A circadian clock in the sinus node mediates day-night rhythms in *Hcn4* and heart rate

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BACKGROUND Heart rate follows a diurnal variation, and slow heart rhythms occur primarily at night.

OBJECTIVE The lower heart rate during sleep is assumed to be neural in origin, but here we tested whether a day-night difference in intrinsic pacemaking is involved.

METHODS *In vivo* and *in vitro* electrocardiographic recordings, vagotomy, transgenics, quantitative polymerase chain reaction, Western blotting, immunohistochemistry, patch clamp, reporter bioluminescence recordings, and chromatin immunoprecipitation were used.

RESULTS The day-night difference in the average heart rate of mice was independent of fluctuations in average locomotor activity and persisted under pharmacological, surgical, and transgenic interruption of autonomic input to the heart. Spontaneous beating rate of isolated (ie, denervated) sinus node (SN) preparations exhibited a day-night rhythm concomitant with rhythmic messenger RNA expression of ion channels including hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4). *In vitro* studies demonstrated 24-hour rhythms in the human HCN4 promoter and the corresponding funny current. The day-night heart rate difference in mice was abolished by HCN block, both *in vivo* and in the isolated SN. Rhythmic expression of canonical circadian clock transcription factors, for example, Brain and muscle ARNT-Like 1 (BMAL1) and Cryptochrome (CRY) was identified in the SN and disruption of the local clock (by cardiomyocyte-specific knockout of *Bmal1*) abolished the day-night difference in *Hcn4* and intrinsic heart rate. Chromatin immunoprecipitation revealed specific BMAL1 binding sites on *Hcn4*, linking the local clock with intrinsic rate control.

Funding sources/Disclosures: This work was supported by a British Heart Foundation Intermediate Basic Science Research Fellowship awarded to Alicia D'Souza (FS/19/1/34035), grants from the British Heart Foundation (RG/11/18/29257; PG/15/16/31330) to Mark Boyett, Halina Dobrzynski and Alicia D'Souza, and grants from the CARIPLO Foundation (ACROSS 2014-0728) and Fondation Leducq (TNE FANTASY 19CV03) awarded to Dario DiFrancesco, Mark Boyett, Halina Dobrzynski and Matteo Mangoni. Cheryl Petit was funded by a grant from the Biotechnology and Biological Sciences Research Council (BB/J003441) awarded to Hugh Piggins. [1]Drs D'Souza and Wang contributed equally to this work. **Address reprint requests and correspondence:** Dr Alicia D'Souza, Division of Cardiovascular Sciences, University of Manchester, 46 Grafton St, Manchester M13 9NT, United Kingdom. E-mail address: alicia.dsouza@manchester.ac.uk.

https://doi.org/10.1016/j.hrthm.2020.11.026

CONCLUSION The circadian variation in heart rate involves SN local clock–dependent *Hcn4* rhythmicity. Data reveal a novel regulator of heart rate and mechanistic insight into bradycardia during sleep.

KEYWORDS Bradycardia; Circadian rhythm; Pacemaking; Sinus node; Vagus nerve

(Heart Rhythm 2021;18:801–810)

Introduction

In humans, the resting heart rate exhibits diurnal rhythms and is higher during the day when we are awake.[1] The heart is therefore primed, anticipating the increase in demand during the awake period. Conversely, slow heart rhythms primarily occur at night during the sleep period.[1] The same occurs in the nocturnal rodent, but in reverse.[1]

For ∼90 years, the day-night difference in heart rate *in vivo* in humans has been attributed to the autonomic nervous system[2] and primarily high vagal tone during sleep.[3] This is in large part based on heart rate variability (HRV) as a surrogate measure of autonomic tone.[3] However, from biophysical analysis of HRV we have previously demonstrated an exponential-like relationship between HRV and heart rate, and changes in HRV observed in humans and animal models are mainly attributable to the accompanying changes in heart rate.[4,5] The involvement of the autonomic nervous system in the circadian rhythm in heart rate has also been previously tested in rodents by acute block of sympathetic and parasympathetic input to the heart. In spontaneously hypertensive rats, Oosting et al[6] demonstrated that the circadian rhythm of heart rate is unaffected by pharmacological block of the autonomic nervous system. Makino et al[7] reported that both sympathectomy and pharmacological block of the parasympathetic nervous system in rats diminishes but does not abolish the circadian variation in heart rate. Knockout of the muscarinic M2 receptor or all 3 β-adrenergic receptors has little or no effect on the circadian rhythm in heart rate in mice.[8,9] Taken together, these data call into question the widely accepted notion that autonomic tone is the *sole* driver of the circadian variation in heart rate. Here we tested the hypothesis that an intrinsic day-night rhythm in the sinus node (SN), the primary pacemaker of the heart, is an important contributor. We have focused on the pacemaker channel hyperpolarization-activated cyclic nucleotide-gated potassium channel 4 (HCN4) as a "first port of call."

Methods

Animal models and associated ethical approval, experimental methods, and statistical comparisons are described in detail in Online Supplemental Methods.

Results

Circadian variation in heart rate is independent of locomotor activity and persists under autonomic blockade

Electrocardiographic (ECG) telemetry in mice showed an *in vivo* day-night difference in mean heart rate and other electrophysiological variables including the PR interval and QRS duration; in Figure 1A, the mean heart rate and other variables are plotted against the zeitgeber time (ZT), in which ZT 0 is taken as the start of the lights-on period. Changes in the heart rate set by the SN were explored: the heart rate was highest at ZT ∼13, and it varied by 76 ± 4 beats/min over the course of 24 hours (Figure 1A; Online Supplemental Table 1). The day-night differences during the 12-hour light/12-hour dark lighting regime (shown by alternating light and dark shading in Figure 1A) were sustained when mice were placed in constant darkness (shown by continuous dark shading in Figure 1A).

Mice are nocturnal, and as expected, lower physical (locomotor) activity was recorded from ZT 0 (lights on) to ZT 12 (lights off) than from ZT 12 to ZT 0; this activity pattern continued in constant darkness (Figure 1A, bottom panel). Physical activity, if sufficiently intense and prolonged, is expected to influence the heart rate via the autonomic nervous system. Various methods were used to test whether the day-night difference in heart rate is an indirect result of the difference in physical activity (via the autonomic nervous system) or is an independent time-of-day effect. During 72 hours of continuous recording in a normal light-dark cycle (Figure 1A), the heart rate and physical activity were averaged for each 5-minute period for each animal. For ZT 0–ZT 12 (day) and ZT 12–ZT 0 (night), individual activity data were then binned into *no activity* (0 arbitrary units [au]) and *high activity* (20–30 au) groups and the corresponding heart rate was recorded. A mixed effects linear model showed that heart rate was significantly higher both at night ($P = .007$) and when mice were active ($P < .0001$). However, the interaction between time and activity was not significant ($P = .27$), which means that in this data set the heart rate difference between night and day does not depend on the activity level. Comparisons were conducted between day and night at each of the activity levels by using a 2-sided 5% test and applying a Sidak multiple comparison adjustment, and this revealed a significant difference ($P = .03$) in heart rate at night vs day in the no activity group. Further details of these statistical tests are available in Online Supplemental Results.

