPer2}::mPER2::LUC knockin mice (a gift from Dr. Joseph Takahashi) that had been back-crossed with the C57BL/6 strain for more than 10 generations were crossed with *Bmal1*+/− and *Bmal2* transgenic (B2Tg) mice. All B2Tg or P_{mPer2}::mPER2::LUC mice used in this study were heterozygous. One to two h before lights-off of LD 12:12, the mouse was sacrificed by cervical dislocation under dim red light, and cultures of SCN, liver, and lung were prepared as previously described [27]. *In vitro* rhythms were analyzed for period, etc., using the analysis software for the LumiCycle (Actimetrics, version 2.31 [27].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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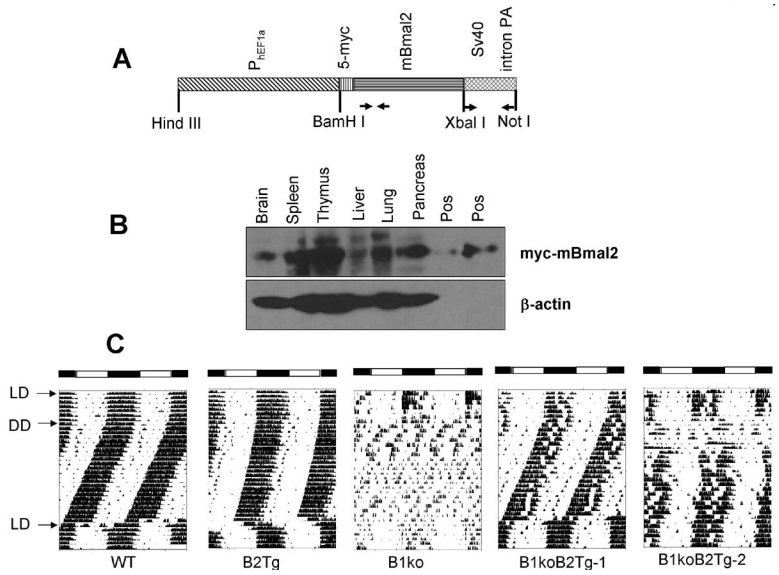

Fig. 1. Constitutive expression of *mBmal2* can rescue locomotor activity rhythms of the *mBmal1*-knockout mouse

(A) Structure of the Bmal2-transgene vector ("5myc-Bmal2"). The coding region of *mBmal2* (cDNA) was fused to an upstream region consisting of 5 tandem repeats of the c-myc sequence (5-myc) and the human EF-1α promoter and to a downstream region composed of the SV40 intron-poly(A) sequence [23]. Primers used for the screening of the transgenic mouse lines are indicated below the construct as arrows.

(B) The expression of 5myc-Bmal2 in the mouse with or without *Bmal2* transgene was analyzed by immunoblotting with an anti-myc-tag antibody. Lysates from HEK293 cells with 5myc-Bmal2 transfection was used as the positive control, and β-actin was immunoblotted as the loading control.

(C) Locomotor activity records. Representative activity records (actograms) are shown in the double-plotted format. Animals were initially in a 12 h light/12 h dark cycle (LD) for at least 10 d and were then transferred to constant darkness (DD). After at least 20 d in DD, the mice were re-entrained to LD. Arrows indicate transitions between different LD cycles. For the B1ko/B2Tg mice, four mice showed a complete behavioral rescue (B1ko/B2Tg-1 is a representative example), while three mice showed partial rescue (e.g., B1ko/B2Tg-2). White and black bars at the top of the actograms indicate the LD cycle when present. Summary data, including power spectra, period, and activity levels, are depicted in Fig. S2.

