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Pregnancy and oestrogen regulate sinoatrial node calcium homeostasis and accelerate pacemaking

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Aims During pregnancy, there is a significant increase in heart rate (HR) potentially associated with an increased risk of arrhythmias or exacerbation of pre-existing cardiac conditions endangering both mother and foetus. Calcium homeostasis plays an important role in regulating automaticity of the sinoatrial node (SAN); however, its contribution to the accelerated HR during pregnancy remains unknown.

Methods and results Using murine SAN cells, we showed that pregnancy increased L-type Ca^{2+} current (I_{CaL}) and $Ca_V1.3$ mRNA expression, whereas T-type Ca^{2+} current (I_{CaT}) and its underlying channel were unchanged. Analysis of SAN intracellular Ca^{2+} oscillations showed that the rate of spontaneous Ca^{2+} transients was significantly higher in pregnant mice along with a higher mRNA expression of ryanodine receptor. Assessment of supra-ventricular arrhythmias using programmed electrical stimulation protocols on anaesthetized mice revealed higher susceptibility in pregnancy. Of note, the modifications associated with pregnancy were reversible following delivery. Furthermore, chronic administration of 17β-estradiol (E_2) to nodal-like human-induced pluripotent stem cell-derived cardiomyocytes (N-hiPSC-CM), control mice, oestrogen-receptor-β knockout (ERKOβ) but not ERKOα mice, accelerated cardiac automaticity, recapitulating the pregnancy phenotype in both mouse and human SAN cell models.

Conclusion Together, these results indicate that pregnancy considerably alters intra-cellular Ca^{2+} homeostasis sustaining faster HR during pregnancy. Importantly, these changes were dependent on an oestrogen receptor α (ERα) mechanism that resulted in increased I_{CaL} and spontaneous Ca^{2+} release from the sarcoplasmic reticulum, highlighting a novel role for oestrogen in regulating HR.

Keywords Heart rate • Calcium handling • Pacemaker • Pregnancy • Sinoatrial node

1. Introduction

Pregnancy brings drastic changes to physiological functions of many organ systems including the vasculature and the heart, which undergo extensive remodelling. Indeed, during normal pregnancy in women, the substantial increase in blood volume, metabolism and foetal needs require the cardiovascular system to prompt major adjustments to accommodate the needs of the developing foetus.[1,2] During pregnancy, the heart rate (HR) gradually increases up to 25% throughout the gestational period. Elevated HR or sinus tachycardia are often seen in pregnant women and are a known risk factor for arrhythmias notably supra-ventricular arrhythmias.[1,3–6] During pregnancy, the incidence of arrhythmias is high and they may occur *de novo* or become exacerbated if an underlying cardiac condition exists.[4,6] Arrhythmia can lead to haemodynamic compromise for the well-being of both the mother and the foetus depending on the symptom severity and the risk associated with potentially recurring arrhythmias during the pregnancy. Furthermore, all therapeutic interventions have the inherent potential to adversely affect the health of the unborn child.[7,8]

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This increase in HR in women suggests that pregnancy directly influences the automaticity of the heart. A major determinant of cardiac automaticity is the spontaneous diastolic depolarization phase of the action potential (AP) in sinoatrial node (SAN) cells where the rate of firing is determined by the slope of the diastolic depolarization.[9–11] Two main mechanisms are thought to regulate spontaneous activity of SAN cells: a voltage and Ca^{2+} clock pacemaking mechanisms. The voltage-sensitive component comprised of several membrane voltage-gated ionic currents including the L-type and T-type Ca^{2+} currents (I_{CaL} and I_{CaT}) along with the pacemaker current (I_f). The activation of I_f initiates the early diastolic depolarization until the activation threshold of I_{CaT} and I_{CaL} is reached. Although I_{CaT} activates more negatively than I_{CaL}, both currents play important roles in the late phase of the diastolic depolarization.[9,12,13] These channels work in concert with a Ca^{2+} cycling mechanism that relies on a spontaneous and rhythmic release of Ca^{2+} from the sarcoplasmic reticulum (SR) during the late diastolic depolarization in the form of Ca^{2+} sparks and transients.[10,14–19] The localized increase in intra-cellular Ca^{2+} initiates membrane depolarization largely through activation of the sodium-calcium exchanger (NCX1).[15,16] The small electrogenic current produced by forward-mode, NCX1 contributes to an acceleration of the late diastolic depolarization and spontaneous activity of the SAN. Intra-cellular Ca^{2+} cycling in SAN cells is also tightly regulated by various Ca^{2+} handling proteins. The major players include the SR-localized ryanodine receptor (RyR2), the SR Ca^{2+}-ATPase pump (SERCA2a) as well as the NCX1, which in concert, regulate SAN automaticity and HR.

In order to explain the increase in HR during pregnancy, several hypotheses related to haemodynamic changes and catecholamines have been put forth. However, the exact mechanisms underlying this increase in HR and the underlying molecular mechanisms of this physiological adaptation have been, until recently, largely unexplored. We proposed the hypothesis that pregnancy leads to an intrinsic electrophysiological remodelling of the SAN resulting in an increased automaticity. Consequently, in our previous study, we showed that pregnant mice reproduce the increased HR observed in human pregnancy and this increase was not secondary to alterations in autonomic tone, arterial blood pressure, or circulating catecholamine levels but was intrinsic to the heart. Specifically, we showed that pregnancy induces an important up-regulation of the pacemaker current I_f in mouse SAN and contributes to faster automaticity.[20] We now report that although block of I_f reduces the rate of the AP, it fails to completely eliminate the increased automaticity observed in SAN cells of pregnant mice, suggesting that other mechanisms are also involved and hinting at a possible role for Ca^{2+} in regulating HR during pregnancy. Accordingly, in light of the fundamental importance of Ca^{2+} in automaticity of the SAN, in this study, we sought to determine the role that intra-cellular Ca^{2+} cycling plays in regulating HR during pregnancy.

