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Aging Disrupts Normal Time-of-day Variation in Cardiac Electrophysiology

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Abstract

Background ––Cardiac gene expression and arrhythmia occurrence have time-of-day variation; however, daily changes in cardiac electrophysiology, arrhythmia susceptibility, and Ca^{2+} handling have not been characterized. Furthermore, how these patterns change with age is unknown.

Methods ––Hearts were isolated during the light (ZT4 and ZT9) and dark cycle (ZT14 and ZT21) from adult (12–18 weeks) male mice. Hearts from aged (18–20 months) male mice were isolated at ZT4 and ZT14. All hearts were Langendorff-perfused for optical mapping with voltage- and Ca^{2+}-sensitive dyes (n=4–7/group). Cardiac gene and protein expression were assessed with real-time PCR (n=4–6/group) and western blot (n=3–4/group).

Results ––Adult hearts had the shortest action potential duration (APD) and Ca^{2+} transient duration (CaTD) at ZT14 (APD_{80}: ZT4: 45.4±4.1ms; ZT9: 45.1±8.6ms; ZT14: 34.7±4.2ms*; ZT21: 49.2±7.6ms, *p<0.05 vs. ZT4 and ZT21; and $CaTD_{80}$: ZT4: 70.1±3.3ms; ZT9: 72.7±2.7ms; ZT14: 64.3±3.3ms*; ZT21: 74.4±1.2ms, *p<0.05 vs. other time points). The pacing frequency at which CaT alternans emerged was faster and average CaT alternans magnitude was significantly reduced at ZT14 compared to the other time points. There was a trend for decreased spontaneous PVCs and pacing-induced ventricular arrhythmias at ZT14, and the hearts at ZT14 had diminished responses to isoproterenol compared to ZT4 (ZT4: 49.5.0±5.6% vs ZT14: 22.7±9.5% decrease in APD, p<0.01). In contrast, aged hearts exhibited no difference between ZT14 and ZT4 in nearly every parameter assessed (except APD_{80}: ZT4: 39.7±1.9ms vs. ZT14: 33.8±3.1ms, p<0.01). Gene expression of KCNA5 (encoding Kv1.5) was increased, whereas gene expression of ADRB1 (encoding β1-adrenergic receptors) was decreased at ZT14 vs. ZT4 in adult hearts. No time-of-day changes in expression or phosphorylation of Ca^{2+} handling proteins (SERCA2, RyR2 and PLB) was found in *ex vivo* perfused adult isolated hearts.

Conclusions ––Isolated adult hearts have strong time-of-day variation in cardiac electrophysiology, Ca^{2+} handling, and adrenergic responsiveness, which is disrupted with age.

Graphical Abstract

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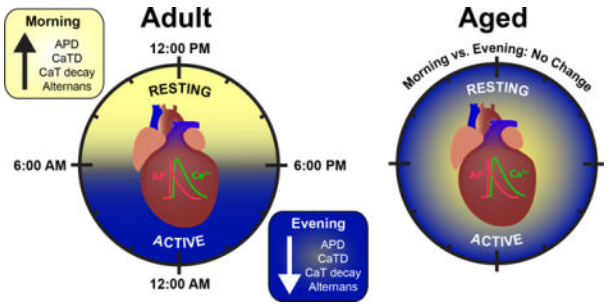
Disclosures: None.



Keywords

circadian rhythm; ventricular arrhythmia; action potential; calcium transients; aging

Journal Subject Terms

Arrhythmias; Electrophysiology; Basic Science Research; Calcium Cycling/Excitation-Contraction Coupling; Physiology

Introduction

Intrinsic circadian rhythms in cells and tissues help organisms anticipate and adapt to time-of-day dependent fluctuations in the environment and activity.[1, 2] Many aspects of the cardiovascular system have daily rhythms, including heart rate, blood pressure, and contractility.[3, 4] Historically, these time-of-day changes in cardiovascular function were primarily attributed to circadian changes in circulating neuro-hormones and autonomic tone.[5,3] However, contemporary studies revealed that cardiomyocytes possess the molecular elements that constitute the circadian clock.[6, 7] This cardiomyocyte molecular clock controls the daily oscillation of numerous genes involved in nearly every aspect of cellular function, including metabolism, contractility, and excitability.[4] Therefore, time-of-day oscillation of cardiac function is likely a result of complex interactions between daily fluctuations in intrinsic cellular properties and extrinsic neuro-humoral inputs.

In addition to heart rate, clinical data indicate that many aspects of cardiac electrophysiology exhibit day-night rhythms, including QT interval,[8] QT interval dispersion,[9] ventricular refractoriness,[10] and incidence of T-wave alternans.[11, 12] Several forms of arrhythmia also show time-of-day dependence. For example, incidence of ventricular tachycardia and fibrillation (VT/VF) tends to peak in the morning,[13] premature ventricular complexes (PVCs) occur more frequently during the day than at night,[14] and bradyarrhythmias and paroxysmal atrial fibrillation are more common at night.[15, 16] Understanding the precise mechanisms by which time-of-day fluctuations in electrophysiology may contribute to arrhythmias in various pathological settings is therefore of utmost importance.

At least 10 different cardiac ion channels or regulatory subunits have been reported to exhibit circadian patterns of gene expression, including voltage-gated Na^+, Ca^{2+}, and K^+ channels, HCN channels, and connexins (reviewed in[17]). The potential functional consequences of these time-of-day fluctuations and their precise role in electrophysiology

and arrhythmogenesis remains an active area of investigation. Furthermore, arrhythmias often involve interactions between intracellular and multi-cellular processes beyond individual ionic currents, including mechanisms of intracellular Ca^{2+} handling, excitation-contraction coupling, adrenergic input and responsiveness, and heterogeneity of conduction and repolarization throughout the heart, to name just a few. However, to our knowledge, there has been no experimental study of time-of-day dependence of any of these properties in the intact heart.

Circadian rhythms change markedly with age,[18] and previous reports indicate that aging can alter circadian patterns of gene expression in the heart.[19] In addition, circadian patterns of sudden cardiac death appear to be altered with age, with a single peak occurring in the morning in aged populations, whereas in middle age, there is another secondary peak in the afternoon.[20, 21] We therefore hypothesized that aging may alter the normal time-of-day variation in cardiac electrophysiology. Thus, the goals of this study were 1) to evaluate whether there are time-of-day changes in intrinsic cardiac electrophysiology, Ca^{2+} handling, and arrhythmia susceptibility in isolated intact hearts, which are devoid of acute, central neuro-humoral inputs, and 2) to determine whether these daily patterns are altered with age.

Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Additional details are available in the Supplementary Materials.

Animals and experimental groups

All procedures involving animals were approved by the institutional animal care and use committee of the University of California, Davis and adhered to the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health. Adult (12–18 weeks) or aged (18–20 month) male C57BL/6 mice were housed in 12-hour light/dark cycle with *ad libitum* access to food and water. Zeitgeber time 0 (ZT0) corresponds to the time at which the lights turn on. For optical mapping experiments, adult mouse hearts were isolated during the resting period (light cycle, ZT4 [n=7] and ZT9 [n=6]) or active period (dark cycle, ZT14 [n=5] and ZT21 [n=4]) and aged hearts were isolated at one resting (ZT4 [n=6]) and one active (ZT14 [n=5]) time point. For Western blots, adult hearts were isolated at ZT4 (n=8) and ZT14 (n=8) and perfused for either 5min or 1hr prior to tissue collection and homogenization (n=4/group). For gene expression, adult hearts were isolated at ZT4 (n=5) and ZT14 (n=5), and aged hearts were isolated at ZT4 (n=6) and ZT14 (n=4). Unequal group numbers are a result of varying experimental success rates after initial randomization of animals.

Dual optical mapping of intracellular Ca^{2+} and transmembrane potential (V_m)

Dual optical mapping was performed as previously described.[22] Hearts were Langendorff perfused with normal Tyrode's solution at 37°±0.5°C with blebbistatin (10 μM). Hearts were loaded with a voltage-sensitive dye (RH237, 10μL of 5 mg/mL in DMSO) and an intracellular Ca^{2+} indicator (Rhod-2AM, 50μL of 1 mg/mL in DMSO containing 10%

pluronic acid). The heart position in the dish was adjusted to image the anterior epicardial surface (including both right and left ventricle). Fluorescence signals were acquired with 2 CMOS cameras (MiCam Ulima-L, SciMedia Costa Mesa, CA) at 1kHz from a 10×10 mm field of view (100×100 pixels). Hearts were paced with an S1-S2 protocol or continuously at progressively faster cycle lengths. Responsiveness to β-adrenergic stimulation was assessed with 1 μM isoproterenol (ISO) added to the perfusate.