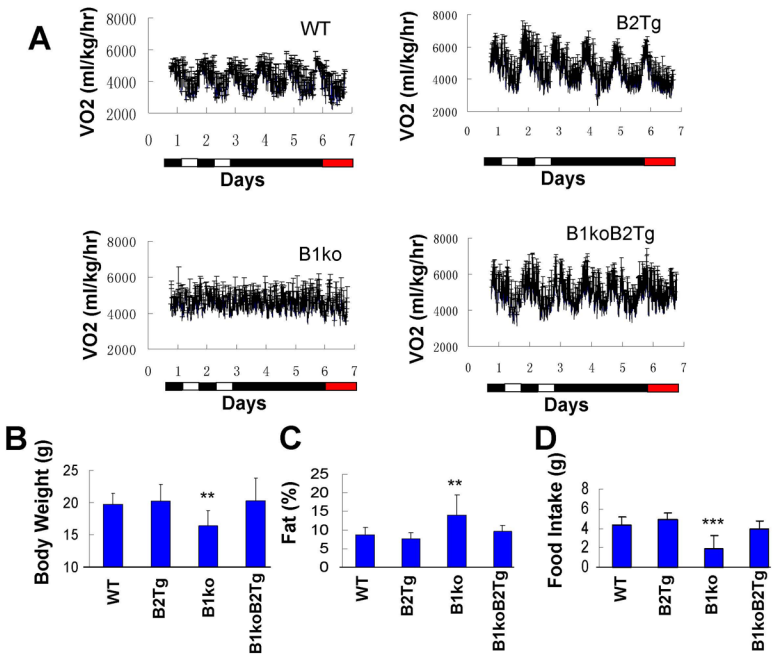

Fig. 2. Expression of *Bmal2* rescues metabolic phenotypes of *Bmal1*-knockout mice

A. Rhythms of oxygen consumption under 2 d of LD 12:12 followed by 3 d of DD, followed by 1 d of red LL. Each bar is the mean±SD (n = 4).

B. Body weight of 8-week-old mice. Each bar represents the mean±SD (n = 8; 4 males and 4 females).

C. Percentage body fat composition measured by NMR. Data are presented as mean±SD (n=8 for each group).

D. Daily food intake. Each bar is the mean±SD of eight mice (4 male and 4 female). For panels B, C, & D: **p<0.01; ***p<0.001. One-way ANOVA, post hoc LSD test.

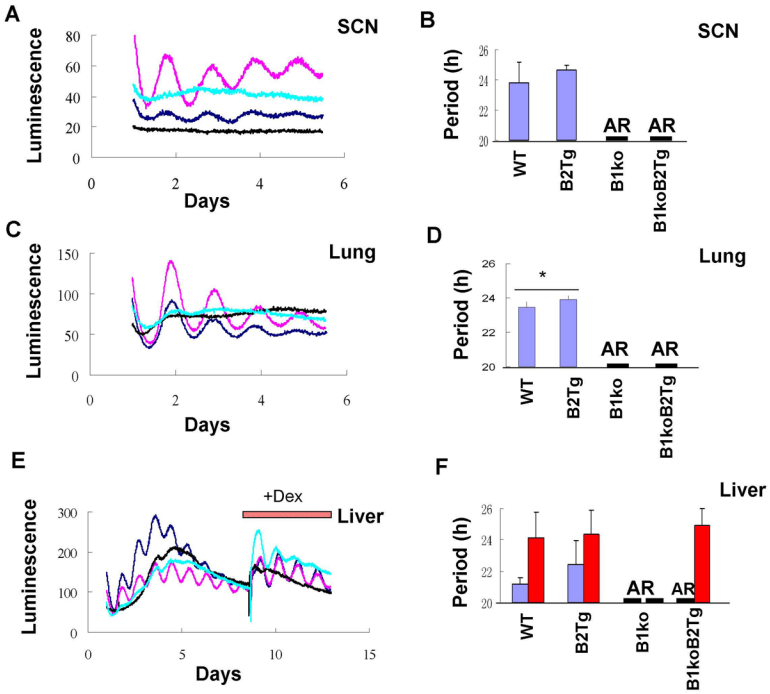

Fig. 3.

Expression of *Bmal2* does not rescue the rhythmicity of B1ko SCN or lung tissues *in vitro*, but can rescue liver rhythmicity after dexamethasone (DEX) stimulation. Panels A, C, E show the raw data of luminescence from tissues of P_{mPer2}::mPer2-Luc knockin mice monitored *in vitro*. Tissue explants were dissected on Day 0 and recorded with a LumiCycle apparatus. Dark blue: WT; Magenta: Bmal2 Tg; Black: Bmal1ko; Light blue: Bmal1ko/Bmal2Tg. Panels B, D, F show the period analyses of those data (AR = arhythmic), plotted as means ± SD (N≥3, * $p<0.05$ by two-tailed T test).