Exposing nocturnal animals to light during their active phase suppresses their locomotor activity, a phenomenon referred to as negative masking.[10,11] Light pulses were used to separate the effects of time-of-day and physical activity. Figure 1B shows the heart rate and activity level in conscious mice measured using telemetry before, during, and after the light pulses (from Figure 1A, which shows the context of the selected data). In constant darkness, a 1-hour light pulse

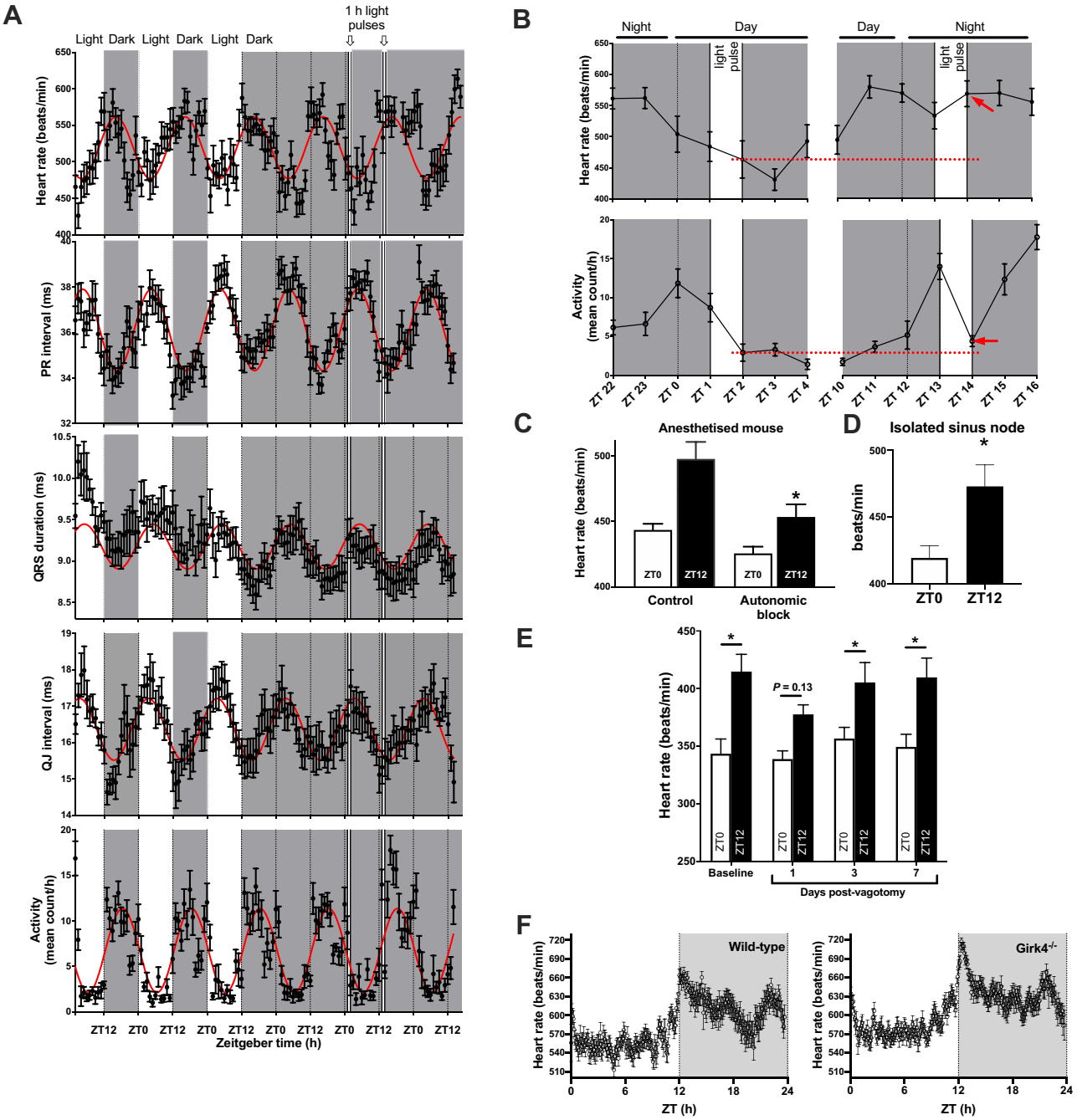

Figure 1 The day-night difference in average heart rate is independent of average physical activity and autonomic tone. **A:** Heart rate, PR interval, QRS duration, uncorrected QJ interval, and physical activity (measured using telemetry) in conscious mice (n = 9) over ~6 days. *Light and dark shaded regions* represent light and dark phases in this and all similar figures. Timing of 1-hour light pulses is shown. In this and all similar figures, data are fit with a standard sine wave (*red*). Values are presented as mean ± SEM in this and all other figures (except in the case of physical activity, for which mean ± SD values are presented). **B:** *In vivo* heart rate and physical activity measured by telemetry at the times shown during 24-hour darkness (subjective day and night is shown) with the exception of a 1-hour light pulse delivered toward the start of the day (left) or night (right). The *dotted red lines* highlight the heart rate and physical activity at the end of the day-time light pulse, and the *red arrows* highlight the heart rate at the end of the nighttime light pulse. From the same experiment as in panel A. **C:** *In vivo* heart rate measured from anesthetized mice at ZT 0 and ZT 12 (n = 9 mice per time point) before (control) and after autonomic block by intraperitoneal injection of 1 mg/kg of atropine and 1 mg/kg of propranolol. **D:** Spontaneous beating rate of the mouse SN isolated at ZT 0 (n =8) and ZT 12 (n = 6). **E:** *In vivo* heart rate at ZT 0 and ZT 12 measured by telemetry in vagotomized rats (n = 7) at baseline (presurgery) and at 1, 3, and 7 days postsurgery. **F:** *In vivo* heart rate over 24 hours measured in telemetrized wild-type control (n = 21) and *Girk4*$^{-/-}$ (n = 23 mice). *$P < .05$. HCN4 = hyperpolarization activated cyclic nucleotide gated potassium channel 4; SN = sinus node; ZT = zeitgeber time.

from ZT 1 to ZT 2 was associated with a baseline level of physical activity and the heart rate was relatively low (Figure 1B). In contrast, a light pulse from ZT 13 to ZT 14 caused physical activity of mice to fall to baseline values whereas the heart rate remained relatively high (Figure 1B). Online Supplemental Figure 1 demonstrates no discernible

relationship between heart rate and physical activity level before, during, and after the light pulses or over 24 hours. Therefore, in this experiment, the average heart rate of the 9 mice was primarily influenced by the time of day rather than the average physical activity level. *In vivo*, the heart rate in the absence of physical activity was obtained by ECG recordings in anesthetized mice at ZT 0 and ZT 12; the heart rate was higher at ZT 12 and increased by 54 ± 14 beats/min from ZT 0 (Figure 1C; Online Supplemental Table 1). It is concluded that in this study the effect of activity on the circadian rhythm in heart rate is not discernible.

