

Circadian rhythms of activity and drinking in mice lacking angiotensin II 1A receptors

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Abstract

Angiotensin II (ANG II) type 1 receptors are found in the mouse suprachiasmatic nucleus (SCN), the site of the circadian pacemaker, but their significance for circadian timekeeping is unknown. We examined circadian rhythms of wheel running and drinking in angiotensin AT_{1a} receptor knockout (KO) mice. Mean daily running and drinking activity were elevated in KO mice under a light–dark (LD) cycle and in constant dark (DD). These increases were confined to the usual active (dark) period, thus, the ‘amplitude’ of running and drinking rhythms was higher in KO mice. The phase of entrainment to LD (measured by the onset of the daily active period) did not differ between groups, either in LD or on the first day of DD (‘unmasked’ phase). KO mice showed a modestly shorter free-running period (τ) in DD. The direction and magnitude of phase shifts to light pulses at two circadian times (CTs) in DD did not differ between groups. Core functions of the circadian system appear intact following AT_{1a} receptor KO. The modestly shorter τ and increased rhythm amplitude in KO mice may be secondary to an effect of the mutation on the level of running and drinking activity. © 2001 Elsevier Science Inc. All rights reserved.

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1. Introduction

The octapeptide angiotensin II (ANG II) serves as the primary effector molecule for the regulation of fluid and electrolyte homeostasis by the renin–angiotensin system [3,28]. These functions are mediated in part by a network of ANG II-releasing and receptive neurons in hypothalamic areas that participate in the regulation of blood pressure, thirst and salt appetite [3,28]. In rats and mice, high concentrations of ANG II receptors [4,9,18,27] and some ANG II-immunoreactive (IR) soma and fibers [17,34] are found within the suprachiasmatic nucleus (SCN), the site of the master pacemaker responsible for the generation of circadian rhythms [12]. The significance of ANG II signaling within this nucleus is currently unknown. However, overexpression of the *renin2* gene in a transgenic rat model is associated with a phase delayed or inverted circadian rhythm of blood

pressure, attenuated circadian and photic induction of the *c-fos* gene in SCN neurons, and attenuated phase shifting of behavioral and cardiovascular rhythms in response to light [14–16]. These findings suggest that dysfunction of the renin–angiotensin system may affect not only the mean level of blood pressure but also the expression of behavioral and cardiovascular circadian rhythms. Whether this is caused by changes in the activity of ANG II within the circadian pacemaker remains to be determined.

Traditionally, studies of the functional role of central peptides have relied on the effects of administration of peptides or antagonists. For example, ANG II directly applied to the SCN in vitro can activate or suppress neuronal firing rates, effects that are blocked by AT₁ receptor antagonists [32]. Recent progress in genetic engineering has made available new experimental models in which peptides or their receptors have been deleted. For the renin–angiotensin system, mouse strains have been developed that lack the synthetic enzymes, the peptides or their receptors [3]. Models in which the AT_{1a} and AT_{1b} angiotensin receptor isoforms are missing exhibit changes in physiological functions related to the actions of ANG II [3,8,11,22,23]. The

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AT_{1a} receptor isoform is the best candidate to mediate cellular actions of ANG II in the SCN [1,2,10,22,31]. Therefore, to evaluate the significance of AT_{1a} signalling for circadian function, we conducted a comprehensive assessment of circadian rhythms in the ANG II AT_{1a} knock-out (KO) mouse.

As master pacemaker, the SCN contains three integrated components; a mechanism for generating circadian oscillations (likely contained within individual SCN neurons [35]), transduction mechanisms by which inputs ('zeitgebers', e.g., photic information from the retina) can entrain or shift the core oscillator, and one or more outputs that confer circadian rhythmicity on other brain systems. Signaling at SCN AT_{1a} receptors could potentially act on SCN 'clock' cells to modulate their rate of circadian oscillation, or to mediate or modulate their responses to one or more zeitgebers. If so, then loss of AT_{1a} signaling should affect all circadian rhythms (behavioral and physiological) in a similar way. Within the SCN, ANG II-IR is found primarily among cells in the dorsomedial region, which contains many output neurons [34]. Thus, AT_{1a} receptors outside of the SCN could potentially mediate coupling between the pacemaker and one or more specific driven processes. If that is the case, then different rhythms may be affected differentially by AT_{1a} KO. As a first step toward evaluating these possibilities, we measured circadian rhythm parameters (phase, period and amplitude) of two behavioral variables (drinking and wheel running activity) in KO and wild-type (WT) mice in the presence and absence of photic and nonphotic zeitgebers.