A/B. suprachiasmatic nuclei (SCN) slices.

C/D. lung slices.

E/F. liver slices; + DEX (100 nM) on Day 8. In panel F, blue histograms represent the recording interval prior to DEX treatment (for WT vs. B2Tg, $p = 0.11$), and red histograms represent the interval during the DEX treatment.

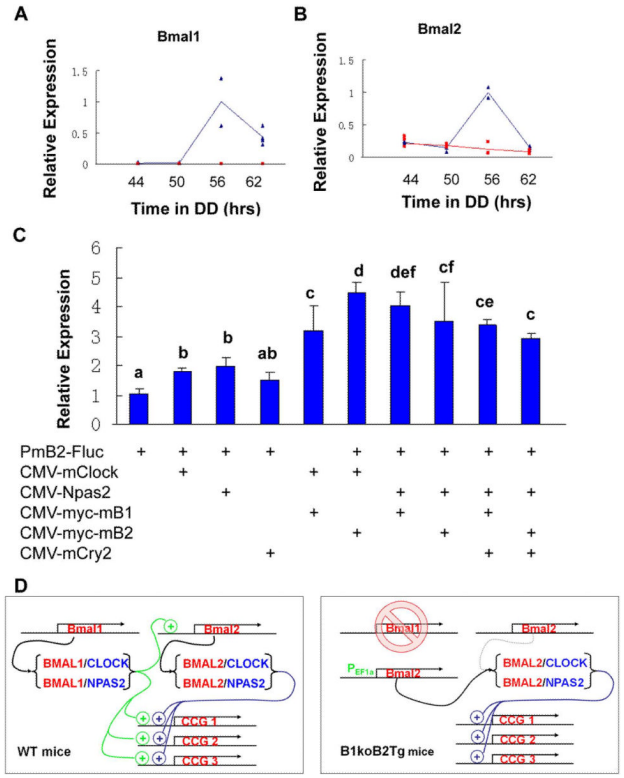

Fig. 4.

The *Bmal2* promoter/enhancer is activated by BMAL1/CLOCK and BMAL1/NPAS2 heterodimers. Levels of *Bmal1*(**A**) and *Bmal2* (**B**) mRNA levels in liver. Transcripts of *Bmal1* and *Bmal2* were quantified by real-time PCR and normalized with reference to *Hprt* mRNA levels as a control. The data were normalized by setting the maximum value of *Bmal1* or *Bmal2* mRNA levels to 1.0. Blue triangles show data from WT mice, while red squares show data from B1ko mice; lines connect the averages at each time point (n = 3 or 4 except for WT at 56 h, where n = 2). **(C)** Effect of CLOCK/NPAS2 and BMAL1/BMAL2 on expression of the *mBmal2* promoter in 3T3 cells. The total amount of plasmid DNA that was transfected was kept constant among by addition of the empty vector DNA when appropriate. Data are mean±SD (n≥4) of firefly luciferase activity (P_{mBmal2}::Fluc) normalized by the *Renilla* luciferase control (P_{CMV}::Rluc). The activity in samples transfected with the reporter construct (i.e., “PmB2-Fluc” without plasmids expressing other proteins) was set as 1.0. Significant differences in pairwise comparisons at the $p < 0.05$ level are indicated by letters. **(D)** Control of circadian gene expression by *Bmal1* and *Bmal2*. Left panel: in wild-type mice, E-box clock-controlled genes (CCGs) are activated by both BMAL1 and BMAL2 proteins in combination with CLOCK and NPAS2. Expression of the *Bmal2* gene is regulated by BMAL1/CLOCK and/or BMAL1/NPAS2 heterodimers. Right panel: in the Bmal1ko/Bmal2Tg mouse, expression of BMAL2 from the transgene (and possibly from the endogenous *Bmal2* gene that is also activated by BMAL2 from the transgene) regulates the expression of the CCGs, thereby rescuing clock and metabolic phenotypes that result from Bmal1/Bmal2 knock-out/down in the Bmal1ko mouse.