Involvement of the autonomic nervous system in mediating the day-night variation in heart rate was tested by blocking cardiac muscarinic and β-adrenergic receptors (using 1 mg/kg of atropine and 1 mg/kg of propranolol; see Online Supplemental Discussion for justification of doses) in anesthetized mice. The day-night difference in heart rate persisted after complete pharmacological autonomic block, although it was reduced in amplitude (Figure 1C; Online Supplemental Table 1). This *intrinsic* day-night difference in SN pacemaking was further confirmed in the isolated, denervated SN dissected at ZT 0 and ZT 12 (Figure 1D). The spontaneous SN beating rate remained higher at ZT 12 than ZT 0—by 55 ± 9 beats/min (Figure 1D; Online Supplemental Table 1). Next, the role of the vagus in setting the diurnal variation in heart rate *in vivo* was studied by 2 loss-of-function approaches: the SN is predominantly innervated by the right vagus[12] and its contribution to heart rate rhythmicity was assessed by unilateral right vagotomy in telemetrized rats. Figure 1E demonstrates that the day-night difference in heart rate persisted on sectioning the right vagus. The influence of vagal signaling (downstream of the muscarinic receptor) on the day-night rhythm was then investigated in *Girk4*$^{-/-}$ mice with genetic ablation of the acetylcholine-activated K^+ current ($I_{K,ACh}$). *Girk4*$^{-/-}$ mice exhibited slightly increased basal heart rates in comparison to wild-type counterparts, which is a typical hallmark of this genetic strain.[13] However, although $I_{K,ACh}$ is acknowledged to be a key mediator of the negative chronotropic effect of vagal stimulation on heart rate, loss of $I_{K,ACh}$ did not perturb the circadian variation in heart rate in *Girk4*$^{-/-}$ mice vs control animals (Figure 1F; Online Supplemental Table 1).

Taken together, these findings demonstrate that the autonomic nervous system is not solely responsible for the day-night difference in heart rate *in vivo*. Intrinsic mechanisms underlying day-night rhythms were thus considered.

Day-night difference in HCN4 channel expression

Pacemaking is the result of the concerted action of ion channels and Ca^{2+}-handling proteins comprising the membrane and Ca^{2+} clocks and messenger RNA (mRNA) for many of these molecules (and key regulatory transcription factors) was measured in the SN by quantitative polymerase chain reaction. Some transcripts, for example, the pacemaker channel *Hcn4* (that carries the pacemaker current I_f), demonstrated a significant day-night difference (Figure 2A; Online Supplemental Table 2). With the exception of Ca^{2+}/calmodulin-dependent protein kinase IIδ (*Camk2d*), a day-night rhythm was not detected in any of the principal components of the Ca^{2+} clock (Figure 2A; Online Supplemental Table 2).

We focused on *Hcn4*, because of its central role in pacemaking in the SN.[14] *Hcn4* mRNA was measured at 4 time points. JTK_Cycle, a statistical software tool for analyzing circadian rhythms,[15] shows that *Hcn4* mRNA displays a robust circadian rhythm and is at a maximum at ∼ZT 20 (JTK_Cycle; $P = .008$) (Figure 2B; Online Supplemental Table 3). To test whether this finding is associated with rhythmic *Hcn4* promoter activity, the 780-bp core promoter region of human *Hcn4*[16] was subcloned into a luciferase reporter construct (*h*Hcn4-Luc). Transient transfection of this construct into C2C12 cells was followed by forskolin treatment to synchronize circadian clocks across cultured cells.[17] As given in Figure 2C, real-time bioluminescence recording (a measure of *Hcn4* promoter activation) revealed a discernible circadian rhythm of *h*Hcn4-Luc (JTK_Cycle; $P = 1.21 \times 10^{-14}$). Interestingly, the phase of *h*Hcn4-Luc in synchronized cultured cells was comparable to HCN4 mRNA expression in the mouse SN (Figure 2B), peaking at circadian time ∼19.5 (circadian time 0 is the time of forskolin treatment).

Expression of HCN4 protein in the SN at ZT 0, ZT 6, and ZT 12 was measured using Western blotting. A representative blot is shown in Figure 2D. HCN4 protein expression was normalized to total protein expression and is plotted against time in Figure 3C; there was a significant change at ZT 6 vs ZT 12. Online Supplemental Figure 2 shows examples of immunolabeling of HCN4 in tissue sections through the SN from wild-type mice culled at ZT 0 and ZT 12; the labeling was brighter, indicating higher expression, at ZT 12. This is confirmed by the mean data in Online Supplemental Figure 2. It is concluded that there is a circadian rhythm in HCN4 protein as well as the *Hcn4* transcript.

Role of HCN4 remodeling in the day-night difference in pacemaking

HCN4 is the primary channel underlying the funny current I_f, and patch clamp experiments on isolated mouse SN cells were carried out to test whether there is a circadian rhythm in I_f. Figure 3A shows representative families of recordings of I_f from SN cells isolated from mice at ZT 0, ZT 6, and ZT 12. The density of I_f was reduced at ZT 6 compared with the earlier and later time points. Mean current density-voltage relationships from n = 38, n = 31 of 38, and n = 24 of 27 cells (at ZT 0, ZT 6, and ZT 12) are shown in Figure 3B; current density at ZT 6 was significantly reduced relative to that at ZT 12 at potentials ≤−90 mV and ZT 0 at ≤−105 mV. Current density at −120 mV is plotted against time in Figure 3D. Current density is calculated from current amplitude and cell capacitance, and these measurements are plotted in Figures 3E and 3F. Cell capacitance did not vary with time, but current amplitude did, explaining the circadian rhythm in the density of I_f (Figures 3D–3F).