Optical mapping data analysis

Data analysis was performed with *Optiq* software (Cairn, UK) as previously described.[22] AP duration (APD) was calculated as 80% (APD_{80}) and 50% (APD_{50}) repolarization minus activation time. Dispersion of APD was calculated as the inner 95th percentile of all APDs in the mapping field of view divided by the mapping area. The time constant of decay (*tau*) of CaT was quantified with an exponential fit of 30–100% recovery of CaT. Recovery of relative Ca^{2+} release in response to premature stimuli was calculated as S2/S1 ratio of CaT amplitude. Conduction velocity (CV) was measured using a polynomial fitting algorithm as previously described.[23] The magnitude and significance of APD and Ca^{2+} alternans were determined using spectral methods as previously described.[24]

Western Blot

Immunoblotting of left ventricular lysates was performed as previously described.[25] Anti-SERCA (1:5000, A010–20, Badrilla), RyR2 (1:2500, ARR002, Alomone), PLB (1:5000, A010–14, Badrilla), PLB-Thr17 (1:5000, A010–13, Badrilla), PLB-Ser16 (1:5000, A010–12, Badrilla), and α-tubulin (1:1000, ab52866, Abcam) were used as primary antibodies. Anti-rabbit or anti-mouse 680nm or 800nm were used as secondary antibodies (IRDyes 680LT or 800CW, Licor Biosciences).

qPCR

RNA was extracted from left ventricles with TRIzol Plus RNA Purification Kit (Invitrogen). Equal amounts of purified RNA were reverse transcribed into cDNA using High-Capacity RNA-to-cDNA kit (Applied Biosystems). The preoptimized TaqMan Gene Expression Assays (Applied Biosystems) were used to quantify each gene expression: CACNA1C Mm01209919_m1, KCNA5 Mm00524346_s1, KCNIP2 Mm00518915_g1, KCND2 Mm00498065_m1, SLC8A1 Mm01232254_m1, ADRB1 Mm00431701_s1, ADRB2 Mm02524224_s1. Real-time PCR was performed with thermal cyclers (Applied Biosystems, 7900HT) in the following conditions: 50°C for 2 min, 95°C for 10min, 40 cycles of 95°C for 15s, and 60°C for 1 min.

Statistics

For recovery of Ca^{2+} release and spectral alternans magnitude, data were first fit with a one phase exponential (nonlinear regression) and then subject to a 2-way ANOVA followed by Tukey's post-hoc test. Premature ventricular complex (PVC) and extra beat (EB) data were analyzed with Fisher's exact test. All other adult data were analyzed with 1-way ANOVA followed by Tukey's post-hoc test unless indicated in figure legend. Aged data were

analyzed with a student's unpaired t-test. Data are presented as mean±SD. $P<0.05$ was considered statistically significant.

Results

Adult hearts exhibit time-of-day variation in AP and Ca^{2+} handling properties *in vitro*

Circadian variation in heart rate and QT interval have been demonstrated *in vivo* in humans and animals.[8, 26] In our study, baseline heart rates in the excised Langendorff-perfused hearts were not significantly different (Table 1). The QT interval is closely related to the ventricular APD, but whether the APD displays time-of-day variation in isolated myocytes or hearts (which are devoid of acute neuro-humoral inputs) has not been assessed. Optical mapping was performed in isolated hearts and data were collected 1 hour after heart isolation. Electrophysiological and Ca^{2+} handling parameters at all four time points are shown in Table 1. There were no time-of-day differences in activation pattern or conduction velocity of adult hearts (Figure 1A, Table 1). However, the effective refractory period (ERP) and APD_{80} showed strong daily variation with ZT14 shorter than the other time points (Figure 1B–D). Example AP traces from hearts at ZT4 and ZT14 demonstrate that APD differences are most prevalent during the plateau and later phases of repolarization, which is also reflected in the time-of-day dependence of $APD_{80–50}$ (Figure 1E–F). APD dispersion tended to have a daily variation, however this did not reach to statistical significance (Figure 1G).

The CaT duration ($CaTD_{80}$) was also shorter at ZT14 compared to the other time points in adult mouse hearts (Figure 2A–B). Similarly, the time constant of Ca^{2+} transient decay (*tau*) was faster at ZT14 compared to ZT4 and ZT21 and tended to be faster than at ZT9, but this did not reach statistical significance (Figure 2C–D). To assess the relative recovery of Ca^{2+} release, the amplitude of the CaT during a premature stimulus (S2) normalized to the amplitude of the CaT at steady state (S1) was compared (Figure 2E). Recovery of Ca^{2+} release was significantly faster at ZT14 than at ZT4 (Figure 2F).

Adult hearts exhibit time-of-day variation in susceptibility to arrhythmogenic cardiac alternans

Because of the marked time-of-day variation in APD and Ca^{2+} cycling, we hypothesized that susceptibility to APD and CaT alternans may also vary with time of day. The average spectral magnitude of both APD and CaT alternans increased as pacing rate increased in all hearts (Figure 3A–B). However, ZT14 was more resistant to both APD and CaT alternans compared to other times of day. For example, at fast pacing cycle lengths (PCLs), ZT14 adult hearts exhibited significantly smaller APD and CaT alternans magnitude compared to ZT4 (Figure 3C–D). Consistent with this, the PCL at which APD alternans emerged tended to be faster at ZT14 (Figure 3E), and the PCL at which CaT alternans emerged was fastest at ZT14 compared to the other time points (Figure 3F).

Time-of-day variation in susceptibility to ventricular arrhythmias

Because ventricular arrhythmias have been shown to exhibit circadian variation in occurrence,[13, 21] we assessed whether the time of day had any influence on inducible or

spontaneous ventricular arrhythmias in isolated adult mouse hearts. No sustained VT/VF was induced at any time point. However, extra beats were induced by a single premature pacing stimulus in some hearts (Figure 4A). The proportion of hearts having extra beats tended to be lower at ZT14, although this was not statistically significant (Figure 4B). Likewise, the proportion of hearts with spontaneous PVCs (Figure 4C) also tended to vary a bit with time of day, but this result was not statistically significant (Figure 4D).

Time-of-day variation is disrupted in aged mouse hearts

There is significant evidence suggesting that aging impacts the normal biological clock.[18] To investigate whether aging affects the time-of-day variation in cardiac electrophysiology and Ca^{2+} handling, we performed similar optical mapping of V_m and intracellular Ca^{2+} on hearts isolated from 18–20-month-old mice (Figure 5A–D). Similar to adult hearts, aged hearts had a longer APD_{80} at ZT4 versus ZT14 (Figure 5E). However, the difference in APD_{80} in the aged group is only 14.8% compared to 30.8% in adult group (ZT4 vs. ZT14: 39.7±1.9ms vs. 33.8±3.1ms and 45.4±4.1ms vs. 34.7±4.2ms for aged and adult, respectively). There was no difference in APD dispersion (Figure 5F) nor ERP (36.7±8.2 ms vs. 34±5.5 ms, ZT4 vs. ZT14, p=NS). There were also no time-of-day differences in any Ca^{2+} handling properties (Figure 5G–I), nor in susceptibility to CaT or APD alternans (Figure 6). Aged hearts showed no differences in the proportion of hearts that had extra beats following a single premature stimulus (ZT4 vs. ZT14: 2/6 vs. 2/5) or in spontaneous PVCs (ZT4 vs. ZT14: 5/6 vs. 5/5). Altogether, these data indicate that aging markedly alters the normal time-of-day variation in cardiac electrophysiology and Ca^{2+} handling properties.

Adult but not aged hearts exhibit daily variation in response to β-adrenergic stimulation

The responsiveness of rodent cardiomyocytes and hearts to β-adrenergic stimulation has been shown to have daily variation, including β-adrenergic-mediated increases in contractility and alterations in intracellular Ca^{2+} handling.[4, 27] To determine if isolated hearts also have time-of-day variation in electrophysiological responses to β-adrenergic stimulation, adult and aged hearts were treated with 1 μM ISO. APD_{80} and $CaTD_{80}$ shortened in all hearts exposed to ISO at all time points (Figure 7). However, in adult hearts, the magnitude of APD shortening was significantly increased at ZT4 compared to ZT14, resulting in a shorter APD_{80} at ZT4 versus ZT14 following ISO (Figure 7A–B). There was a similar trend for CaTD shortening in response to ISO (Figure 7C). In contrast, aged hearts responded similarly to β-adrenergic activation at ZT4 and ZT14 (Figure 7D–F).