2. Materials and methods

2.1. Animals and housing

The ANG II AT_{1a} receptor KO was created by homologous recombination in embryonic stem cells by methods previously described [8]. F2 generation mice with KO (*Agt1a* $-/-$) and WT (*Agt1a* $+/+$) alleles were generated from crosses of (129 $\times$ C57BL/6)F1 *Agt1a* $+/-$ parents. Genotypes were determined by Southern blot analysis of DNA isolated from tail biopsies. Eight KO and six WT mice were received at the age of 7 months, adapted to the laboratory for 1 week, and then housed for 142 days in standard polypropylene cages with wire floors, 17 cm running wheels and drinkometers. Running and drinking contacts were monitored continuously by computer and stored to disk at 10-min intervals. Drinking was determined by contact of the mouse with the tip of the sipper tube, which closed a circuit, generating a 5-V pulse recorded by custom interface. Sipper tube contacts are proportional to drinking activity, but do not constitute a measure of absolute fluid intake. Rodent chow pellets were provided ad libitum.

2.2. Procedures

The mice were maintained in a 12:12 light–dark (LD) cycle (150 lx/0 lx) for 28 days, and then in constant dark (DD) for the remainder of the study. On Day 25 (± 1) of DD, the mice were exposed to a 10-min, 4-lx light pulse at circadian time (CT) 16 or 23. CT12 is defined as the onset of the primary bout of wheel running for that circadian cycle; CT16 is therefore 4 circadian hours after activity onset, where 1 circadian hour = the duration of the circadian cycle (τ)/24. On Day 55 (± 1), the mice were exposed to a second light pulse, such that all mice received one pulse at each of the two circadian phases (counterbalanced for order). Light pulses were provided by a 7-W white incandescent bulb. During the light pulse, individual cages were covered to prevent light leakage. On Day 68 (± 2) of DD, the running wheels were locked, and then unlocked on Day 104 (± 2). Cages were serviced with the aid of an infrared viewer.

2.3. Data analyses

Data were transferred to a Macintosh for display and analyses using a Matlab (Mathworks, MA) customized program (Biological Rhythms Software; available from the author for correspondence). Drinking and wheel running data were displayed in the form of actograms and average waveforms. Drinking data from one WT mouse were lost due to intermittent drinkometer failure.

The following parameters were quantified: (1) mean daily amount of running or drinking in LD and DD; (2) L/D ratios; (3) α/ρ ratios (where ' α ' is the duration of the active period, and ' ρ ' the duration of the rest period), in both LD and DD; (4) phase angle of entrainment to LD (ψ_{LD} , defined here as the difference in minutes between the onset of the main nocturnal bout of running or drinking and lights-off); (5) activity onset time on the first day of DD (an 'unmasked' measure of ψ_{LD} , i.e., a measure in which the behavioral suppressing effects of light are removed); (6) free-running τ in DD, with and without wheel access; and (7) phase shifts to light pulses at two circadian phases.

To quantify α and ρ , 7-day blocks of data in LD or DD were averaged with a folding period set to 24 h in LD, and to the free-running τ in DD. Thresholds defining the onset and end of α were set to 80% of the mean value for that block of days. To quantify ψ_{LD} , the average onset of activity in 7-day blocks of data was expressed in minutes relative to the time of lights-off. In DD, τ was obtained from the slope of the regression line fit to activity onsets for blocks of 7–14 days. An onset was defined as the first 10-min bin in which running or drinking activity exceeded 50 counts, after an interval of 240 min in which counts were below this threshold. Phase shifts were quantified by comparing the displacement of regression lines fit to blocks of data before and after the light pulses. The first 2–3 days after the light pulse were excluded, to ensure that phase shifts were complete. Data were evaluated statistically using independ-

ent *t* tests or repeated-measures ANOVA, with the Student–Newman–Keuls method used for pairwise comparisons. Means $\pm$ S.E.M. are reported.