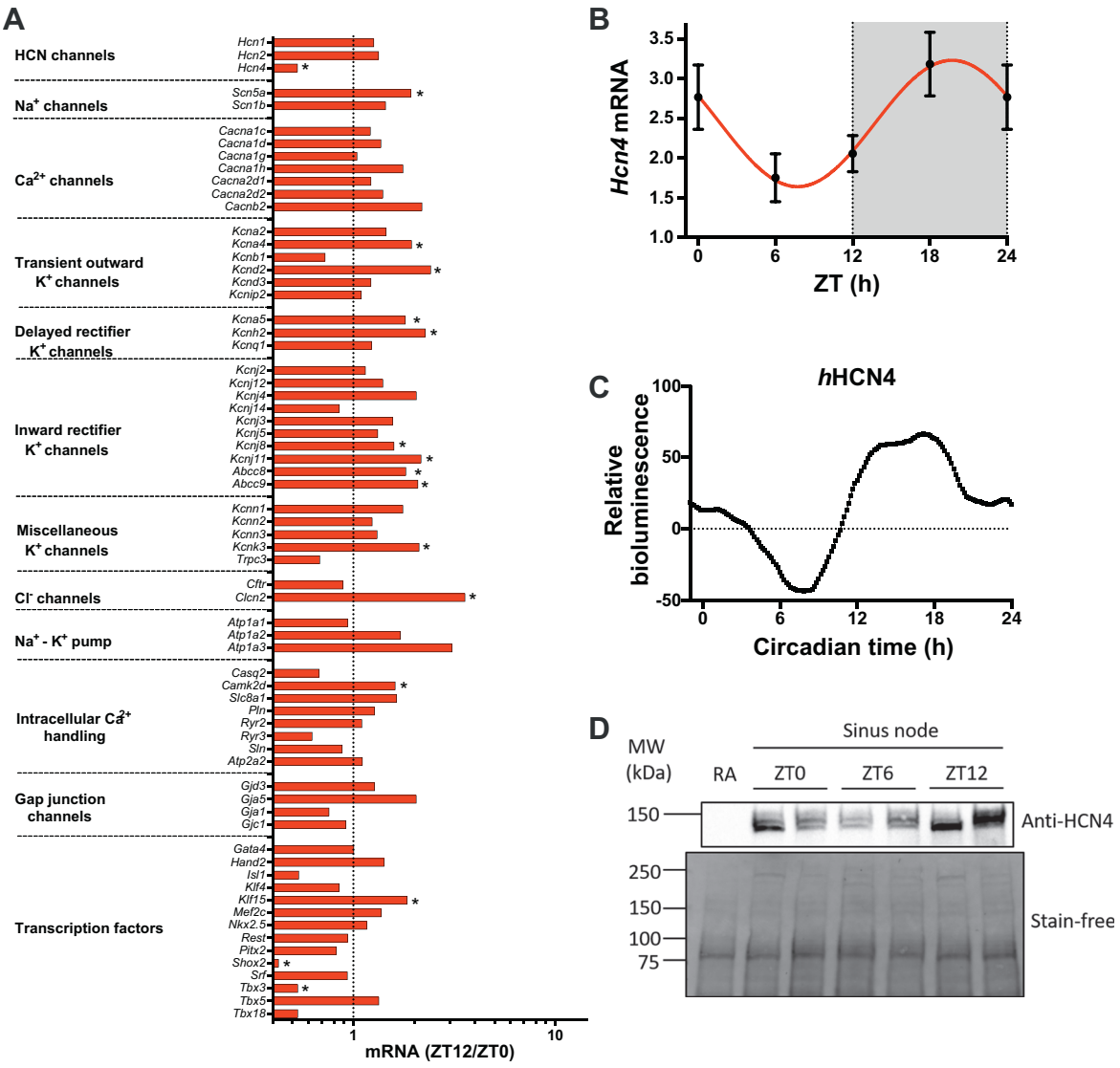

Figure 2 Day-night rhythms in SN ion channel profile and *Hcn4*. **A:** Relative expression of transcripts in the SN at ZT 12 (vs ZT 0; n = 7/9 mice). The *vertical line* corresponds to 1, that is, no change. Values <1 correspond to a decrease at ZT 12 and >1 an increase, *$P<0.05$. **B:** Expression of *Hcn4* mRNA (normalized to the expression of *Tbp* and *Ipo8*) in the SN at 4 time points over 24 hours (n = 6 mice at each time point). **C:** Representative LumiCycle data (Actimetrics, Wilmette, Illinois) (in arbitrary units) showing human *Hcn4* promoter activity over 24 hours. **D:** Representative HCN4 Western blot from the SN dissected at ZT 0, ZT 6, and ZT 12. A right atrial tissue sample (RA) is also shown as a negative control; as expected, there is no HCN4 expression. Corresponding stain-free total protein gel was used for quantification shown in the lower panel. HCN4 = hyperpolarization activated cyclic nucleotide gated potassium channel 4; mRNA = messenger RNA; MW = molecular weight; SN = sinus node; ZT = zeitgeber time.

To test whether rhythms in HCN4 and I_f contribute to the day-night difference in heart rate, HCN4 was blocked *in vivo* and in the isolated SN (the efficacy and selectivity of blockers used are considered in Online Supplemental Discussion). Telemetrized mice received an intraperitoneal injection of 6 mg/kg of ivabradine to block HCN4 and thus I_f.[18] Block of I_f decreased the heart rate as expected (Figures 4A and 4B), and the effect of ivabradine on heart rate was greater at ZT 12 than at ZT 0 (Figures 4A and 4B). Furthermore, as shown by ECG recordings in Figure 4A and the summary data in Figure 4B, the presence of ivabradine abolished the day-night rhythm in heart rate *in vivo*. Analogous results were obtained *in vitro* by application of 2 mM Cs^+ to the isolated SN.[19] Once again, block of I_f by 2 mM Cs^+ decreased beating rate as expected, but the Cs^+ effect was greater at ZT 12 than at ZT 0 (Figures 3C and 3D). In the presence of Cs^+, the day-night difference in beating rate was abolished (Figure 3D). These results suggest that HCN4 and I_f participate in the circadian rhythm in the intrinsic heart rate as well as heart rate *in vivo*.