Time-of-day variation in gene expression

To assess whether time-of-day changes in gene expression were altered with age, several genes contributing to the mouse AP and repolarization were evaluated. KCND2 (α-subunit of the transient outward potassium channel, Kv4.2, $I_{to,f}$), KCNIP2 (β-subunit of $I_{to,f}$), CACNA1C (L-type Ca^{2+} channel, Cav1.2, $I_{Ca,L}$), and NCX1 (Na^+-Ca^{2+} exchanger) were not different between ZT4 and ZT14 (Table 2). However, KCNA5 (Kv1.5, I_{Kur}) gene expression was significantly upregulated at ZT14, which is consistent with the shorter APD_{80} observed at ZT14 in adult hearts. In aged hearts, daily variation in KCNA5 gene expression was no longer present. However, NCX1 expression was decreased at ZT14 in aged but not adult hearts (Table 2). Consistent with decreased responsiveness to ISO at ZT14 in adult hearts,

ADRB1 (β1-adrenergic receptor) gene expression was significantly reduced at ZT14 in adult hearts. Aged hearts showed a similar trend for decreased ADRB1 gene expression at ZT14, but this did not reach statistical significance.

Time-of-day variation in Ca^{2+} handling protein expression and phosphorylation

Adult hearts demonstrated significantly shorter $CaTD_{80}$ and faster CaT decay (*tau*) (Figure 2) at ZT14. One of the most appreciated mechanisms for acceleration of CaT decay is via phosphorylation of phospholamban (PLB). Therefore, PLB phosphorylation was assessed at S16 (PKA site) and T17 (CaMKII site) in adult hearts following either 5 min of perfusion (to wash blood from the tissue) or following 1 hour of perfusion (to match the perfusion time at which optical mapping data were collected). PLB phosphorylation at S16 was essentially non-detectable in both periods following either 5 min or 1 hour of perfusion (Figure 8A–B). Following 5 min of perfusion, PLB phosphorylation at T17 was increased at ZT4. However, after 1 hour of perfusion, PLB phosphorylation at T17 was not different between ZT4 and ZT14 (Figure 8C–D). These data suggest that following 1 hour of perfusion (time point that matched optical mapping data collection), PLB phosphorylation levels at both sites are similar; thus, the daily variation in CaT decay is not likely a consequence of phosphorylation status. Total PLB protein levels were also not different between ZT4 and ZT14, and expression of SERCA2A and RyR were also similar (Figure 8E–G).

Discussion

In this study, we showed for the first time, pronounced day-night variation in cardiac electrophysiology and Ca^{2+} handling properties in isolated perfused mouse hearts, which are devoid of acute central neuro-humoral inputs. We found that adult mouse hearts had shorter APD, ERP, and CaTD, faster CaT decay, and decreased susceptibility to APD and CaT alternans at ZT14 (early active period) compared to the other time points investigated. For a majority of electrophysiological parameters investigated, the remaining three time points were not significantly different from one another. Adult mouse hearts were also less responsive to β-adrenergic stimulation at ZT14, and tended to have decreased spontaneous and inducible arrhythmias. These results are not only important for understanding circadian mechanisms of cardiac arrhythmias, but may also have implications for experimental rigor and reproducibility of *ex vivo* electrophysiological data collected at different time points throughout the day and night. Interestingly, with the exception of slightly prolonged APD_{80} at ZT4, the difference in electrophysiology and Ca^{2+} handling properties between ZT14 and ZT4 was absent in isolated aged (18–20 month) mouse hearts.

Consistent with previous reports, we observed elevated gene expression of KCNA5 (encoding Kv1.5, I_{Kur}) in the active (dark) period (ZT14).[28, 29] Although changes in gene expression do not necessarily indicate corresponding changes in protein expression or current, an increase in I_{Kur} is consistent with the shorter APD_{80} observed at ZT14. Daily variation in KCNA5 gene expression was absent in the aged hearts, and the time-of-day APD difference was also markedly reduced with age. Therefore, time-of-day variation in KCNA5 gene expression may be one potential mechanism contributing to the phenotype observed here. I_{Kur} is a major repolarizing current in the human atria, and due to its

differential expression in human atria versus ventricles, I_{Kur} may be an important target for the pharmacological treatment of atrial fibrillation.[30] Thus, the role of circadian variation of I_{Kur} in atrial fibrillation and its potential as a chronotherapeutic drug target may be an important area for future study.

Although previous reports have shown circadian oscillations in both KCND2[28, 29] and KCNIP2,[26] we did not see differences in expression of these genes at the two timepoints examined in the present study. It is possible that time-of-day differences in expression would have been observed with more time points. However, the fact that early repolarization and APD_{50} were not different at these same time points support the notion that there may not be large differences in $I_{to,f}$ at the timepoints assessed here.

We hypothesized that following 1 hour of *ex vivo* perfusion, hearts would reach a steady-state and would not have significant residual neuro-humoral activation. Consistent with this hypothesis, PKA and CaMKII phosphorylation of a common target (PLB) was similar between ZT4 and ZT14 in adult hearts, indicating that the daily variation in $CaTD_{80}$ and decay time (*tau*) are not likely a consequence of residual catecholamine stimulation or phosphorylation status. This is in contrast to freshly isolated hearts, in which time-of-day changes in phospho-PLB have been reported.[31] Total protein levels of PLB, SERCA2A, and RyR were also not different between ZT4 and ZT14 in adult hearts. The mechanisms underlying the time-of-day variation in Ca^{2+} handling properties is therefore an important area for further investigation and may involve circadian variation in post-translational modifications or intracellular signaling mechanisms not investigated here. However, given the strong bi-directional feedback between the AP and CaT, it is possible that daily alterations in intracellular Ca^{2+} handling are a secondary consequence of time-of-day changes in the AP.

Time-of-day variation in cardiac electrophysiology

It is well known that many aspects of human cardiac electrophysiology have circadian variation, including heart rate, QT interval, QT interval dispersion, and ventricular refractoriness.[8–10] Several *in vivo* studies in mice have also reported circadian oscillations in heart rate and QT interval, with slower heart rates and longer QT intervals during the resting period (day).[26, 32, 33] Although closely related to the ventricular APD, the QT interval is also influenced by heart rate and autonomic tone, and to our knowledge, no study has directly measured action potential properties from isolated cells or hearts at different times of day. Our data show that in isolated mouse hearts paced at identical rates, APD was longer in the early resting (ZT4) versus early active (ZT14) period. We also observed the shortest ERP in the early active period (ZT14), and these data are consistent with *in vivo* patient data[10].

Time-of-day variation in Ca^{2+} handling and cardiac alternans

We observed strong time-of-day variation in Ca^{2+} handling properties, including CaTD, *tau*, and relative recovery of Ca^{2+} release that were all accelerated at ZT14 versus other time points in adult hearts. We and others have shown that acceleration of either sarcoplasmic reticulum (SR) Ca^{2+} release or reuptake can significantly decrease Ca^{2+} alternans magnitude.[24, 34] Consistent with these observations, faster Ca^{2+} cycling at ZT14 was

associated with a decrease in the amplitude and susceptibility to arrhythmogenic alternans compared to ZT4. Only a couple of studies have assessed the circadian variation in alternans in patients, and both observed reduced incidence of alternans during the resting period, opposite of our results.[11, 12] One explanation for these observations is that in patients at rest, heart rates are typically sufficiently low as to not induce alternans, whereas in our study, identical pacing rates were used to evoke alternans during the resting and active periods. However, Martin-Yebra et al. demonstrated that even when similar heart rates were compared, patients with congestive heart failure still had lower amplitude of alternans during the resting period.[12] Interestingly, in our study, aged hearts did not show a time-of-day dependence of alternans severity. Thus, the mechanisms underlying the circadian oscillation in alternans, how this relates to arrhythmia susceptibility, and how underlying factors are altered with age and/or cardiovascular disease will be an important area for future study.

Time-of-day variation in β-adrenergic responses

We observed strong time-of-day differences in the electrophysiological responses to the β-adrenergic agonist isoproterenol in adult but not aged mouse hearts. In fact, the time-of-day difference in APD was actually reversed following isoproterenol administration (longer APD at ZT14 versus ZT4 in adult hearts with isoproterenol). This was associated with a significant difference in mRNA expression of ADRB1 (encoding β1-receptors) at ZT4 versus ZT14 in adult hearts. Aged hearts showed a similar trend in β1-receptor mRNA, however this did not reach statistical significance. Although the magnitude of electrophysiological responses may be due in part to receptor expression levels, other downstream signaling mechanisms may also be involved (e.g., adenylyl cyclase, cAMP, PKA) and these factors were not investigated in the present study. Likewise, responses to sympathetic nerve activity rather than circulating agonists might reveal novel mechanisms of neuro-humoral control of cardiac electrophysiology throughout the day.