3. Results

3.1. General

Wheel running and drinking rhythms of typical KO and WT mice are depicted in double-plotted actogram format in Fig. 1. Visual inspection revealed robust circadian organ-

ization of running and drinking activity in both LD and DD in all animals, with no obvious differences between groups.

3.2. Mean levels of running and drinking activity in LD and DD

KO and WT mice exhibited comparable levels of running and drinking during the first week in LD, but the KO mice ran more with each week [$F(1,12) = 3.14$, $P = .007$], such that by the last week in LD, they averaged as a group $11\,660 \pm 2631$ wheel revolutions/day, compared to 5013 ± 1342 revolutions/day in the WT group ($P < .05$; Fig. 2a). Mean drinking

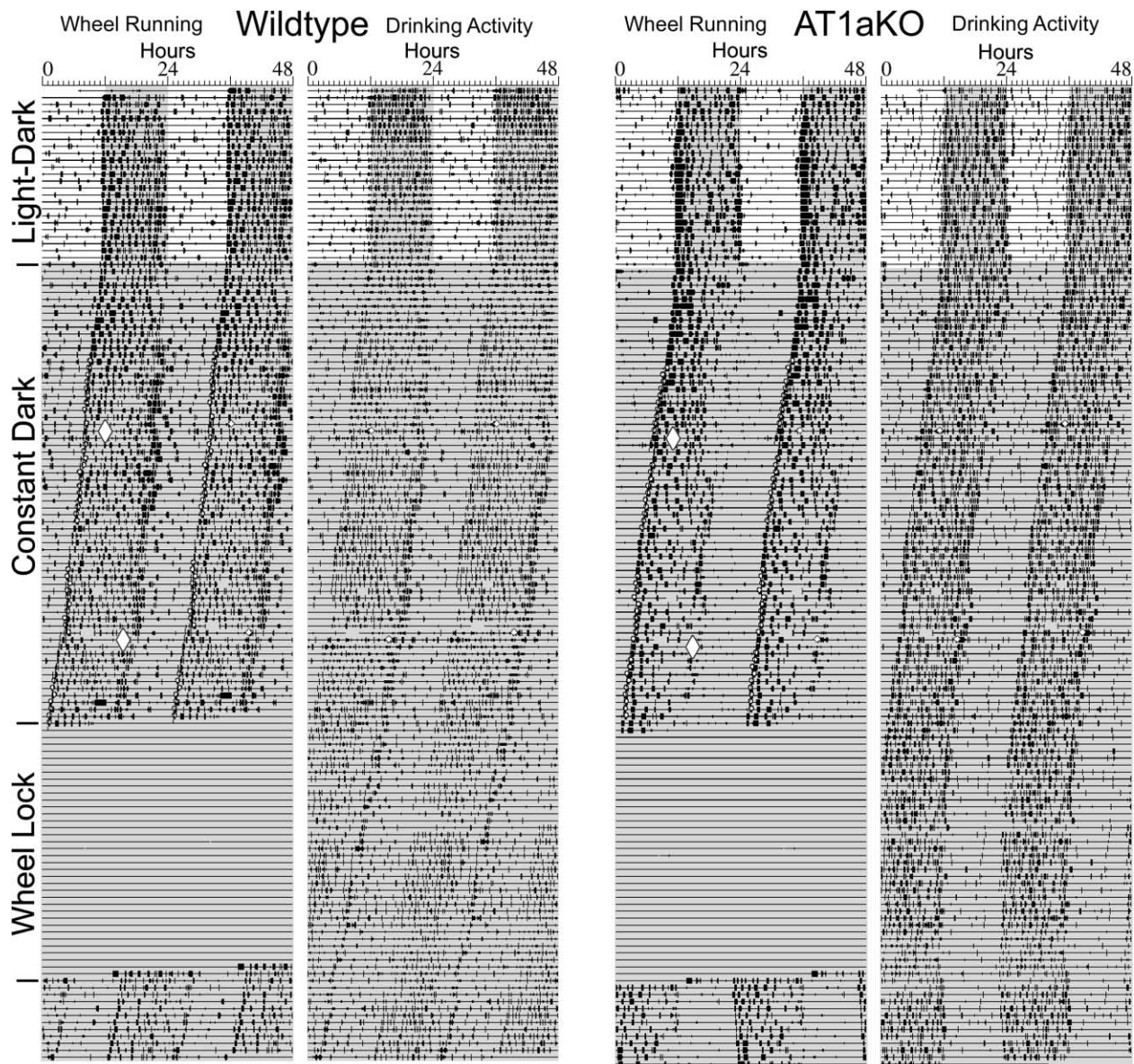


Fig. 1. Wheel running and drinking activity of one representative WT mouse and one KO mouse. Each line represents two consecutive days plotted from the left in 10-min bins. Consecutive days are also aligned vertically (i.e., the records are double-plotted to facilitate inspection of non-24-h rhythms). Vertical bars indicate bins when