2. Methods

An expanded methods section is available in the Supplementary material online.

2.1 Animals

CD-1 female non-pregnant (NP, 2–4-month-old), pregnant (P, 18–19 gestation days), and post-partum (PP, 1–2 days post-delivery) mice were used. A subset of experiments was carried out using 2–4-month-old oestrogen-receptor α or β knockout female mice (ERKOα and ERKOβ) and their littermate controls (Wild-type C57BL/6 female mice). This study was performed in accordance to the guidelines of the Canadian Council on Animal Care (Ottawa, Canada) and the *Guide for the Care and Use of Laboratory Animals* published by the US NIH (NIH Publication No 85-23, revised 2011); the Montreal Heart Institute Animal Care Committee approved all experiments (approval reference number 2012-80-02 and 2015-80-04/05).

2.2 Sinoatrial cell isolation

Mice were anaesthetized by inhalation of 2% isoflurane and then killed by cervical dislocation. Isolated mouse SAN cells were obtained using previously published protocols.[20]

2.3 Culture of human-induced pluripotent stem cell-derived cardiomyocytes

Human-induced pluripotent stem cell-derived cardiomyocytes (hiPSC-CM) (iCell® Cardiomyocytes^2) were purchased from Cellular Dynamics International and used following manufacturer's instructions.

2.4 Cellular electrophysiology

Spontaneous APs were recorded using perforated-patch clamp technique (nystatin, 350 ng/mL) in current-clamp mode. Recordings of Ca^{2+} currents were obtained using the voltage-clamp technique in whole-cell configuration.

2.5 *In vivo* HR assessment

HR were recorded from anaesthetized mice using RR intervals obtained from surface electrocardiograms (ECGs) in Lead I configuration.

2.6 Programmed electrical stimulation protocols

An octapolar electrophysiology catheter (1.9F) (Transonic Scisense Inc.) was introduced into the heart of anaesthetized mice via the right jugular vein. Bipolar recordings were obtained from the distal two electrode pairs. The induction of supra-ventricular arrhythmias was tested using burst stimulation protocols.

2.7 Quantitative real-time polymerase chain reactions

Total RNA extraction and quantitative real-time polymerase chain reactions (qPCR) were conducted using adaptation of previously published protocols.[20,21] Primers for the various genes were designed and sequences are presented in the Supplementary material online, *Table S1*.

2.8 Calcium transients

Spontaneous and caffeine-induced Ca^{2+} transients were obtained using adaptation of published protocol.[21] Isolated SAN cells were pre-incubated with Fluo-4 AM (5 μM). Ca^{2+} transients were acquired by live imaging using a Zeiss LSM 710 laser scanning microscope.

2.9 Statistical analysis

All data are presented as mean ± standard error of the mean (SEM). '*n*' represents the number of cells and '*N*' the number of mice. A *P*-value <0.05 was considered significantly different. Statistical analyses are detailed in the Supplementary material online.

3. Results

3.1 Increased I_f only partially explains the faster SAN automaticity in pregnancy

We previously reported that up-regulation of I_f during pregnancy contributes to an acceleration of spontaneous AP firing rate in isolated mouse SAN cells.[20] In order to determine the functional impact of I_f on pacing rate of SAN cells, we applied the specific HCN channel blocker ivabradine. *Figure 1* presents typical examples of spontaneous AP recorded in mice in absence and presence of ivabradine, along with measurements of diastolic depolarization rate and rate of spontaneous SAN AP. Consistent with our previous report,[20] *Figure 1B* shows that the diastolic depolarization rate was higher in SAN cells of P mice compared with NP mice. Application of ivabradine resulted in a similar reduction in NP (20 ± 5%) and P (16 ± 3%, $P = 0.5$) mice, indicating that I_f, which plays a crucial role in the early phase of the diastolic depolarization was successfully blocked in both groups. The fact that the diastolic depolarization was still faster in P mice in presence of ivabradine suggests that I_f accounts for some but not all the increased diastolic depolarization rate in pregnancy. As shown in *Figure 1C*, the rate of spontaneous SAN AP was elevated in P mice under baseline conditions and although ivabradine decreased the rate in both groups, the reduction was significantly larger in NP mice (18 ± 2%) compare with P (9 ± 1%, $P = 0.005$). Thus, when I_f is blocked, cardiac automaticity is reduced to a lesser extent and remains higher in P mice, strongly suggesting that mechanisms other than I_f are also playing an important role in the pregnancy-induced enhanced automaticity. The AP threshold and the maximum diastolic potential that also contribute to SAN automaticity were measured and found to be unaffected by ivabradine in both groups (see Supplementary material online, *Figure S1*).

Considering Ca^{2+} homeostasis is a fundamental mechanism of pacemaking in the SAN, we devised the next series of experiments to determine the role of Ca^{2+} currents and Ca^{2+} handling functions in the increased SAN automaticity and consequent acceleration of HR during pregnancy.