Impact of aging on time-of-day variation in cardiac electrophysiology

It is known that aging can alter the circadian pattern of gene expression in the heart.[19] However, how aging impacts circadian rhythms in electrophysiological function has not been previously examined. In our study, we found that nearly all of the significant electrophysiological and Ca^{2+} handling differences present in adult mouse hearts between ZT4 and ZT14 were absent in aged hearts. Because only two time points were investigated in the aged hearts, it is possible that circadian variation of cardiac electrophysiology is present, but is phase-shifted with age. Either loss or shift of this normal rhythm (which may lead to misalignment with the central clock or with external environmental cues) could be detrimental and may contribute to cardiovascular dysfunction or disease.[35, 36] Indeed, in mice, cardiac-specific deletion of the endogenous clock has been shown to impact cardiac metabolism, reduce cardiac contractility, increase susceptibility to arrhythmias, and lead to a significantly shorter life span.[4, 33, 37] It is interesting to speculate whether potential cardiac clock disruptions caused by aging lead to similar cardiovascular dysfunction. Interestingly, it has been shown that patients who died from chronic heart failure had blunted circadian variability of the QT interval (heart rate corrected) compared to normal subjects or survivors.[38, 39] This may suggest that preserving or restoring the circadian clock during aging may have cardiovascular benefits.

Implications for experimental rigor & reproducibility

In adult hearts, we observed that most electrophysiological and Ca^{2+} handling parameters were significantly different at ZT14 compared to the other three time points. This result cautions against collecting data from isolated hearts or myocytes in the evening hours if it is to be compared with daytime experimental data. Likewise, our results indicate that electrophysiological interventions, pharmacological therapies, arrhythmia susceptibility, etc., experimentally tested during the daylight hours in mice do not fully reflect the response in the normal active period of the animal. This also cautions against directly translating results obtained in mice during the light phase (their resting period) to those obtained in larger species (e.g., rabbit, canine, human), which are most often collected during the light phase (active period) for these species.

Conclusions

In summary, we report strong time-of-day variation in cardiac electrophysiology and Ca^{2+} handling properties in isolated hearts from adult mice, and these differences are altered with age. These results are not only important for understanding circadian mechanisms of cardiac arrhythmias, but may also have significant implications for experimental rigor and reproducibility, as well as for translation of *ex vivo* electrophysiological data collected at different times throughout the day and night.

Limitations

We investigated four time points in adult hearts, and therefore time-of-day variations seen in this study might be underestimated. Aging altered the differences between ZT4 and ZT14; however, it is possible that differences still exist, but are phase-shifted. Likewise, we only assessed young adult (12–18 weeks) and a single aged time point (18–20 months), so we do not know at what age, precisely, these alterations occur. Unequal n-numbers for optical mapping groups are a result of varying experimental success rates after initial randomization of animals to groups. Isolated heart cannulation/perfusion success rate at ZT21 (4:00AM) was unusually low, and we do not know whether this is biologically meaningful. Neither gene nor protein expression levels are necessarily reflective of ion channel/transporter function. However, the targets uncovered here may represent important candidates. All data are from the ventricular myocardium, and circadian variation in sino-atrial and atrial electrophysiology remains an essential area for future investigation. Likewise, more reductionist studies (i.e., isolated myocytes) will be necessary to reveal underlying electrophysiological mechanisms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms

ADRB1	β1-adrenergic receptor
ADRB2	β2-adrenergic receptor
APD	action potential duration
APD Alt. Thres.	APD alternans threshold
APD_{80} Disp.	APD_{80} dispersion
Ca^{2+} Alt. Thres.	Ca^{2+} alternans threshold
Ca^{2+} release recov.	recovery of Ca^{2+} release
CACNA1C	calcium voltage-gated channel subunit α1C
CaT	Ca^{2+} transient
CaTD	Ca^{2+} transient duration
CV	conduction velocity
EB	extra beat
ERP	effective refractory period
HR	heart rate
ISO	isoproterenol
KCNA5	potassium voltage-gated channel subfamily A member 5
KCND2	potassium voltage-gated channel subfamily D member 2
KCNIP2	potassium voltage-gated channel interacting protein 2
NCX1	Na^{+}/Ca^{2+}exchanger 1
PCL	pacing cycle length
PLB	phospholamban
PVC	premature ventricular complex
RyR2	ryanodine receptor 2
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SLC8A1	solute carrier family 8 member A1 / NCX1
VF	ventricular fibrillation
VT	ventricular tachycardia
ZT	zeitgeber time

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What Is Known

- Heart rate, ECG properties, and arrhythmia occurrence all have strong time-of-day variation, which may be influenced in vivo by circulating neuro-hormones and autonomic tone.
- Multiple cardiac ion channels and associated regulatory subunits also have daily variation in gene expression and may contribute to the time-of-day oscillation in electrophysiological parameters.

What the Study Adds

- Ex vivo Langendorff-perfused mouse hearts, which are devoid of acute neuro-humoral inputs, exhibit strong time-of-day variation in action potential properties, Ca2+ handling parameters, and arrhythmogenic cardiac alternans.
- Aging disrupts the time-of-day variation in nearly all electrophysiological parameters assessed.

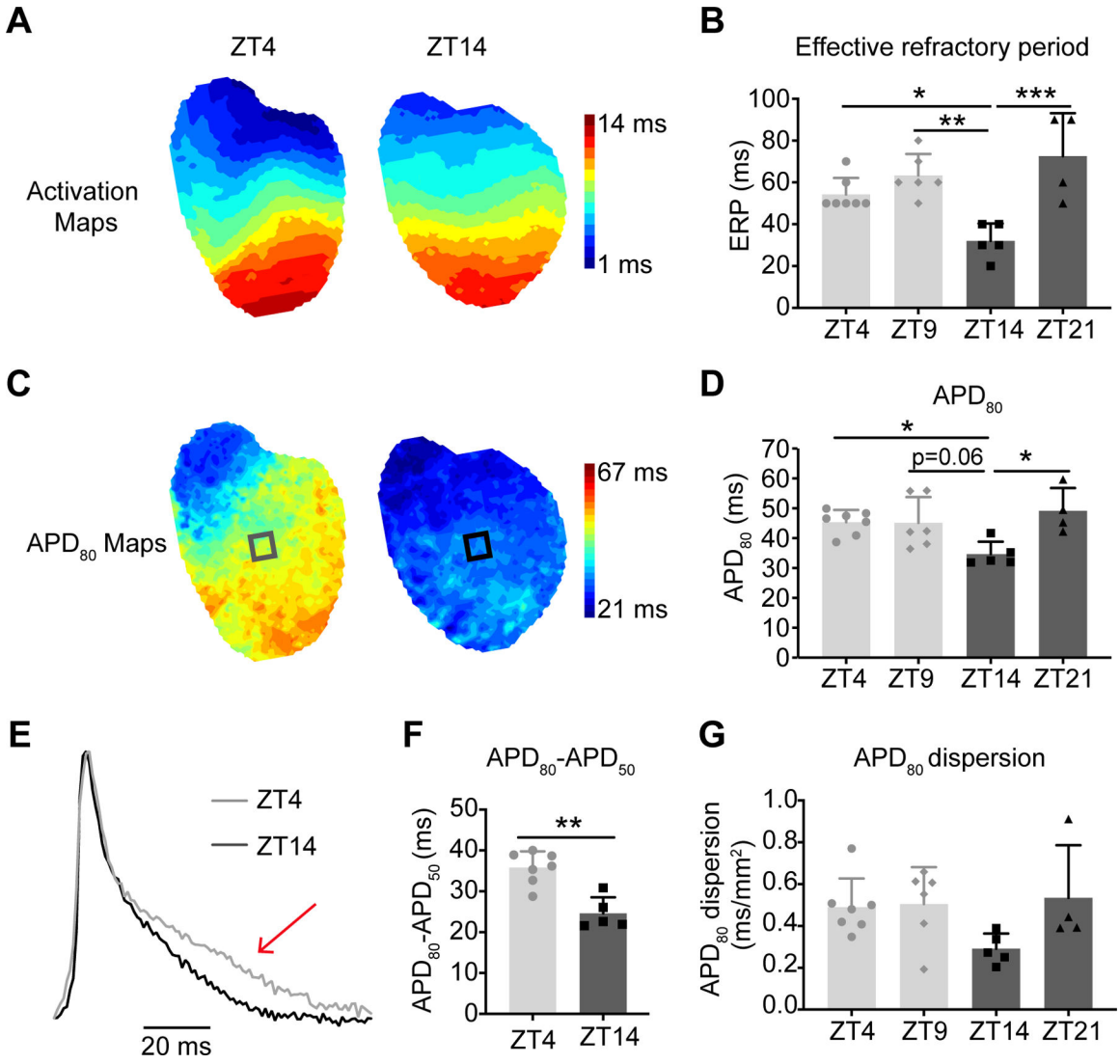

Figure 1.