activity counts were registered. Four levels of activity are represented (single point = 0 counts, 3-point bar = 1–9 counts, 5-point bar = 10–19 counts, 7-point bar = 20+ counts). The first 28 days were conducted in LD 12:12, and the remaining days in DD (lights-off indicated by shading). Running wheels were locked for ~6 weeks as indicated along the vertical axis. Light pulses in DD are denoted by opaque diamonds. Opaque circles indicate computer-detected activity onsets to which regression lines were fit to measure τ and phase shifts.

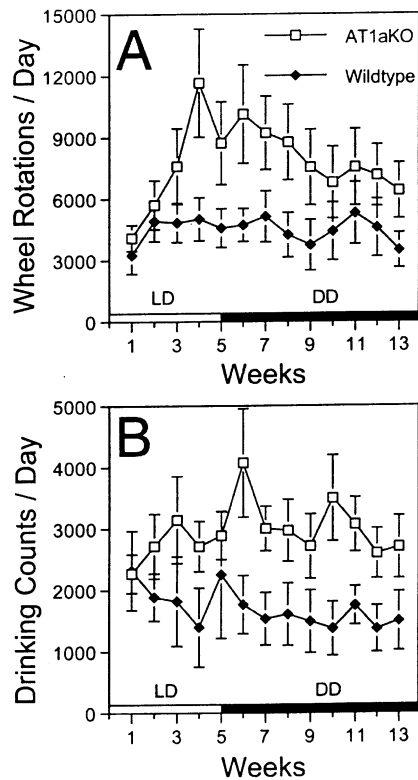


Fig. 2. Weekly averages of running (A) and drinking (B) in WT (solid diamonds) and KO (squares) mice in LD and DD.

contacts/day were also substantially greater in the KO by the last week of LD (2707 ± 403 vs. 1382 ± 648 contacts/day, respectively; Fig. 2b), but there was sufficient variability that this difference constituted only a trend [$F(1,31) = 1.54$, $P > .05$].

During the first 9 weeks in DD, the KO group averaged more running than the WT group each week and overall (7992 ± 2764 vs. 4608 ± 1065 revolutions/day, respectively).