3.2 Influence of pregnancy on Ca^{2+} currents

To determine if pregnancy caused any change in Ca^{2+} current density, we compared Ca^{2+} currents in isolated SAN cells. As previously reported, I_{CaL} activates around -50 mV and peaks at -10 mV in SAN cells.[22,23] The typical recordings and *I-V* curves of I_{CaL} shown on *Figure 2A*, indicate that current density of I_{CaL} was significantly increased in SAN cells from P (at -10 mV, -7.1 ± 0.6 pA/pF) compared with NP (-5.2 ± 0.4 pA/pF, $^*P = 0.047$) mice. We also measured I_{CaL} in PP mice in order to determine whether delivery would reverse the increase in current density. Representative current recordings and mean *I-V* curves (*Figure 2A*) demonstrate comparable I_{CaL} current densities between the PP and NP group. On the other hand, I_{CaT}, which was obtained through subtraction of I_{CaL} from total Ca^{2+} currents (*Figure 3A*), remained comparable in SAN cells from NP (at -45 mV, -4.9 ± 0.6 pA/pF) and P (-5.7 ± 1.0 pA/pF, $P = 0.45$) mice. Overall, the voltage-clamp data show that pregnancy specifically increases I_{CaL} in the SAN, making it a likely contributor to faster automaticity of the SAN during pregnancy. In addition, this up-regulation was completely reversible 24–48 h following delivery. The mean cell capacitances were 37.0 ± 3.2 pF in NP ($n = 16$), 40.3 ± 2.5 pF in P ($n = 16$) and 43.2 ± 4.7 pF in PP ($n = 8$) ($P = 0.7$).

The mean *I-V* curves of T-, L-type, and total Ca^{2+} currents are illustrated on the same graphs in Supplementary material online, *Figure S2* for non-pregnant and pregnant mice in order to appreciate the relative importance of I_{CaT} and I_{CaL} for the total Ca^{2+} current in both groups.

Kinetic analysis of the current showed that the steady-state activation properties of I_{CaL} *I-V* relationship as well as its time to peak and macroscopic inactivation were comparable between the three groups (Supplementary material online, *Table S2*), indicating that a change in the kinetics properties of the current cannot explain the increased density of I_{CaL} in SAN cells from pregnant mice.

3.3 Transcriptional up-regulation of L-type Ca^{2+} channels by pregnancy

In the following set of experiments, mRNA expression of Ca^{2+} channel α-subunits in whole SAN tissue was evaluated using qPCR analysis. Specifically, we examined both isoforms of L-type Ca^{2+} channels ($Ca_V1.2$ and $Ca_V1.3$) as well as the major isoform of T-type Ca^{2+} channels $Ca_V3.1$. Results shown on *Figure 2B* indicate that mRNA expression of $Ca_V1.3$ but not $Ca_V1.2$ was significantly increased in the P group compared with NP. These data support the notion that $Ca_V1.3$ contributes more significantly than $Ca_V1.2$ to the diastolic depolarization in SAN cells given that the current generated by $Ca_V1.3$ activates more rapidly and at lower membrane potentials than $Ca_V1.2$.[22,23] Consistent with the voltage-clamp data on I_{CaT}, mRNA abundance of $Ca_V3.1$ were not affected by pregnancy (*Figure 3B*), thereby indicating that pregnancy induces a selective transcriptional up-regulation of $Ca_V1.3$ leading to increased I_{CaL} density in the SAN. Interestingly, qPCR analysis in PP mice (*Figure 2B*) also reveals that upon delivery, $Ca_V1.3$ expression reverts to NP levels indicating, in clear concordance with the voltage-clamp data, a key role of pregnancy in transcriptionally regulating $Ca_V1.3$ L-type Ca^{2+} channels in the SAN.

3.4 Pregnancy accelerates spontaneous Ca^{2+} transient rate

We, then, determined whether pregnancy altered intra-cellular SAN Ca^{2+} handling. Using SAN cells isolated from NP, P, and PP mice that were loaded with the Ca^{2+} probe Fluo-4, we examined spontaneous Ca^{2+} transients. *Figure 4A* illustrates representative Ca^{2+} transient recording from the various groups. As shown on this figure, the rate of spontaneous transients in SAN cells from P mice was significantly increased (19.1 ± 1.3%) compared with NP mice whereas the amplitude of the transients was unaltered. Furthermore, there was a decrease in the Ca^{2+} transient time-to-peak and the time to 90% decay during pregnancy. The results also show that the rate and time-to-peak were reversed in the PP group, highlighting again the specific effect of pregnancy in regulating Ca^{2+} homeostasis.

Figure 4B illustrates representative examples of caffeine-induced Ca^{2+} transients in SAN cells from NP and P mice. The amplitude of the caffeine-induced Ca^{2+} transient (total SR Ca^{2+} content) and the fractional release, the amount of Ca^{2+} released (spontaneous Ca^{2+} transient) divided by the total SR Ca^{2+} content, were similar in both groups. These data are consistent with the unchanged Ca^{2+} transient amplitude as the amplitude of the transients depends largely on SR Ca^{2+} content.

3.5 Influence of pregnancy on gene expression of Ca^{2+} handling proteins

The mRNA levels of the major Ca^{2+} handling proteins expressed in mouse SAN were analysed to determine whether Ca^{2+} handling