Time-of-day variation of action potentials in adult hearts. **A**. Example maps of activation at ZT4 and ZT14. **B**. Mean effective refractory period. **C**. Example maps of action potential duration (APD_{80}) at ZT4 and ZT14. **D**. Mean APD_{80}. **E**. Example optical APs at ZT4 and ZT14 from the regions indicated in the corresponding boxes in (**C**). Red arrow indicates the divergence between the two traces at later stages of repolarization. **F**. Mean APD_{80} minus APD_{50}. **G**. Mean APD_{80} dispersion. Pacing cycle length = 150ms. ZT4 n= 7; ZT9 n=6; ZT14 n=5; ZT21 n=4. *p<0.05; ** p<0.01; ***p<0.001.

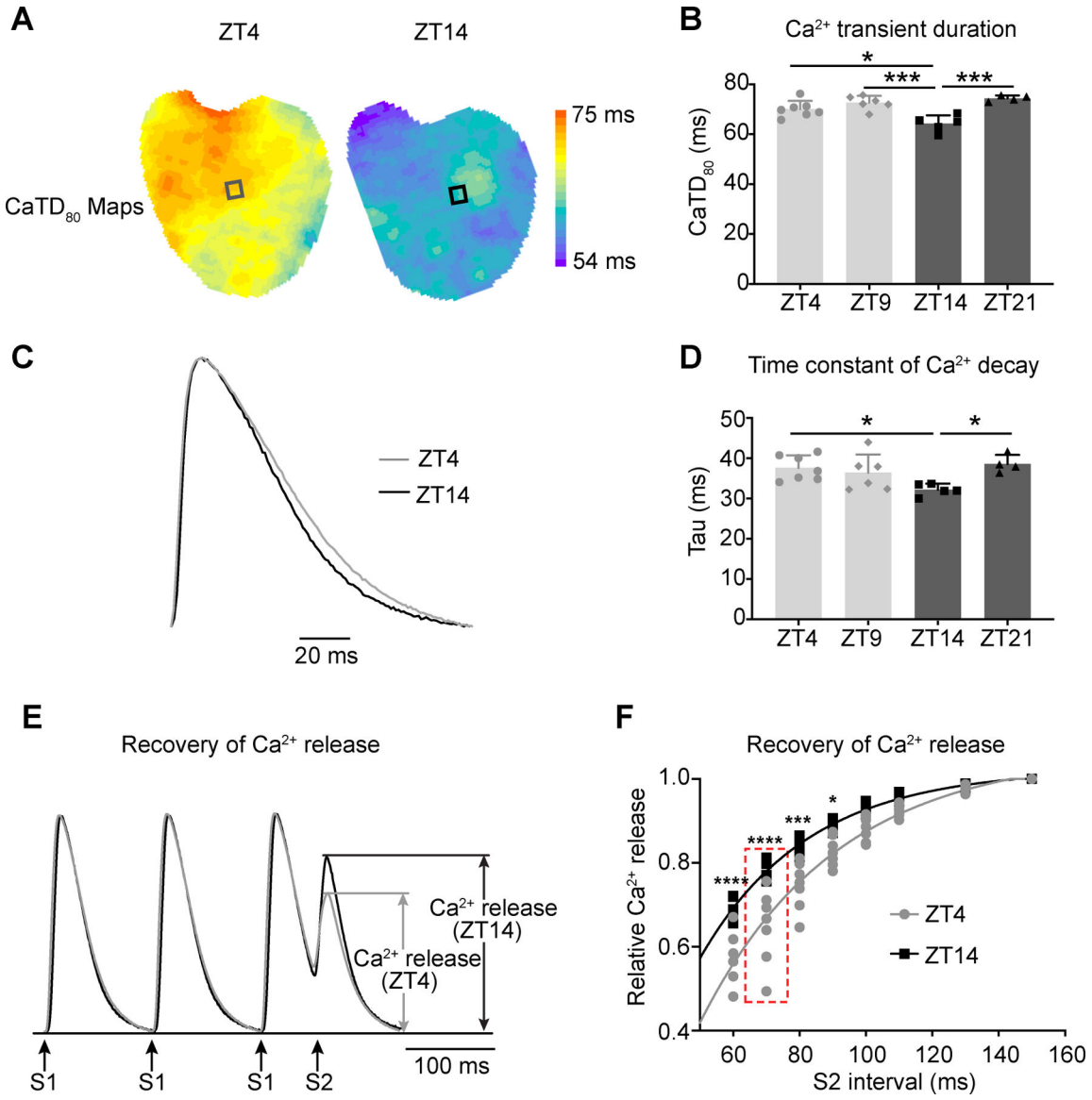

Figure 2.

Time-of-day variation in Ca^{2+} handling properties in adult hearts. **A**. Example maps of Ca^{2+} transient duration at ZT4 and ZT14. **B**. Mean Ca^{2+} transient duration. **C**. Example optical Ca^{2+} transients at ZT4 and ZT14 from the regions indicated with boxes in (**A**). **D**. Mean time constant of Ca^{2+} transient decay (*tau*). **E**. Example Ca^{2+} transients at ZT4 and ZT14 at S1=150ms and S2=70ms demonstrating differential recovery of Ca^{2+} release. **F**. Recovery of Ca^{2+} release (S2/S1) at ZT4 and ZT14. Parameters in (**B**) and (**D**) were measured at a pacing cycle length = 150ms. ZT4 n=7 (except n=6 at S2=60ms due to loss of capture); ZT9 n=6; ZT14 n=5; ZT21 n=4. *p<0.05; *** p<0.001; ****p<0.0001.

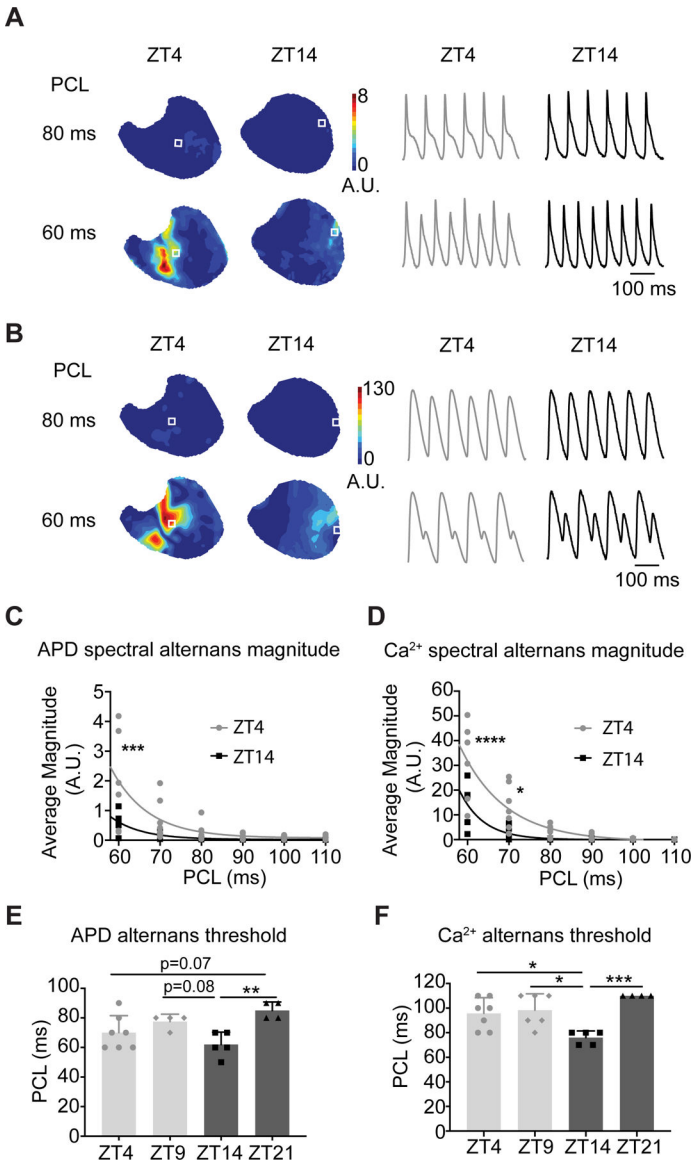

Figure 3.