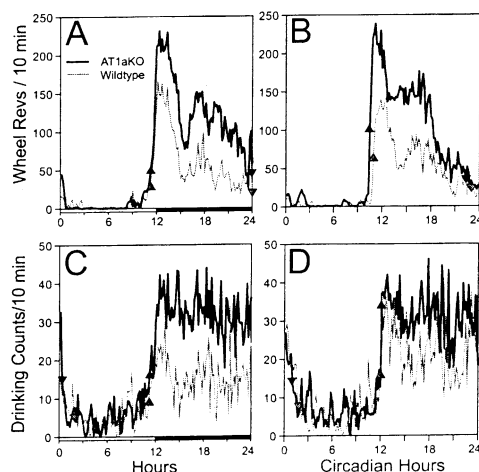


Fig. 3. Group mean waveforms of running (A, B) and drinking (C, D) during the last week of LD (A, C; corresponds to Week 4 in Fig. 2) and the first week of DD (B, D; corresponds to Week 5 in Fig. 2). The heavy bar along the horizontal axis denotes lights out in LD.

However, with a prominent outlier in both groups, an effect of genotype was evident only as a trend [$F(1,48) = 2.33$, $P > .05$]. The KO group also averaged more drinking contacts each week and overall in DD [3153 ± 418 vs. 1598 ± 389 contacts/day; $F(1,82) = 5.24$, $P = .04$; Fig. 2b].

3.3. Waveform

A plot of the waveform of the daily rhythms of running and drinking during the last week of LD (Fig. 3A and C) and the first week of DD (Fig. 3B and D) revealed that group differences in mean levels were confined entirely to α (Fig. 3). KO and WT mice did not differ in the mean duration of α (defined using wheel running data) in either LD (750 ± 13 vs. 800 ± 66 min, respectively) or DD (716 ± 58 vs. 823 ± 50 min, respectively). However, there was a trend for more intense running within α in the KO mice in both LD (151 ± 33 vs. 60 ± 13 wheel revolutions/10 min; $t = 2.02$, $P = .068$) and the first week of DD (117 ± 44 vs. 61 ± 18 revolutions/10 min; $P > .1$).

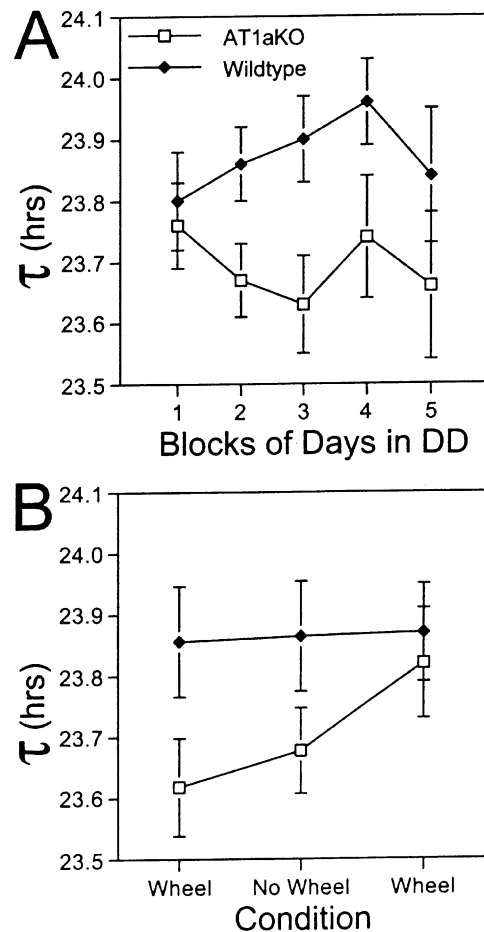


Fig. 4. (A) Group mean free-running τ of the wheel running rhythm in 10-day blocks of DD, in WT (solid diamonds) and KO (squares) mice. (B) Group mean τ of the drinking rhythm before, during and after ~ 6 weeks with running wheels locked.

Despite these differences in the amount of daily running, the average waveforms were similar in shape in the two groups (Fig. 3). Activity tended to be concentrated early in α , but considerable amounts were evident throughout α . Bimodal and trimodal patterns were occasionally evident, and one mouse in each group consistently exhibited maximal running at the end of α in both LD and DD. This range of waveforms is similar to that obtained for other WT mouse strains (e.g., C57BL/6J and BALB/c) [8,21].

The waveform of the drinking rhythm was also quite similar in the two groups (Fig. 3). Compared to running, drinking contacts were more evenly distributed throughout α , although individual mice did show weak bimodal and trimodal patterns in parallel with their wheel running rhythms.