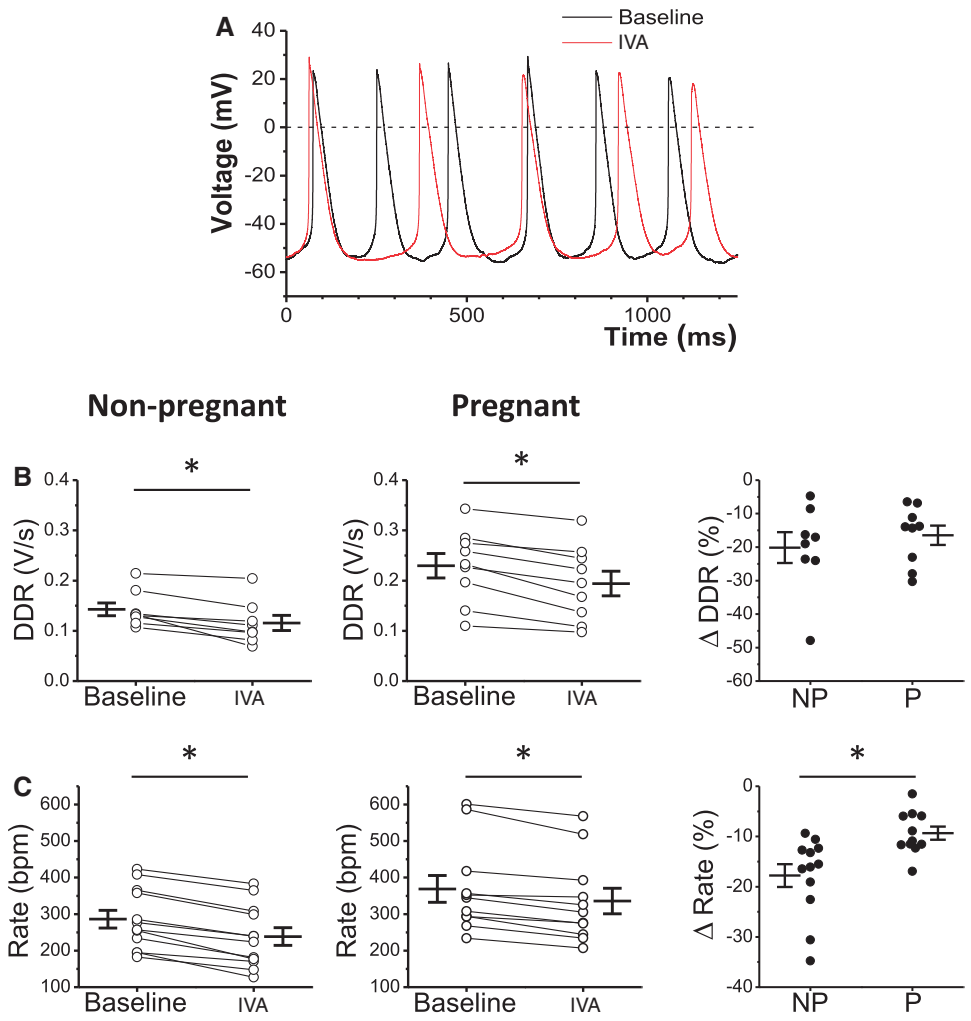

Figure 1 Ivabradine differentially reduces AP rate in isolated SAN cells. (*A*) The effects of ivabradine (IVA, 3 μM) on AP rate are shown for SAN cells isolated from NP mice. (*B*) Scatter plots summarize diastolic depolarization rate (DDR) before and after application of IVA to SAN cells from NP to P mice. Results show a significant reduction in DDR with IVA in both groups (NP: $n = 9/N = 8$; P: $n = 10/N = 9$). Mean change in DDR was obtained by averaging the differences from individual cells before and after IVA (Right). (*C*) Scatter plots show a significant reduction in AP rate with IVA in NP and P mice; however, the reduction was greater in NP mice (*right panel*) (NP: $n = 15/N = 12$; P: $n = 12/N = 11$) $^*P < 0.05$ by contrasts from two-way repeated measures analysis of variance (ANOVA).

components were altered. *Figure 5* shows that the mRNA expression of RyR2 was significantly increased in SAN from P mice compared with NP mice, whereas expression of NCX1, SERCA2a, and phospholamban (PLB) were unaffected. In addition, we noted that the effects of pregnancy on RyR2 expression were also reversed to non-pregnancy levels following delivery whereas all the other genes remained stable in PP mice (*Figure 5*). Taken together, the findings of the last experiments suggest that pregnancy enhances the rate of SR Ca^{2+} release as indicated by the smaller time-to-peak values through a transcriptional up-regulation of RyR2 in the SAN. The faster time to 90% decay was not associated with transcriptional up-regulation of NCX1, SERCA2a, or PLB in the P group suggesting that other mechanisms may be involved such as functional modulation by second messengers. Translational or post-translational modifications of one or many proteins involved in the Ca^{2+} cycling could also be implicated.

3.6 Cellular and *in vivo* electrophysiological studies

The *in vivo* HR analysis obtained by ECG measurements shown on *Figure 6A* revealed that P mice had a significantly higher HR compared with the NP, consistent with our previous study.[20] Furthermore, the PP had HR values comparable to the NP, indicating a return to baseline rates shortly after delivery. Interestingly, a similar observation was made at the cellular level (*Figure 6B*). When the rate of spontaneous AP was measured, PP mice had an AP pacing rate of 306 ± 27 b.p.m. ($n = 8$) that was comparable to the previously reported NP values (292 ± 13 b.p.m.).[20] These data show that 24–48 h following delivery there was a rapid reversal in automaticity of the SAN and a drop from the P rates (330 ± 12 b.p.m.).[20]

Furthermore, programmed *in vivo* electrophysiological stimulations were used to determine whether the increase in SAN automaticity, we observed in P mice might contribute to an increased supra-ventricular arrhythmia risk, a well observed phenomenon in pregnant women.[6]