Cardiac alternans in adult hearts. **A-B**. Example APD (**A**) and CaT (**B**) alternans maps at a pacing cycle length (PCL) of 80ms (top) and 60ms (bottom) at ZT4 and ZT14 and corresponding optical traces from regions indicated by the boxes on maps. **C-D**. Average APD (**C**) and CaT (**D**) alternans magnitude vs. PCL at ZT4 and ZT14. **E-F**. The longest PCL at which APD (**E**) and CaT (**F**) alternans first emerge. ZT4 n=7 (except n=6 at PCL=60ms due to loss of capture); ZT9 n=4 for APD, n=6 for CaT (two hearts did not have significant APD alternans prior to loss of capture); ZT14 n=5; ZT21 n=4. *p<0.05; **p<0.01; *** p<0.001; ****p<0.0001.

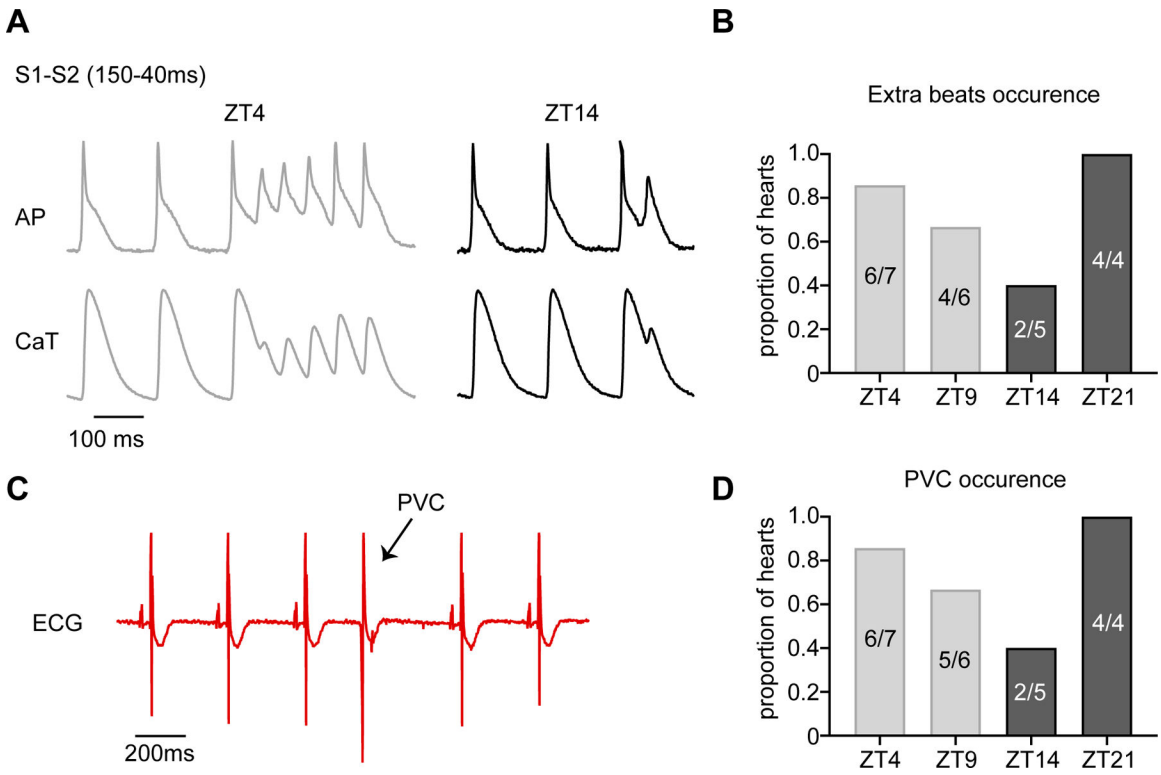

Figure 4.

Ventricular arrhythmias in adult hearts. **A**. Example optical AP (top) and CaT (bottom) with premature stimulus (S2) at ZT4 (left) and ZT14 (right). **B**. The proportion of hearts having extra beats induced by premature stimuli. **C**. Example ECG recording demonstrating a typical premature ventricular complex (PVC). **D**. The proportion of hearts with spontaneous PVCs in each group. ZT4 n= 7; ZT9 n=6; ZT14 n=5; ZT21 n=4. There were no statistical differences between groups.

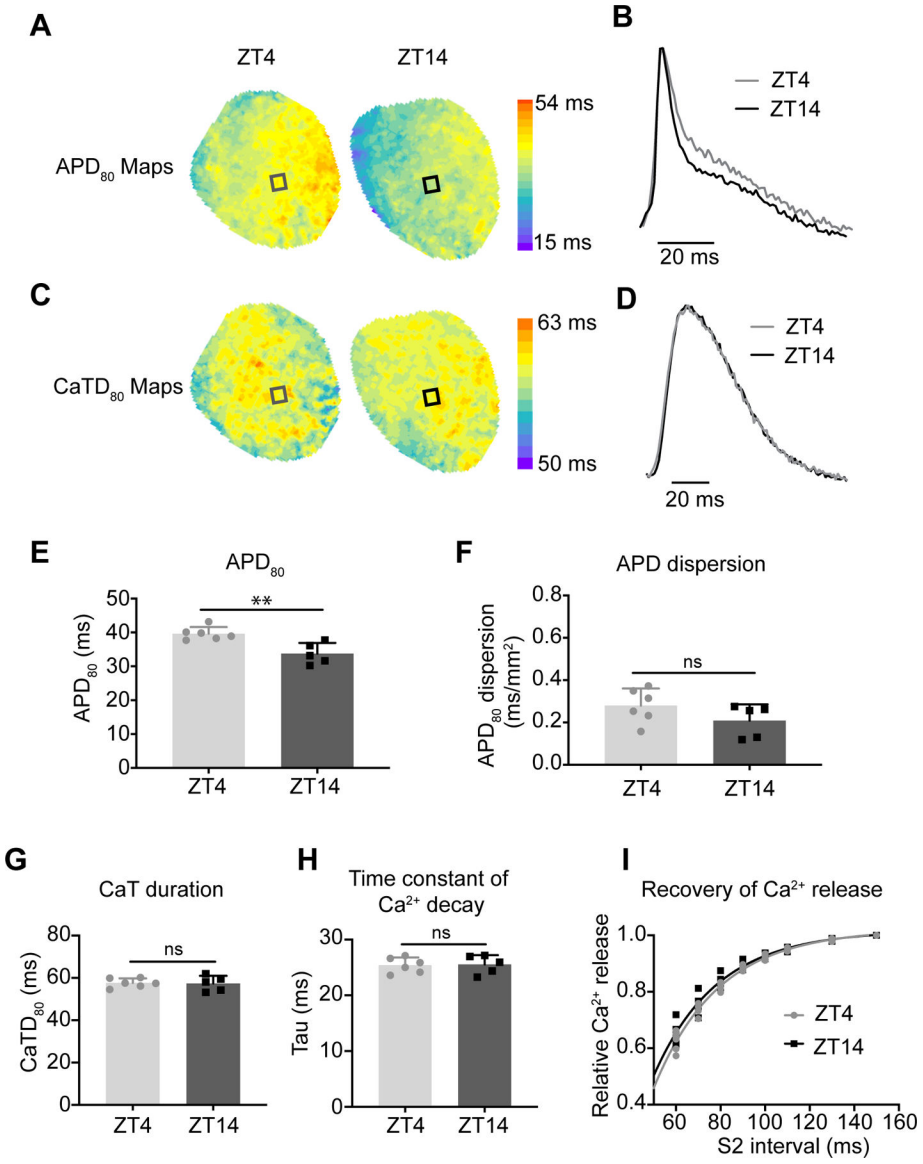

Figure 5.

Time-of-day variation in AP and CaT properties in aged hearts. **A-B**. Example maps of APD_{80} (**A**) and the corresponding optical AP traces (**B**) from regions indicated with boxes in (**A**) at ZT4 and ZT14. **C-D**. Example maps of $CaTD_{80}$ (**C**) and the corresponding optical CaTs (**D**) at ZT4 and ZT14. **E-F**. Aged hearts exhibited shorter APD_{80} at ZT14 (**E**) and similar APD_{80} dispersion (**F**) between ZT4 and ZT14. **G-I**. Aged hearts have similar Ca^{2+} transient duration (**G**), time constant of Ca^{2+} decay (*tau*, **H**) and recovery of Ca^{2+} release (**I**) in ZT4 versus ZT14. ZT4 n= 6; ZT14 n=5. **p<0.01.