3.4. Phase angle of entrainment to LD

The onset of the main bout of wheel running occurred in advance of lights-off by 29 ± 23 min in the KO group and 27 ± 17 min in the WT group. The onset of enhanced 'nocturnal' drinking activity began 35 ± 10 min before lights-off in KO mice and 24 ± 12 min before lights-off in WT mice. Neither of these differences were significant.

Light suppresses activity and drinking in nocturnal rodents, and can thus mask the true phase at which the circadian pacemaker entrains to LD. An unmasked measure of ψ_{LD} was obtained using activity onset on the first day of DD. In KO mice, activity and drinking onsets on the first day of DD occurred, on the average, 33 ± 21 and 30 ± 4 min, respectively, before the usual time of lights-off. In WT mice, running and drinking onsets occurred 16 ± 16 and 26 ± 5 min, respectively, before the usual time of lights-

off. Differences between groups or between LD and DD were not significant, indicating little masking effect of LD on ψ_{LD} , and no differential effect of genotype.

3.5. Amplitude measures

Amplitude can be defined as the magnitude of variation of a rhythm across different phases of its cycle. One amplitude measure that is frequently reported is the L/D ratio. This measure can be used to compare groups that do not differ in ψ_{LD} , as was demonstrated above for running and drinking rhythms. Group mean L/D ratios for running were lower (i.e., a greater proportion of total running was confined to the dark) during each week in KO mice (significant for Week 3, $t = 2.28$, $P = .04$), but over all days in LD the group difference was marginal (0.16 ± 0.05 vs. 0.26 ± 0.04 , $t = 1.70$, $P = .10$).

L/D ratios for drinking activity tended to be higher in both groups, by comparison with the L/D ratios for running (i.e., sipper tube contacts were less strictly nocturnal). Group mean drinking ratios in KO mice were lower by Week 4 of LD (0.24 ± 0.03 vs. 0.50 ± 0.05 , respectively, during the last week; $t = 3.98$, $P = .003$), but over all days in LD the group difference was evident only as a weak trend (0.28 ± 0.04 vs. 0.39 ± 0.06 ; $t = 2.10$, $P = .18$).

3.6. Free-running τ in DD, with and without wheel access

KO mice as a group exhibited a shorter free-running τ in DD, as measured using wheel running data [$F(1,12) = 6.44$; $P = .026$; Fig. 4a]. The group difference was in this direction during each of five 10-day segments over the first 60 days in