A peripheral circadian clock in the SN

Circadian clocks are endogenous oscillators that generate transcriptional rhythms. In the ventricles, there is an intrinsic circadian clock as well as a circadian rhythm in some ion channels.[1] Quantitative polymerase chain reaction showed that many canonical circadian clock genes are also present in the SN and their expression varies in the expected manner

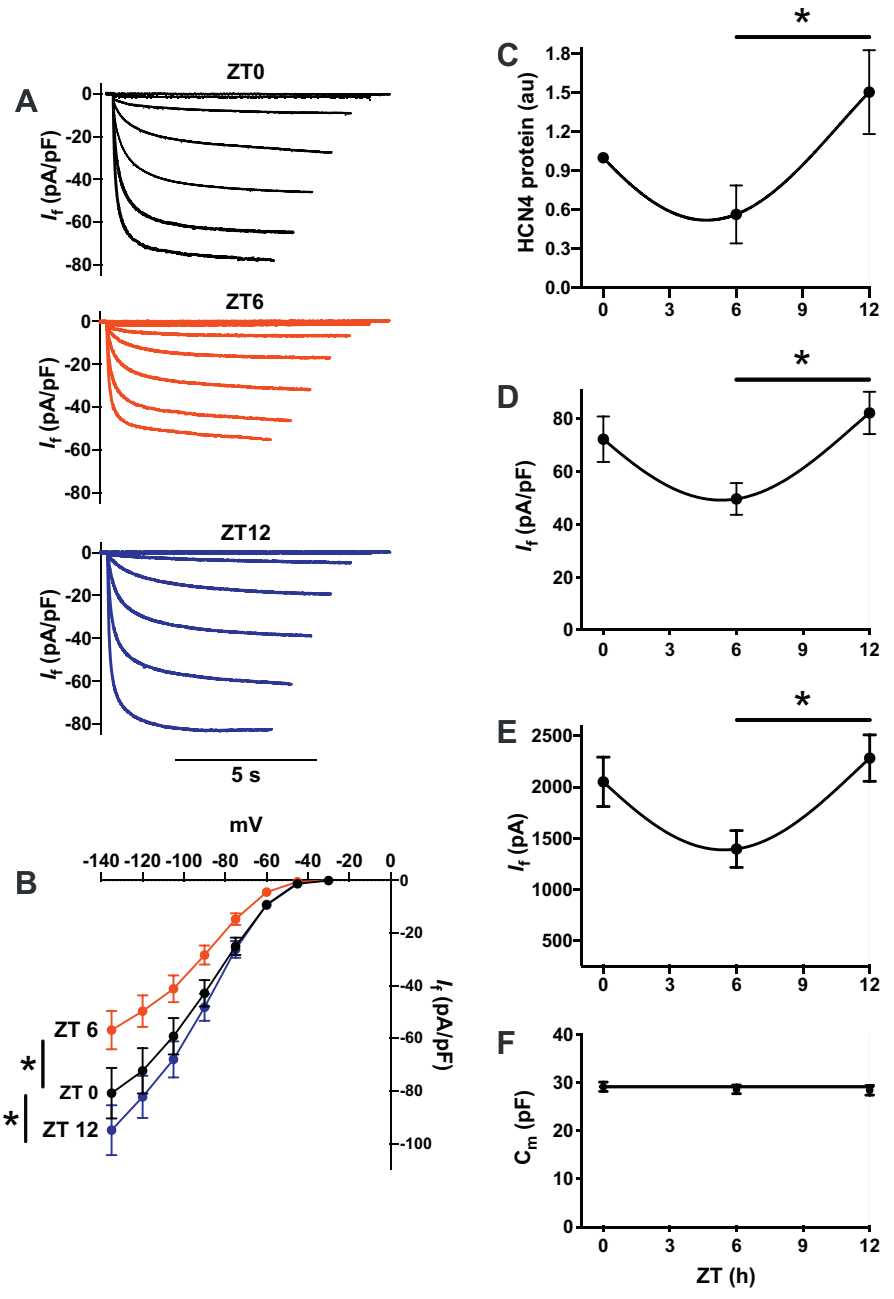

Figure 3 Day-night rhythm in I_f. **A:** Families of recordings of I_f made from SN cells isolated at ZT 0, ZT 6, and ZT 12. **B:** Current-voltage relationships for I_f recorded from SN cells isolated at ZT 0 (n = 38 cells per 4 mice), ZT 6 (n = 38 cells per 3 mice), and ZT 12 (n = 27 cells per 5 mice). **C:** HCN4 protein expression from the Western blot at ZT 0 (n = 10), ZT 6 (n = 5), and ZT 12 (n = 10). Data pooled from 2 sets of independent experiments and protein expression are normalized to those at ZT 0. **D:** Density of I_f at −120 mV at ZT 0 (n = 38 cells per 4 mice), ZT 6 (n = 38 cells per 3 mice), and ZT 12 (n = 27 cells per 5 mice). **E:** Amplitude of I_f at −120 mV at ZT 0, ZT 6, and ZT 12 (same data as in panel D). **F:** Cell capacitance at ZT 0, ZT 6, and ZT 12 (same data as in panel D). *$P < .05$. HCN4 = hyperpolarization activated cyclic nucleotide gated potassium channel 4; I_f = funny current; SN = sinus node; ZT = zeitgeber time.

from ZT 0 to ZT 12 (Figure 5A). mRNA expression of two key circadian clock transcripts—Brain and muscle ARNT-Like 1 (BMAL1) and Circadian locomotor output cycles kaput (CLOCK) —were measured in the SN at 4 time points (BMAL1 and CLOCK form a heterodimer). Figure 5B shows that they demonstrated a robust daily rhythm and were at a maximum at ∼ZT 0 (Online Supplemental Table 1). This suggests that a functional intrinsic circadian clock is contained in the SN. This was subsequently confirmed by measuring the bioluminescence in the isolated SN of *Per1*::-LUC mice carrying a luciferase gene reporting the activity of *Per1*, a key circadian clock component (Figure 5C). *Per1*-driven bioluminescence fluctuated in the expected circadian manner, and this periodicity was lost in $Cry1^{-/-}Cry2^{-/-}$ mice lacking a functional clock[20] (Figure 5C).