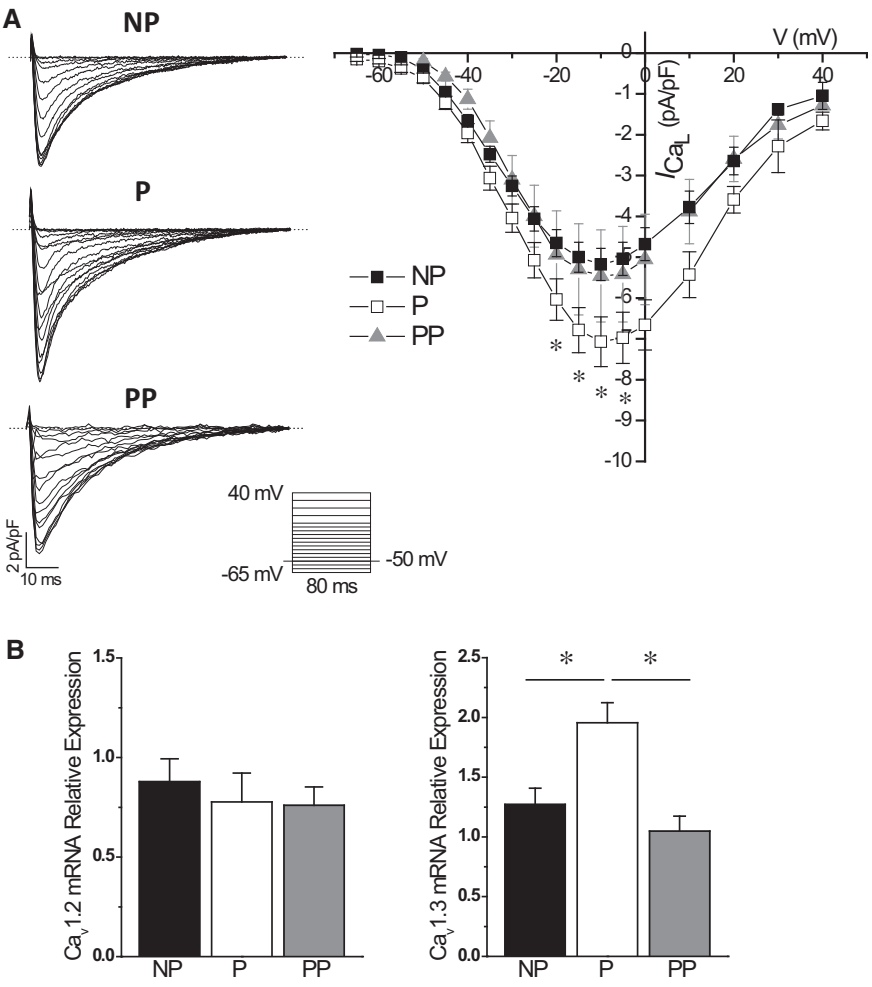

Figure 2 Pregnancy increases I_{CaL} density and $Ca_V1.3$ mRNA expression in mouse SAN. (*A*) Typical I_{CaL} traces recorded from SAN cells of NP, P, and PP mice (*inset shows protocol*). Corresponding mean *I-V* relationships show increased I_{CaL} density in P ($n = 16/N = 11$) compared with NP ($n = 16/N = 9$) and PP mice ($n = 8/N = 2$) (*$P = 0.036$ vs. NP and 0.010 vs. PP, mixed-effects model). (*B*) qPCR results showing increased relative mRNA expression of $Ca_V1.3$ in SAN from P compared with NP and PP mice (*$P < 0.05$, one-way ANOVA with Tukey *post hoc* test), whereas $Ca_V1.2$ mRNA expression is similar in all groups ($n = 3$, $N = 5$–6 SAN/sample) (P = NS, one-way ANOVA with Tukey *post hoc* test).

Our *in vivo* data obtained with programmed electrical stimulation protocols on *Figure 6C* shows that the probability of inducing a sustained supra-ventricular arrhythmia were increased by ~50% in P mice compared with NP. Data also show that in PP mice the arrhythmia inducibility was reduced, indicating that when the mechanisms involved in the increased SAN automaticity go back to baseline, the arrhythmia susceptibility is also normalized (*Figure 6C*).

3.7 Oestrogen treatment recapitulates the pregnancy-induced increased HR

It is known that during the post-partum period rapid and major changes in hormonal status occur.[24] Indeed, herein, we noted a fast drop in plasma E_2 levels occurring 24–48 h post-delivery. *Figure 7A* shows that P mice had a significantly higher plasma E_2 concentration (23.3 ± 5.0 nM) whereas concentration in PP mice was reduced to 2.2 ± 0.3 nM, similar to NP control levels (2.7 ± 0.7 nM). Importantly, this drop in E_2 also coincided with the rapid reversal in electrophysiological and Ca^{2+} handling parameters that were observed in PP mice. With the simultaneous occurrence of these phenomena, we next sought to determine the potential role of E_2 as a rate-altering hormone and underlying molecular trigger of the changes observed during pregnancy. Accordingly, we first administered E_2 (30 µg *per os* twice daily) to NP mice for a period of four consecutive days. Following the treatment period, surface ECG recordings were performed and blood samples withdrawn from the animals. Data on *Figure 7A* show that E_2 administration significantly increased plasma E_2 concentrations up to pregnancy levels. Furthermore, ECG data obtained from these same mice (*Figure 7B*) showed an important acceleration of HR (NP = 514 ± 15 b.p.m.; NP + E_2 = 583 ± 21 b.p.m., $P = 0.001$). Thus, by increasing plasma E_2 to concentrations found during pregnancy, HR was accelerated by ~13%, as observed in pregnant animals. Overall, the data show that E_2 is a potent and rapid regulator of HR, whereby its administration to control animals is sufficient to recapitulate the pregnancy-induced increase in HR.