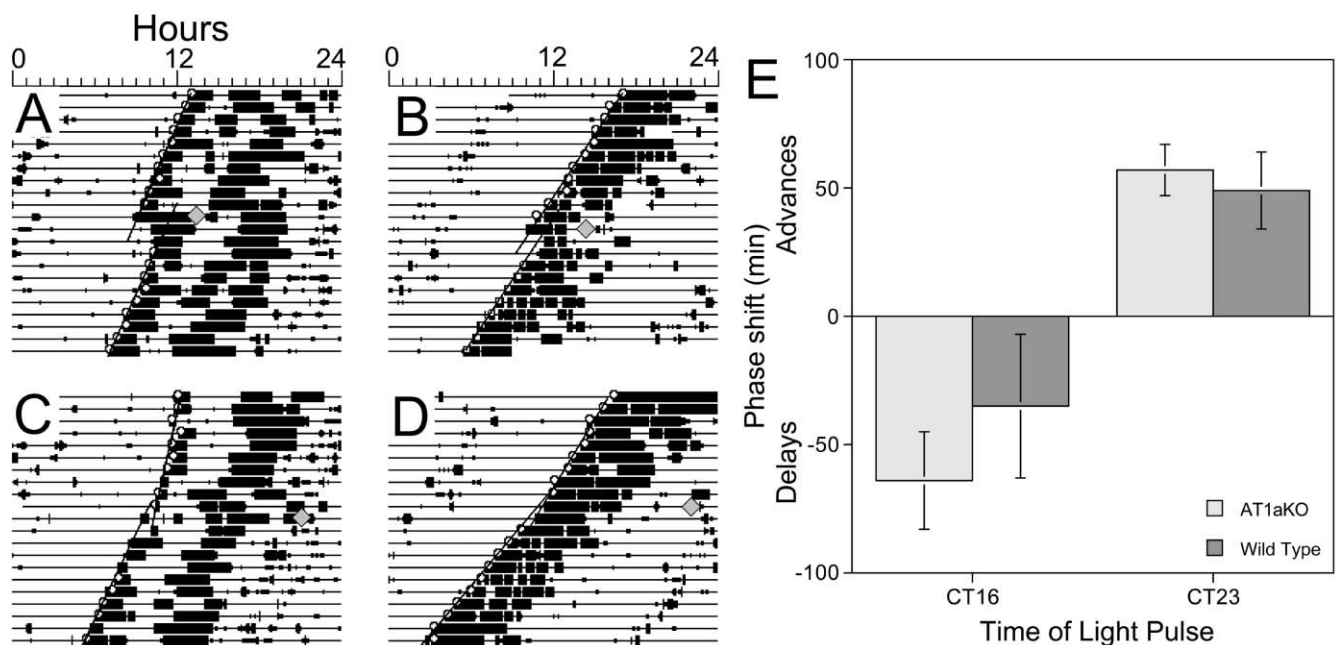


Fig. 5. Single-plotted actograms illustrating representative phase shifts in response to light pulses (shaded diamonds) at CT16 (A, B) and CT23 (C, D), in KO (B, D) and WT (A, C) mice. Plotting conventions as in Fig. 1. Group means ($\pm$ S.E.M.) are plotted in panel E.

DD, and was significant during the second and third segments ($P < .05$). Running wheels were locked from ~Day 65 to 104, a procedure that has been shown to lengthen τ in rodents [19,24,36], but this had no significant effect on τ , as measured using drinking data, in either group (Fig. 4b).

3.7. Phase shifts to light pulses

Light pulses at CT13, on the average, induced small phase delay shifts, while light pulses at CT23 induced small phase advance shifts. There were no significant group difference in shift magnitude (Fig. 5).

4. Discussion

Daily rhythms of physiology and behavior can be characterized by their timing, waveform and amplitude. Timing refers to the periodicity of the rhythm, and to the steady-state position of the rhythm relative to entraining stimuli (e.g., the LD cycle) or to other rhythms. Timing reflects intrinsic properties of the driving pacemaker, i.e., its period and endogenous rhythm of sensitivity to resetting stimuli. Waveform and amplitude refer to topological features of the overt rhythm and can be influenced by many factors. Although the pacemaker likely has an amplitude dimension [13,29], neither the waveform nor the amplitude of overt rhythms necessarily reflect intrinsic properties of the driving pacemaker (e.g., a mouse with no wheel has a zero amplitude running rhythm, but not necessarily a zero amplitude pacemaker).

In the present study, we found mixed evidence for a role of AT_{1a} receptors in the timing of running and drinking rhythms, and more consistent evidence for an effect on the amplitude of these rhythms. The phase angle of entrainment to LD did not differ significantly between groups, either in LD or on the first day of DD (an ‘unmasked’ measure). However, the KO group showed a modestly shorter τ in DD. In both LD and DD, the waveforms of running and drinking rhythms were qualitatively similar in the two groups, but the amount of running and drinking within α , and, therefore, the α/ρ and L/D ratios tended to be higher in the KO group.

One interpretation of these data is that loss of signaling at AT_{1a} receptors in KO mice shortens the τ and increases the amplitude of the circadian oscillation generated within the SCN clockworks. Alternatively, the mutation may alter parameters of overt rhythms by affecting processes upstream or downstream from the molecular gears of the clock. We will argue here that the τ and amplitude differences between groups are likely secondary to effects of the mutation on processes outside of the clock.