Role of the SN circadian clock in setting the day-night difference in *Hcn4* and intrinsic heart rate

To investigate a possible link between the local circadian clock in the SN and the circadian rhythm in the intrinsic heart

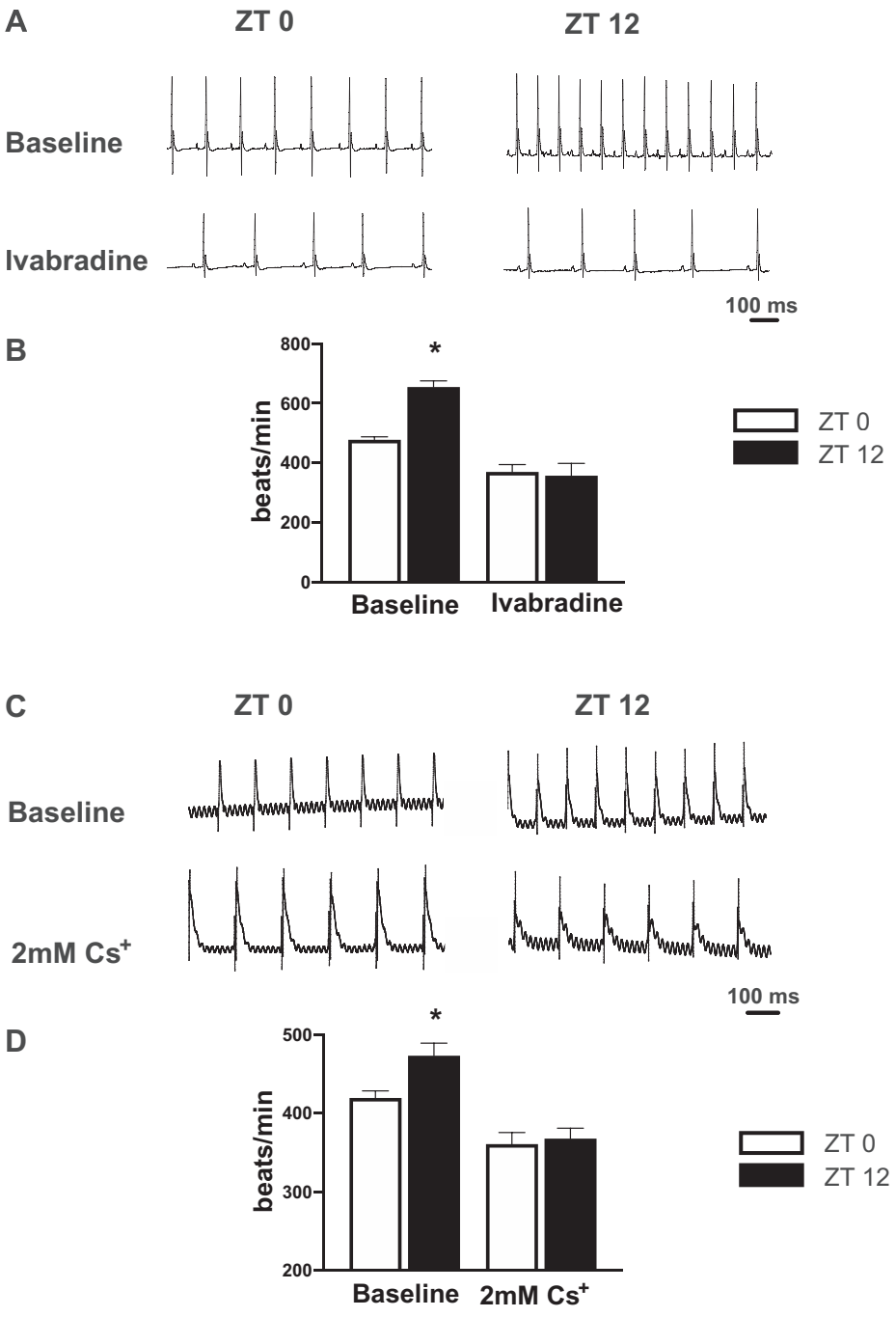

Figure 4 Effect of HCN4 block on the heart rate. **A:** Representative electrographic recordings from telemetrized mice obtained at ZT 0 and ZT 12 at baseline and after the application of 6 mg/kg of ivabradine. **B:** *In vivo* heart rate of wild-type mice measured at ZT 0 and ZT 12 before and after the administration of 6 mg/kg of ivabradine (n = 3 mice per time point). **C:** Representative extracellular potential recordings from the SN isolated at ZT 0 and ZT 12 at baseline and after the application of 2 mM Cs^+. **D:** Intrinsic heart rate of the isolated SN at ZT 0 and ZT 12 before and after the application of 2 mM Cs^+ (n = 8 mice at baseline and 6 mice for Cs^+ at ZT 0; n = 6 mice at baseline and 7 mice for Cs^+ at ZT 12). *$P < .05$. HCN4 = hyperpolarization activated cyclic nucleotide gated potassium channel 4; SN = sinus node; ZT = zeitgeber time.

rate, experiments were conducted on cardiomyocyte-specific *Bmal1* knockout mice. Knockout of *Bmal1* is known to disrupt the circadian clock[21] and previous studies have identified SN dysfunction on cardiomyocyte-specific *Bmal1* deletion.[22] Figure 6A confirms that in these animals, *Bmal1* was effectively knocked out in the SN. Figure 6B shows that this disrupted the circadian rhythm in the expression of *Clock* mRNA—evidence that the SN circadian clock had been disrupted as expected (Online Supplemental Table 4). The intrinsic heart rate was measured in the isolated SN; *Bmal1* knockout mice presented with lower intrinsic heart rates vs wild-type mice and the normal day-night variation in intrinsic heart rate seen in wild-type mice was absent in *Bmal1* knockout mice (Figure 6C; wild-type data from Figure 1D). Furthermore, whereas there was a circadian difference in the reduction in the intrinsic heart rate on block of I_f by Cs^+ in wild-type mice (the effect of Cs^+ was greater at ZT 12 than at ZT 0), in *Bmal1* knockout mice, once again this