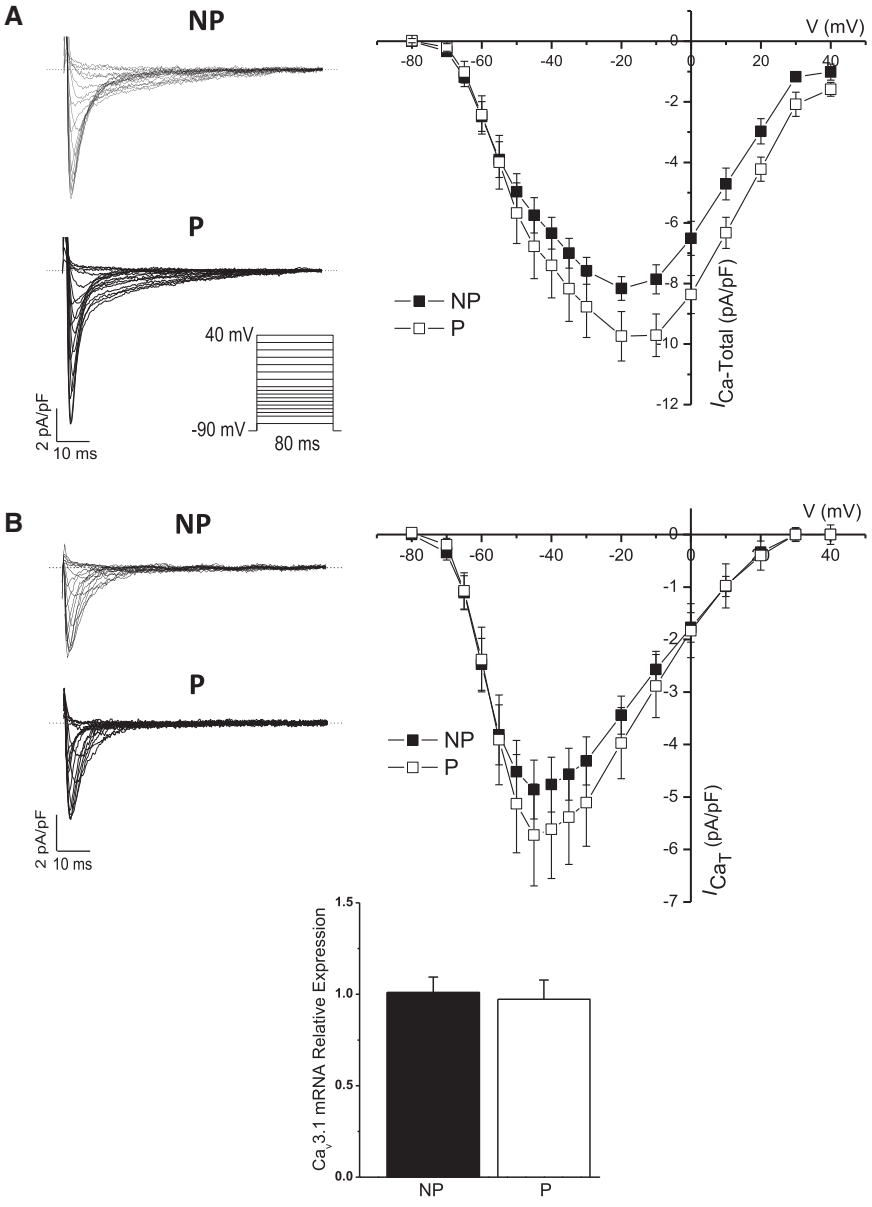

Figure 3 Pregnancy does not alter I_{CaT} in mouse SAN. (*A*) Typical examples of total Ca^{2+} current ($I_{Ca\text{-}Total}$) traces from SAN cells of NP and P mice (*inset shows protocol*) and corresponding mean *I-V* curves of $I_{Ca\text{-}Total}$. (*B*) Representative examples of I_{CaT} traces obtained by subtracting I_{CaL} from $I_{Ca\text{-}Total}$ for each cell. Corresponding mean *I-V* relationships for I_{CaT} reveal that the current density was similar in both groups ($I_{Ca\text{-}Total}$ and I_{CaT}: NP: $n = 15/N = 8$; P: $n = 15/N = 9$). Consistently, $Ca_V3.1$ mRNA expression was comparable in SAN tissues from NP to P mice (NP: n=3; P: n=4; N = 5–6 SAN/sample) ($P > 0.8$, unpaired Student's *t*-test).

3.8 Oestrogen fails to accelerate HR in ERKOα mice

We then used both ERKOα and ERKOβ mice in order to test whether the action of E_2 are mediated by oestrogen receptors while also establishing which receptor subtype is implicated in the regulation of HR. Applying the same E_2-treatment protocol, data in *Figure 7B* show that in ERKOβ, E_2 treatment resulted in a ∼14% increase in HR (ERKOβ = 511 ± 15 b.p.m.; ERKOβ + E_2 = 580 ± 10 b.p.m., $P = 4 \times 10^{-4}$), similar to results found with E_2-treated NP mice (*Figure 7B*) and wild-type littermates (Supplementary material online, *Figure S3*). However, when E_2 was administered to ERKOα mice, despite the dramatic elevation in plasma E_2 levels (*Figure 7A*), there was no significant increase in HR (ERKOα = 520 ± 16 b.p.m.; ERKOα+E_2 = 530 ± 21 b.p.m.). Overall, these results show that E_2 significantly increases HR in NP and ERKOβ mice but not ERKOα, implicating the E_2-ERα pathway in the regulation of HR.

3.9 Oestrogen accelerates automaticity of nodal-like human-induced stem cell-derived cardiomyocytes (N-hiPSC-CM)

To further investigate the role of E_2 as a rate-altering hormone, we treated N-hiPSC-CM with E_2 for 48–72 h. Following treatment, spontaneously beating, N-hiPSC-CM were selected and AP were recorded.