There are two reasons for suggesting that the τ difference between groups was secondary to processes upstream from the clock. First, the τ difference was quite modest, and the two τ distributions overlapped. Other mutations known to affect τ in mice and hamsters have much more dramatic

effects, on the order of 1–4 h, with little or no overlap in the distributions characteristic of WT and mutant genotypes [30,33]. Second, τ is shortened in WT mice, rats and hamsters by access to a running wheel, or by increased use of a wheel [19,20,24,36]. This effect of wheel running on τ defines an input pathway (i.e., from processes upstream from the clock) by which behavior can modulate cycling of the clock (reviewed in Ref. [21]). The magnitude of this effect in mice is small, and is similar to the between-groups differences in τ evident in the present study. A parsimonious explanation, then, is that the KO mice displayed a shorter mean τ because, on the average, they ran and drank somewhat more than WT. Neither group showed a significant change of τ when the running wheels were locked, but both groups ran less during this latter portion of the experiment, making it less likely that an effect of wheel access on τ would be detected. Moreover, the group τ difference at that time was not significant.

Group mean differences in rhythm amplitude are likely also secondary to an effect of the mutation on the levels of running or drinking, rather than on the amplitude of the pacemaker. If pacemaker amplitude was increased in KO mice, we would expect to see smaller phase shifts in response to light pulses, based on evidence that the magnitude of phase shifts in response to resetting stimuli is inversely related to pacemaker amplitude (e.g., Refs. [13,29]). Instead, the ‘trend’, if any, was in the opposite direction. KO mice may tend to show higher amplitude running and drinking rhythms because the mutation promotes both of these behaviors, while the circadian pacemaker and the presence of light in LD continue to limit the expression of these behaviors to the usual active (lights-off) phase of the circadian cycle.

It is interesting to note that activity and drinking are reportedly decreased in the AT_2 receptor KO mouse [7]. If our explanation of τ and amplitude differences in AT_{1a} KO mice is correct, we would expect circadian rhythm amplitude to be lower and τ to be longer in AT_2 KO mice. Consistent with this prediction, it has recently been shown that the amplitude of cardiovascular and locomotor activity rhythms is attenuated in the AT_2 KO mouse [6], although τ has yet to be measured, to our knowledge.

Previous studies indicate that alterations of the renin–angiotensin system, particularly of the proteins or the receptors, alters fluid balance [3,23]. Genetic deficiency of angiotensinogen or AT_{1a} receptors produced mice with high urine volume and increased fluid intake [23,26]. The increased level of drinking spout contacts in KO mice in the present study thus likely represents increased fluid intake. By contrast, the increased level of wheel running activity in KO mice is a novel result for which we do not yet have an interpretive framework.

The results of this study demonstrate that the core mechanisms for the generation of circadian rhythms and their entrainment by LD are intact in mice lacking AT_{1a} receptors. A conservative interpretation of these data is that

the AT_{1a} receptor plays little or no role in the circadian timekeeping functions of the SCN, suggesting that this nucleus may have other functions distinct from its role as pacemaker. This interpretation is subject to the usual caveats concerning the KO technique. For example, a role for signaling at AT_{1a} receptors within the circadian system could be obscured by compensatory developmental processes or by polymorphism in the genetic background related to the use of two different mouse strains to produce the F2 homozygous null mutants [3,5]. Complimentary experimental approaches will be needed to gain a comprehensive view of the function of ANG II within the SCN. In addition, it remains possible that ‘hands’ of the clock other than running and drinking are more significantly affected by the mutation. For example, ANG II-containing neurons in the dorsomedial SCN could convey timing information to AT_{1a} receptors on cell populations elsewhere that couple the clock to other specific regulated variables. Loss of such signaling could create a state of internal desynchrony in which some hands of the clock are selectively ‘broken’ (loss of rhythmicity) or ‘shortened’ (attenuated amplitude), while others cycle with normal amplitude. Other hands of particular interest include those associated with functioning of the cardiovascular system, such as the daily rhythms of heart rate and blood pressure, which are known to be altered in association with cardiovascular disease processes and genetic manipulation of the renin–angiotensin system [6,14,25].

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