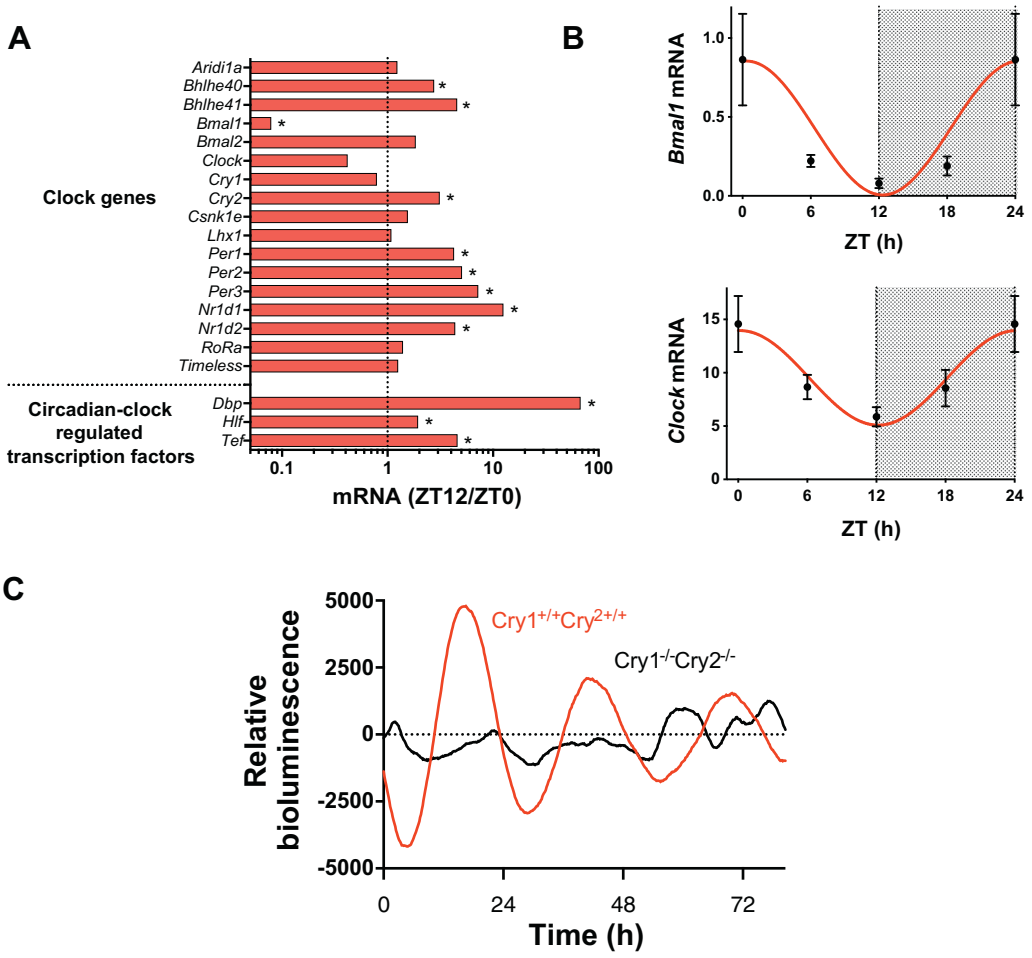

Figure 5 An intrinsic circadian clock in the SN. **A:** Relative expression of transcripts encoding key circadian clock components in the SN at ZT 12 vs ZT 0. The vertical line corresponds to 1, that is, no change. Values <1 correspond to a decrease at ZT 12 and >1 an increase (n = 7/9 mice). **B:** Expression of *Bmal1* and *Clock* mRNA in the mouse SN at 4 time points over 24 hours (n = 6 at ZT 0, n = 6 at ZT 6; n = 7 at ZT 12 and n = 8 at ZT 18). **C:** Representative *Per1* activity (reported by luciferase bioluminescence) in the isolated SN from *Per1*::LUC mice with a $Cry1^{+/+}Cry2^{+/+}$ or $Cry1^{-/-}Cry2^{-/-}$ background. *$P < .05$. mRNA = messenger RNA; SN = sinus node; ZT = zeitgeber time.

pattern was lost (Figure 6D); this suggests that there is a circadian variation in I_f, which is lost on disrupting the local circadian clock. Consistent with these observations, the ZT 0 to ZT 12 variation in both *Hcn4* transcript and HCN4 protein (determined by immunolabeling) in wild-type mice was lost in *Bmal1* knockout mice (Figures 6E and 6F; Online Supplemental Figure 2).

These findings suggest that the SN circadian clock controls intrinsic pacemaker activity via *Hcn4* transcriptional regulation. Mechanisms by which BMAL1 may regulate *Hcn4* were therefore investigated *in vitro*. The CLOCK::-BMAL1 heterodimer acts as a transcriptional enhancer by binding to E-box binding sites in the promoter, intron, or exon of a gene.[23] rVISTA 2.0 was used to identify canonical E-box binding sites on *Hcn4*.[24] Eight sites on *Hcn4* and 20 kb of its 5′ flanking region were identified (Figure 6G). *In vitro* chromatin immunoprecipitation was used to test whether BMAL1 specifically binds to these sites. Chromatin immunoprecipitation enrichment for E-box binding sites D and G (within introns of the *Hcn4* gene) (Figure 6G) were identified (Figure 6H). These data reveal potential direct interactions between the local SN clock and *Hcn4*.

Discussion

For the first time, we show that the diurnal rhythm in heart rate in rodents cannot be fully attributed to oscillations in autonomic tone and reveal a day-night variation in intrinsic SN pacemaker activity. We define time-of-day variation in the expression of key pacemaking ion channels and ascribe particular physiological relevance to rhythmic *Hcn4* and associated I_f remodeling in setting the day-night variation in heart rate. Finally, we link, for the first time, SN pacemaking to an intrinsic circadian clock and propose a new role for BMAL1 as a transcriptional regulator of *Hcn*4.

This study has shown that there is a day-night difference in intrinsic SN pacemaker activity. Day-night differences in HCN4 mRNA and protein and I_f (Figures 2 and 3) accompanied the day-night variation in heart rate *in vivo* and intrinsic SN beating rate (Figure 1). Block of HCN4 and I_f by