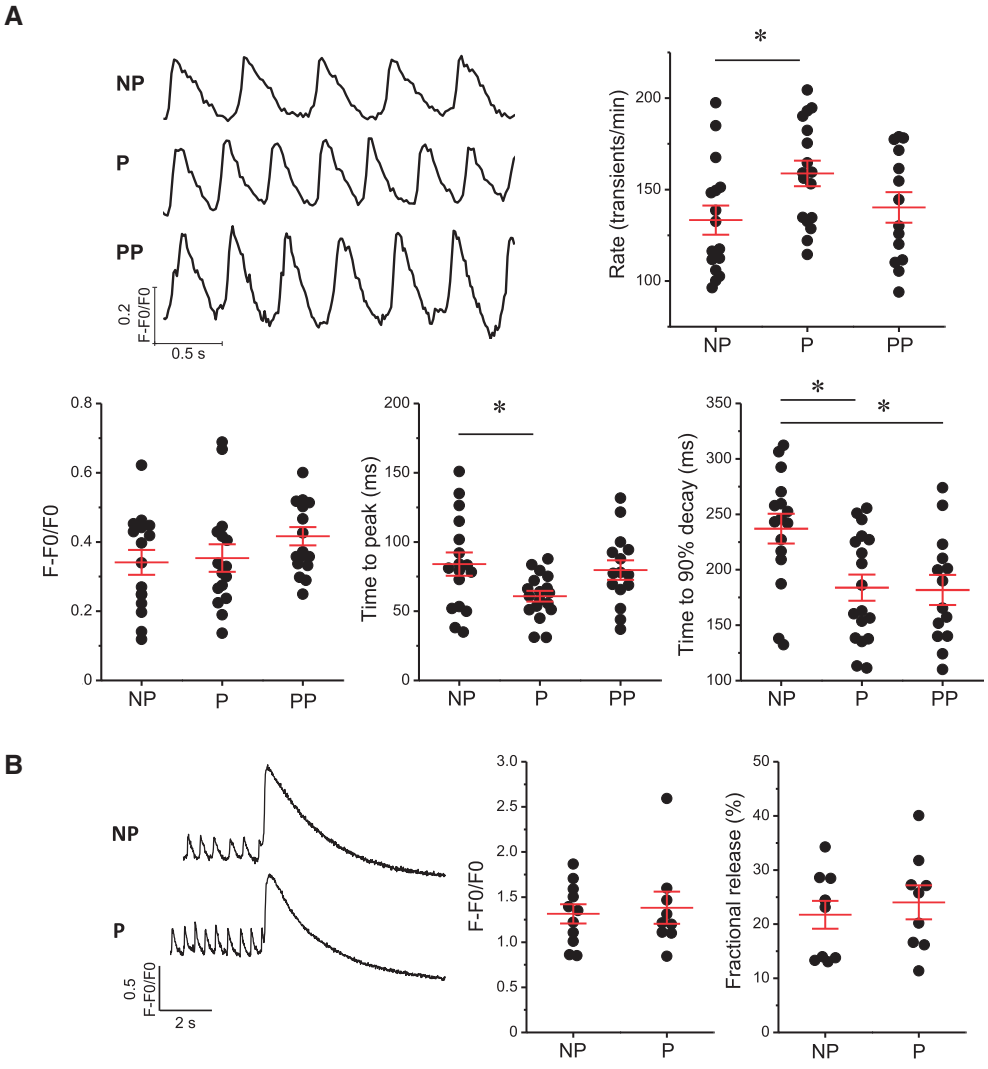

Figure 4 Spontaneous Ca^{2+} transient parameters obtained from NP, P, and PP mice. (*A*) Typical examples of spontaneous transients are shown on the top (left) panel obtained from a SAN cell loaded with Fluo-4AM. Scatter plots summarize data for transients rate, spontaneous Ca^{2+} transient amplitude [$(F\text{-}F_0)/F_0$), time-to-peak, and time to 90% decay] for NP ($n = 16–17/N = 6$), P ($n = 16–18/N = 7$), and PP ($n = 14–16/N = 6–7$) mice (*$P < 0.05$, mixed-effects model). Results show an increased transient rate, faster time-to-peak, and time to 90% decay in P mice (*B*). Left: typical examples of caffeine-induced transient in NP and P mice. Scatter plot data indicate that caffeine-induced Ca^{2+} transient amplitude (SR Ca^{2+} content) and fractional release for NP ($n = 10–11/N = 5$) and P ($n = 9/N = 5$) mice were similar between NP and P mice.

AP parameters including AP duration, rate, and configuration that features spontaneous depolarization phase were all consistent with previously published reports of N-hiPSC-CM phenotypes.[25–27] Analysis of spontaneous AP rate from control (CTL) and E_2-treated cells show that E_2 significantly increases AP rate. Indeed, as shown on *Figure 7C*, AP rate for control cells was 79.0 ± 2.2 b.p.m. compared with 99.6 ± 5.5 b.p.m., $P = 0.006$) for E_2-treated cells. Furthermore, analysis of the diastolic depolarization phase revealed that the diastolic depolarization rate was significantly increased in E_2-treated cells (53.1 ± 4.2 mV/s) compared with baseline (27.3 ± 0.9 mV/s, $P = 0.0004$). The higher diastolic depolarization rate is consistent with our findings showing an up-regulation of several major players implicated in the diastolic depolarization. Since the effects of E_2 on HR from *in vivo* experiments translated to individual N-hiPSC-CM, this demonstrates that E_2 is capable of regulating pacemaking in nodal cells. These results support the notion that the observed effects of E_2 administration *in vivo* are not due to peripheral or indirect actions of E_2 but to a direct modulation of SAN function. Equally important, findings obtained with the N-hiPSC-CM indicate that the effects of E_2 are also applicable to human nodal cells whereby E_2 might be a major player in increasing automaticity and an underlying mechanism to the rapid rates observed during pregnancy in women.