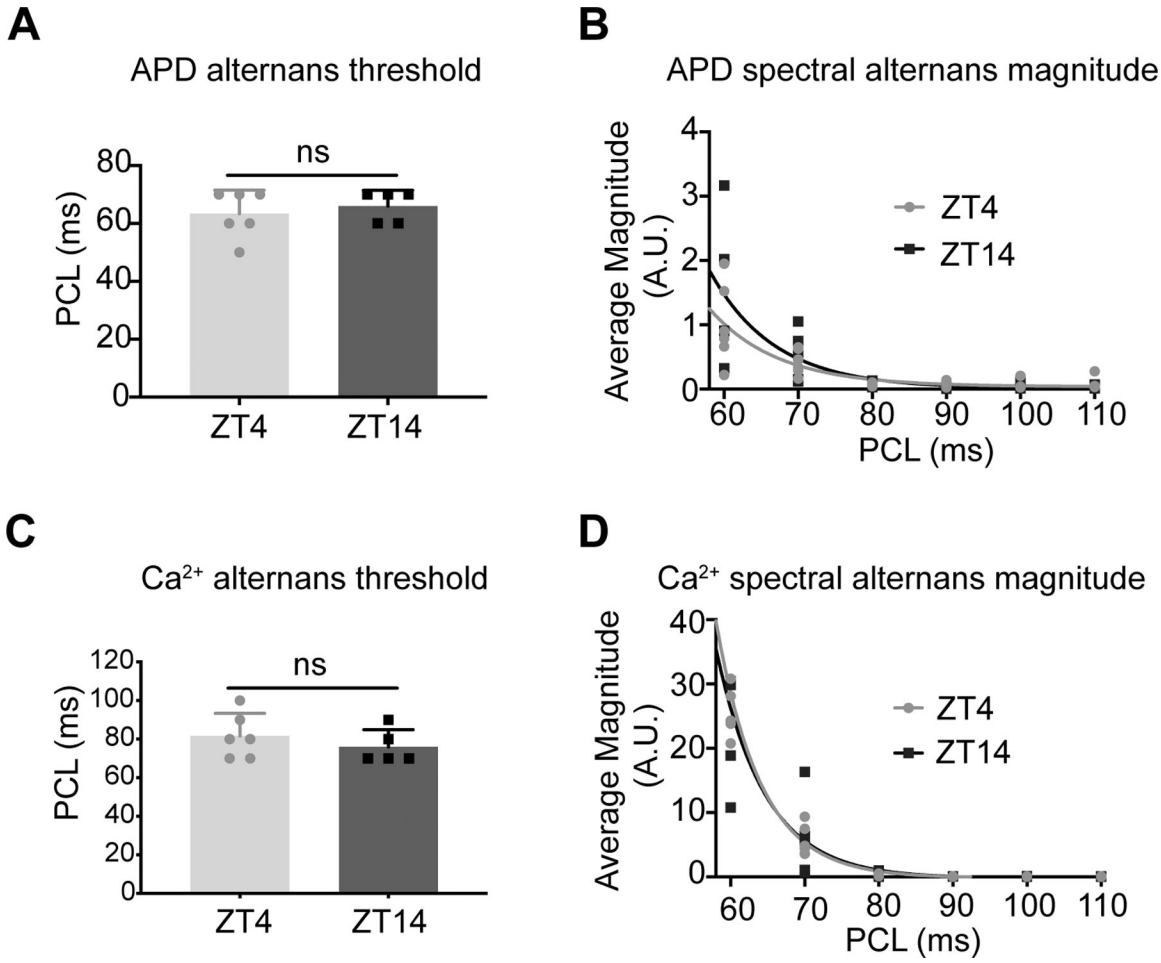

Figure 6.

Cardiac alternans in aged hearts. **A-B**. Aged hearts exhibit similar APD alternans thresholds (**A**) and spectral alternans magnitude (**B**) at ZT4 and ZT14. **C-D**. Aged hearts exhibit similar Ca^{2+} alternans thresholds (**C**) and spectral alternans magnitude (**D**) in both periods. ZT4 n= 6; ZT14 n=5.

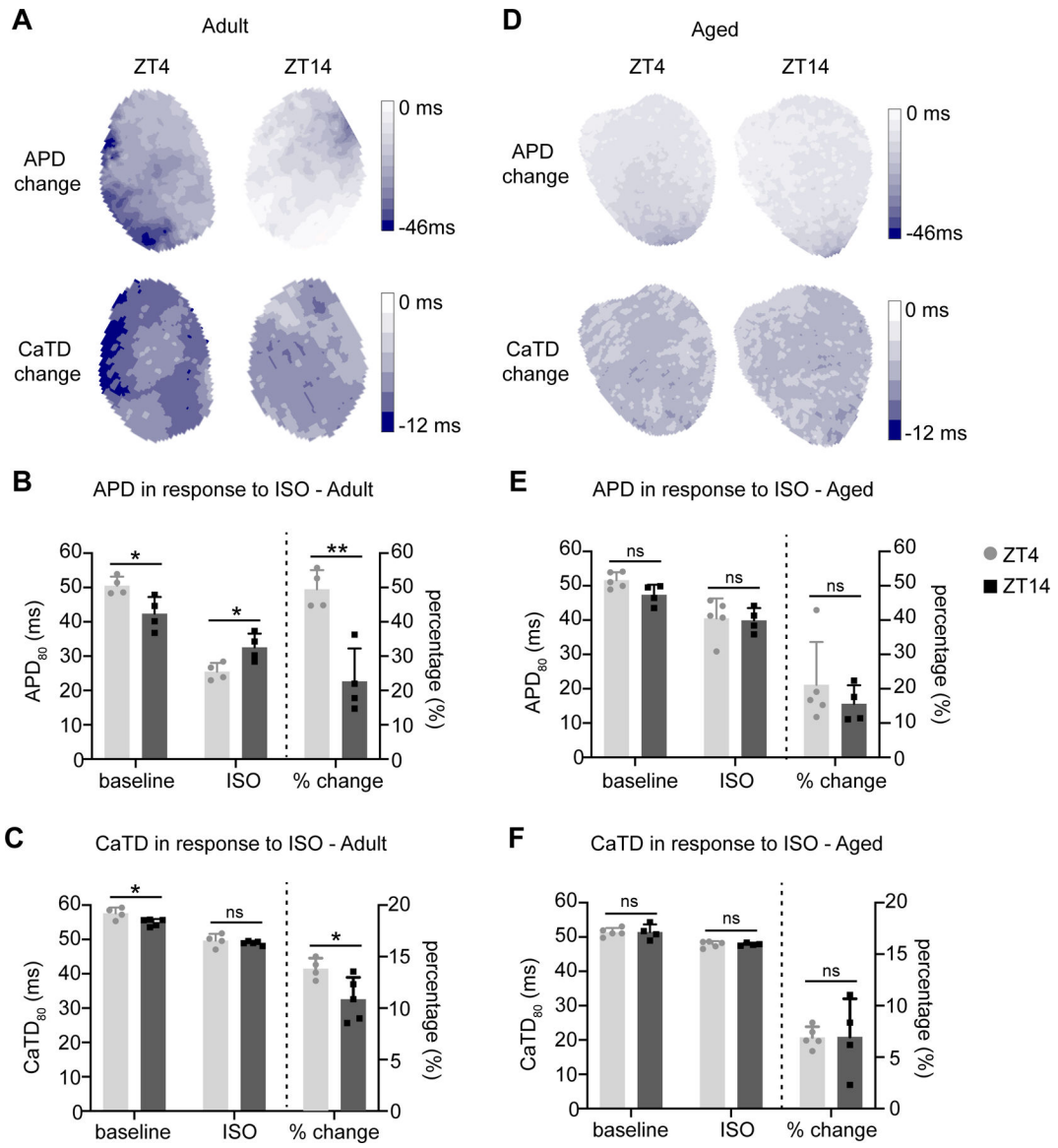

Figure 7.