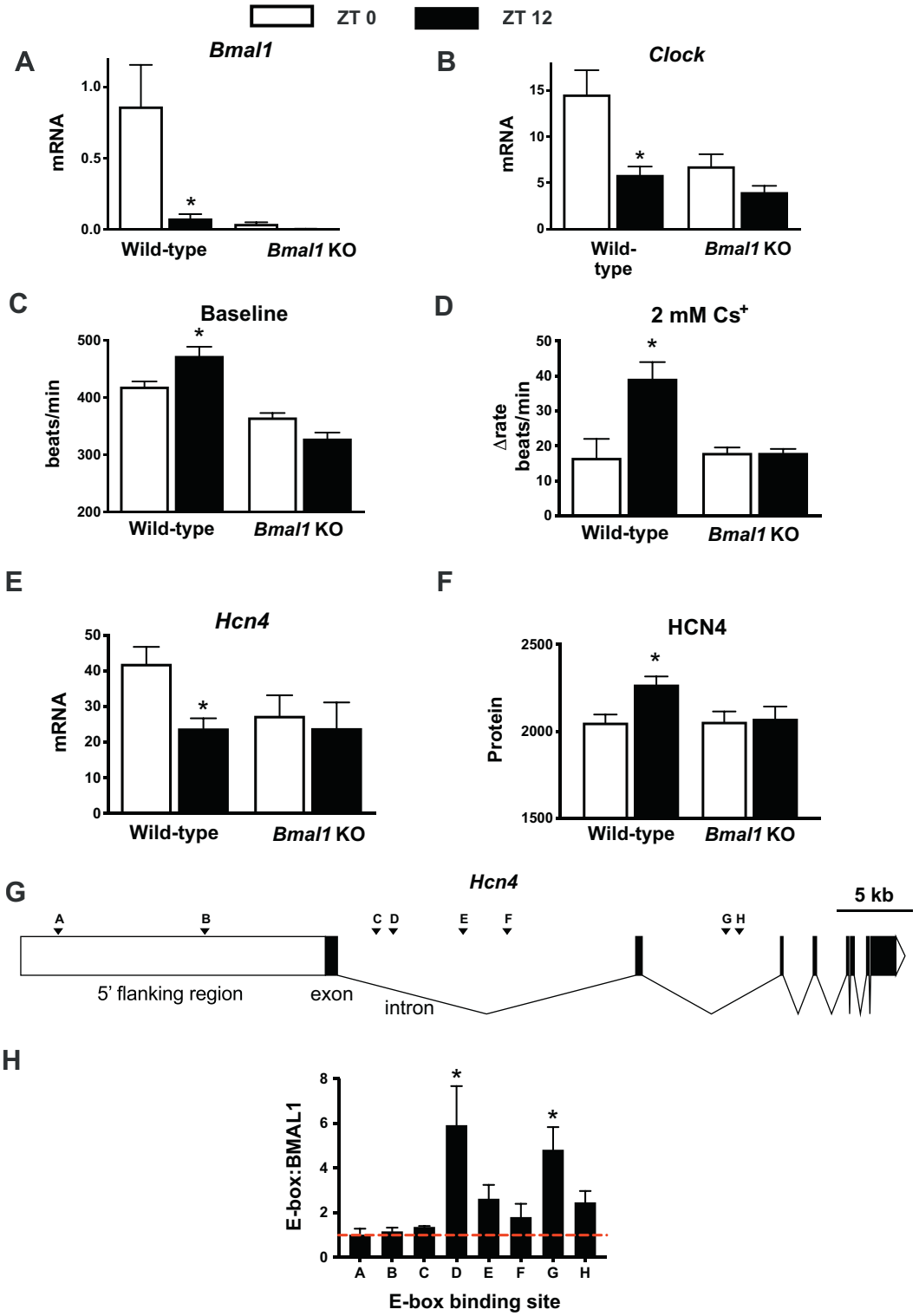

Figure 6 BMAL1 modulates intrinsic heart rate and HCN4. **A and B:** Expression of *Bmal1* (panel A) and *Clock* (panel B) at ZT 0 and ZT 12 in the SN of wild-type and *Bmal1* KO mice (n = 6 at ZT 0 and n = 7 at ZT 12 for wild-type mice; n = 5 at ZT 0 and n = 6 at ZT 12 for *Bmal1* KO mice). **C:** SN beating rate at ZT 0 and ZT 12 from wild-type (n = 8 at ZT 0 and n = 6 at ZT 12) and *Bmal1* KO (n = 3 per time point) mice. **D:** Change in SN beating rate at ZT 0 and ZT 12 from wild-type (n = 8 at ZT 0 and n = 6 at ZT 12) and *Bmal1* KO (n = 3 per time point) mice on application of 2 mM Cs^+. **E and F:** Expression of *Hcn4* mRNA (panel E; n = 6 at ZT 0 and n = 7 at ZT 12 for wild-type mice; n = 5 at ZT 0 and n = 6 at ZT 12 for *Bmal1* KO mice) and HCN4 protein (panel F; determined by immunohistochemistry; in arbitrary units; n = 56 sections at ZT 0 and n = 42 sections at ZT 12 for wild-type mice; n = 38 sections at ZT 0 and n = 46 sections at ZT 12 for *Bmal1* KO mice; 3 mice per group) in the SN of mice at ZT 0 and ZT 12. **G:** Diagram of *Hcn4* gene with 20 kb of the 5′ flanking region showing the position of 8 potential E-box binding sites (panels A–H). **H:** Eight potential E-box binding sites pulled down on immunoprecipitation of His-tagged BMAL1 from Cos-1 cells transfected with His-tagged *Bmal1*. Data are normalized to immunoprecipitation from untransfected control cells; the *red dashed line* equals 1 and is the baseline level. *$P < .05$ (binding site of interest vs binding site A; n = 2 replicate experiments). HCN4 = hyperpolarization activated cyclic nucleotide gated potassium channel 4; KO = knockout; mRNA = messenger RNA; SN = sinus node; ZT = zeitgeber time.

ivabradine *in vivo* and Cs^+ in the isolated SN was more pronounced at ZT 12 and HCN4 block abolished the day-night difference in heart rate *in vivo* and *ex vivo* (Figure 4). Data consistent with the time-of-day dependence of HCN4 block have been obtained from patients: in patients with inappropriate sinus tachycardia or ischemic heart disease and heart failure, ivabradine causes a large decrease in heart rate during the day and a slight decrease at night.[25,26] Nevertheless, I_f may not be the only mechanism involved: there was a day-night difference in calcium/calmodulin dependent protein kinase II delta (Camk2d/CaMKIIδ) expression and various K^+ channels, particularly ether-a-go-go related gene (ERG; *Kcnh2)* (Figure 2A). Further study is warranted on the relative importance of these alterations in controlling SN pacemaking. Please refer to Online Supplemental Discussion for detailed limitations of the study.

This is the first report of a functioning circadian SN clock (Figure 5): key clock components were identified and many showed expected rhythms and phase relationships, for example, *Bmal1* was downregulated, but *Cry2*, *Per1*, and *Per2* were upregulated at ZT 12 vs ZT 0 (Figure 5A). This study provides the first evidence that *Hcn4* is under local clock control, as cardiac *Bmal1* knockout suppressed transcript abundance and abolished the day-night rhythm in both message and protein levels (Figures 6E and 6F). Functional BMAL1-binding E-box sites on *Hcn4* (Figures 6G and 6H) were identified, and this is the first clue as to how the local clock exerts transcriptional control of pacemaking. Akin to the mouse, human *Hcn4* also includes 1 E-box consensus site within 5 kb of the 5′ flanking region and 4 sites within the first intron (Online Supplemental Figure 3). There is further discussion of the phasing of the circadian clock, *Hcn4*, HCN4, and I_f in Online Supplemental Information.

Conclusion

This study has shown that a local clock–driven day-night difference in intrinsic SN pacemaking contributes to the day-night difference in heart rate *in vivo*. Our findings provide new mechanistic insight into the fundamental question of why the heart rate of a mammal is lower when asleep and may explain the nocturnal occurrence of bradyarrhythmias in the human.

Appendix

Supplementary data

Supplementary data associated with this article can be found in the online version at https://doi.org/10.1016/j.hrthm.2020.11.026.

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