Adult and aged hearts have different responses to β-AR stimulation. **A**. Example maps of APD change (top) and CaTD change (bottom) in adult hearts in response to 1μM isoproterenol. **B**. Left axis: APD_{80} before (baseline) and after isoproterenol (ISO) at ZT4 and ZT14 in adult hearts. Right axis: The %APD change with isoproterenol in adult hearts. ZT4 n=4; ZT14 n=4. **C**. Left axis: $CaTD_{80}$ before (baseline) and after isoproterenol (ISO). Right axis: The %CaTD change with isoproterenol. ZT4 n=4; ZT14 n=5. **D**. Example maps of APD and CaTD change in aged hearts. **E-F** Aged hearts had similar APD_{80} (**E,** ZT4 n=5; ZT14 n=4) and $CaTD_{80}$ (**F,** ZT4 n=5; ZT14 n=4.) responses to 1μM isoproterenol at ZT4 and ZT14. Pacing cycle length = 100ms. Data graphed on the left y-axis were analyzed by 2-way ANOVA followed by Sidak post-hoc test and data on the right y-axis were analyzed by student's unpaired t-test. *p<0.05; ** p<0.01.

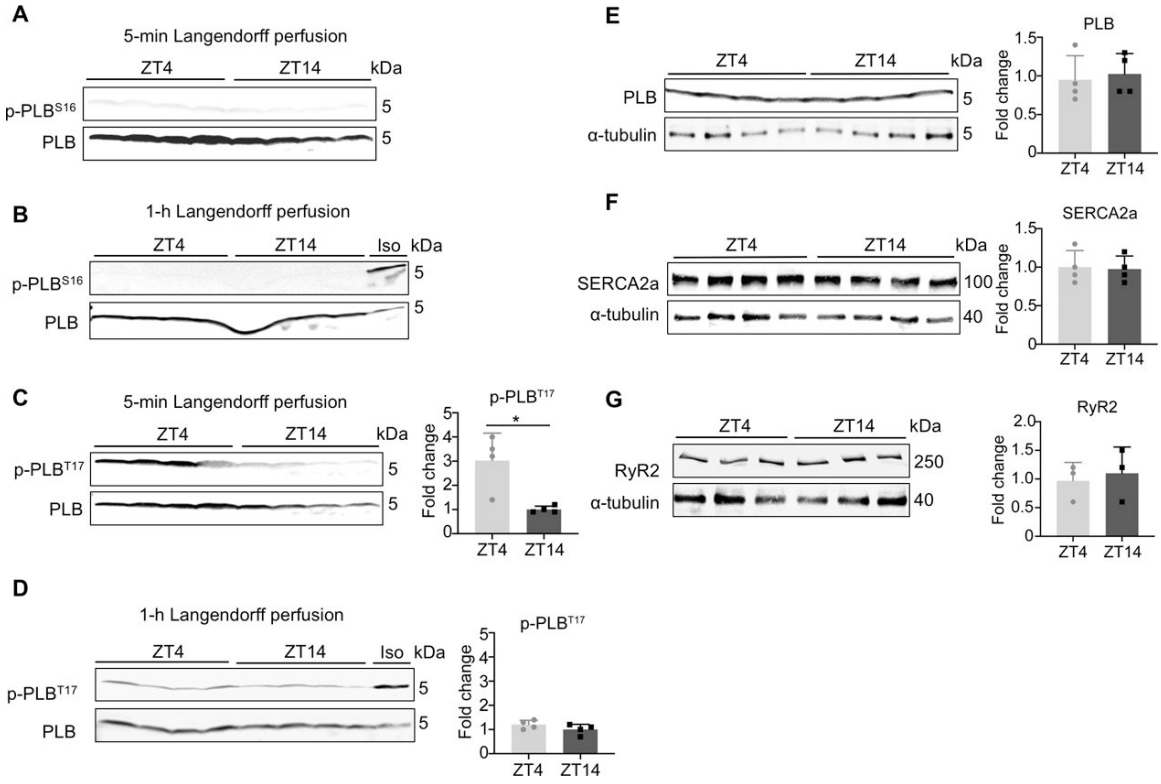

Figure 8.

Ca^{2+} handling protein expression or phosphorylation at ZT4 and ZT14 in adult hearts. **A-B**. Phosphorylation of PLB at serine16 was essentially non-detectable following 5min perfusion (**A**) and 1hr perfusion (to match optical mapping data collection timepoint, **B**). Band labeled 'Iso' in (**B**) was a single heart perfused with isoproterenol for a positive control for pPLB. **C-D**. PLB phosphorylation was higher at the Thr17 site at ZT4 vs. ZT14 following 5min perfusion (**C**). However, phosphorylation levels were reduced and were not different between ZT4 and ZT14 following 1hr of perfusion (**D**). Band labeled 'Iso' in (**D**) was a single heart perfused with isoproterenol for a positive control for pPLB. **E-G**. Total PLB (**E**), SERCA2a (**F**), and RyR2 (**G**) expression. α-tubulin was used as loading control. ZT4 n=4; ZT14 n=4; except RyR (**G**) where ZT4 n=3; ZT14 n=3. *p<0.05.

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Table 1.

Time-of-day variation of electrophysiological parameters in adult hearts.

Parameter	ZT4	ZT9	ZT14	ZT21
HR (bpm)	236.2±53.0	276.8±47.7	247.6±62.7	305.4±15.2
CV (cm/s)	47.1±2.8	47.2±4.8	49.8±2.4	45.2±3.6
APD_{80} (ms)	45.4±4.1 *	45.1±8.6	34.7±4.2	49.2±7.6 *
APD_{80} Disp. (ms/mm^2)	0.49±0.14	0.51±0.18	0.29±0.073	0.53±0.25
APD_{50} (ms)	9.6±1.9 ††	11.2±4.7 †	10.1±0.6	16.9±2.2 *
APD_{80-50} (ms)	35.8±4.0 **	33.9±4.2 *	24.6±3.9	32.3±5.8
ERP (ms)	54.3±7.9 *	63.3±10.3 **	32.0±8.4	72.5±20.6 ***
CaT rise (ms)	6.3±0.5 ###,††††	8.2±1.1 ***	5.9±0.2	9.0±0.8 ****
Ca^{2+} release recov. (AU)	0.83±0.035	0.78±0.050 **	0.89±0.015	0.76±0.053 ***
$CaTD_{80}$ (ms)	70.1±3.3 *	72.7±2.7 ***	64.3±3.3	74.4±1.2 ***
CaT decay (*tau*) (ms)	37.6±3.1 *	36.4±4.5	32.2±1.5	38.6±2.2 *
APD Alt. Thres. (ms)	70.0±11.6	77.5±5.0	62.0±8.4	85.0±5.8 **
Ca^{2+} Alt. Thres. (ms)	95.7±12.7 *	98.3±13.3 *	76.0±5.5	110.0±0.0 ***
PVC occurrence	6/7	5/6	2/5	4/4
EB occurrence	6/7	4/6	2/5	4/4

Data are mean±SD. Except for PVC and EB occurrence, data were analyzed with 1-way ANOVA followed by Tukey's multiple comparison test.

p<0.001 vs. ZT9;

* p <0.05,

** p<0.01,

*** p<0.001,

**** p<0.0001 vs. ZT14;

† p<0.05,

†† p<0.01,

††††p<0.0001 vs. ZT21.

PVC and EB occurrence were analyzed with Fisher's exact test. HR, heart rate; CV, conduction velocity; APD, action potential duration; APD_{80} Disp., APD_{80} dispersion; ERP, effective refractory period; CaTD, Ca^{2+} transient duration; CaT decay, Ca^{2+} transient decay; Ca^{2+} release recov., recovery of Ca^{2+} release; APD Alt. Thres., APD alternans threshold; Ca^{2+} Alt. Thres., Ca^{2+} alternans threshold; PVC, premature ventricular complex; EB, extra beats.

Table 2.

Time-of-day variation in gene expression in adult and aged hearts.

	Adult mice		Aged mice	
Gene	**% change (ZT14 vs. ZT4)**	**p**	**% change (ZT14 vs. ZT4)**	**p**
CACNA1C ($I_{ca,L}$ α1c-subunit)	6.6±5.1	ns	7.3±9.0	ns
KCND2 ($I_{to,f}$ α-subunit)	12.7±9.7	ns	−5.1±14.1	ns
KCNIP2 ($I_{to,f}$ β-subunit)	−1.4±3.7	ns	5.1±7.6	ns
KCNA5 (I_{Kur} α-subunit)	37.2±1.4	p<0.05	−3.5±14.8	ns
SLC8A1 (NCX1)	−4.4±4.0	ns	−20.3±3.8	p<0.001
ADRB1 (β1-adrenoreceptor)	−16.0±6.5	p<0.05	−17.7±11.0	p=0.15
ADRB2 (β2-adrenoreceptor)	−12.3±9.3	ns	−4.0±19.5	ns

Data are mean±SD and were analyzed with an unpaired student's t-test (Adult mice: n=5/group; Aged mice: ZT4 n=6, ZT14 n=4). Gene expression changes are expressed as percentage change relative to ZT4. 18s was used as an internal control.