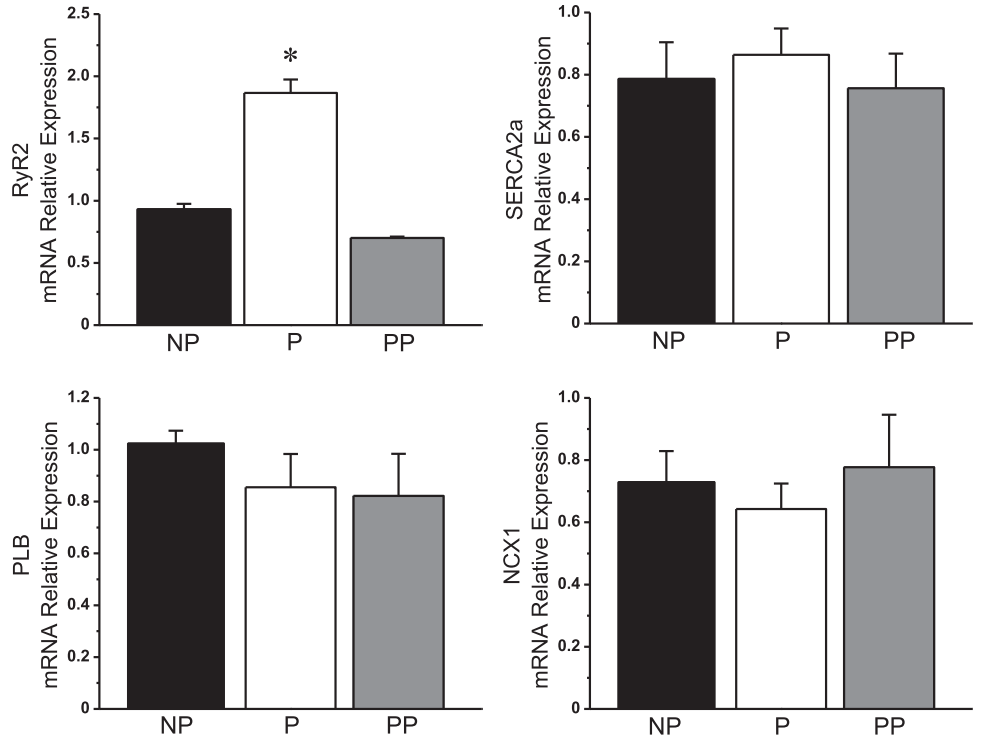

Figure 5 qPCR analysis of Ca^{2+} handling genes show a significant increases of RyR2 mRNA expression during pregnancy. Relative mRNA expression of RyR2, SERCA2a, PLB, and NCX1 in whole SAN tissues from NP, P, and PP mice are shown. All graphs represent $n = 3–5$ samples from 5 to 6 pooled SANs (*$P < 0.01$, one-way ANOVA with Tukey *post hoc* test).

4. Discussion

4.1 Summary of main findings

During pregnancy, cardiac remodelling is considered an essential physiological adaptation that helps provide adequate supply of nutrients and oxygen to the growing foetus.[1] Along with the structural changes such as hypertrophy, there is an up to 25% increase in resting HR[28] indicating that effects of pregnancy also extend to cardiac electrophysiological parameters. While investigating these changes, we have previously found that the increase in HR in pregnant mice was independent of changes in autonomic tone, blood pressure, or circulating catecholamines and was instead attributable to cardiac electrical remodelling.[20] Specifically, we showed that there was a significant increase in the density and expression of the pacemaker current I_f that was associated with faster automaticity of the SAN.[20] In this study, we show that blockade of I_f, using the selective HCN channel blocker ivabradine, affected NP and P mice differently. Indeed, while the SAN cells firing rate was reduced in both groups, following the application of ivabradine, P mice had a rate that remained significantly higher, indicating that pacemaking mechanisms other that I_f also contribute to maintain an elevated HR during pregnancy. Herein, we report that Ca^{2+} homeostasis is also regulated to support an accelerated HR. Our data show that, in the SAN, pregnancy induces a transcriptional up-regulation of $Ca_V1.3$ and RyR2 channels. This up-regulation resulted in an increase in I_{CaL} current density along with a faster Ca^{2+} extrusion from the SR measured during spontaneous Ca^{2+} transients. The increase in both $Ca_V1.3$ and RyR2 may be critical to sustain the faster HR by allowing a quicker spontaneous transient rate through accelerated Ca^{2+} entry and subsequent intra-cellular Ca^{2+} release.

4.2 Role of I_{CaL} in the increased pacemaker activity in pregnancy

Of note, there was no increase in $Ca_V1.2$ expression in the SAN of pregnant mice and I_{Ca_L} peaked at a relatively hyperpolarized voltage (-10 mV). These observations are consistent with a greater contribution of $Ca_V1.3$ than $Ca_V1.2$ to the pacemaker activity of the SAN cells. Although $Ca_V1.2$ plays a major role in the upstroke phase of the SAN AP, its physiological role in pacemaking is thought to be modest.[22,23,29] Indeed, as previously reported $Ca_V1.3$ constitutes the major L-type Ca^{2+}channel isoform in the diastolic depolarization of the mouse SAN AP and in support of this observation, previous studies have shown that deletion of $Ca_V1.3$ resulted in bradycardia and dysrhythmic SAN pacemaking. Furthermore, $Ca_V1.3$ was demonstrated to play a critical role in modulating intra-cellular Ca^{2+} dynamics by regulating Ca^{2+} release from the SR.[22,23,29] Although it was shown that $Ca_V1.3$ deletion resulted in an inhibition of Ca^{2+} transients, it is very likely that the increase in $Ca_V1.3$ we observed here, along with the increase in RyR2, serves the opposite purpose: increasing Ca^{2+} transient rate and robustness of the $Ca_V1.3$/RyR2 coupling, thereby sustaining the elevated HR during pregnancy. In support of this notion, Torrente *et al.*[22] recently reported that $Ca_V1.3$ channels co-localize with RyR2 in mouse SAN cells, which would favour the Ca^{2+}-induced Ca^{2+} release from the SR.

The increased Ca^{2+} inflow mediated through higher levels of $Ca_V1.3$ directly contributes to depolarize the pacemaker cells faster to trigger the next AP leading to elevated HR. Furthermore, an increase in I_{CaL} will also trigger a RyR2-dependent Ca^{2+} release from the SR, which in turn activates NCX1, generating a depolarizing current that increases the

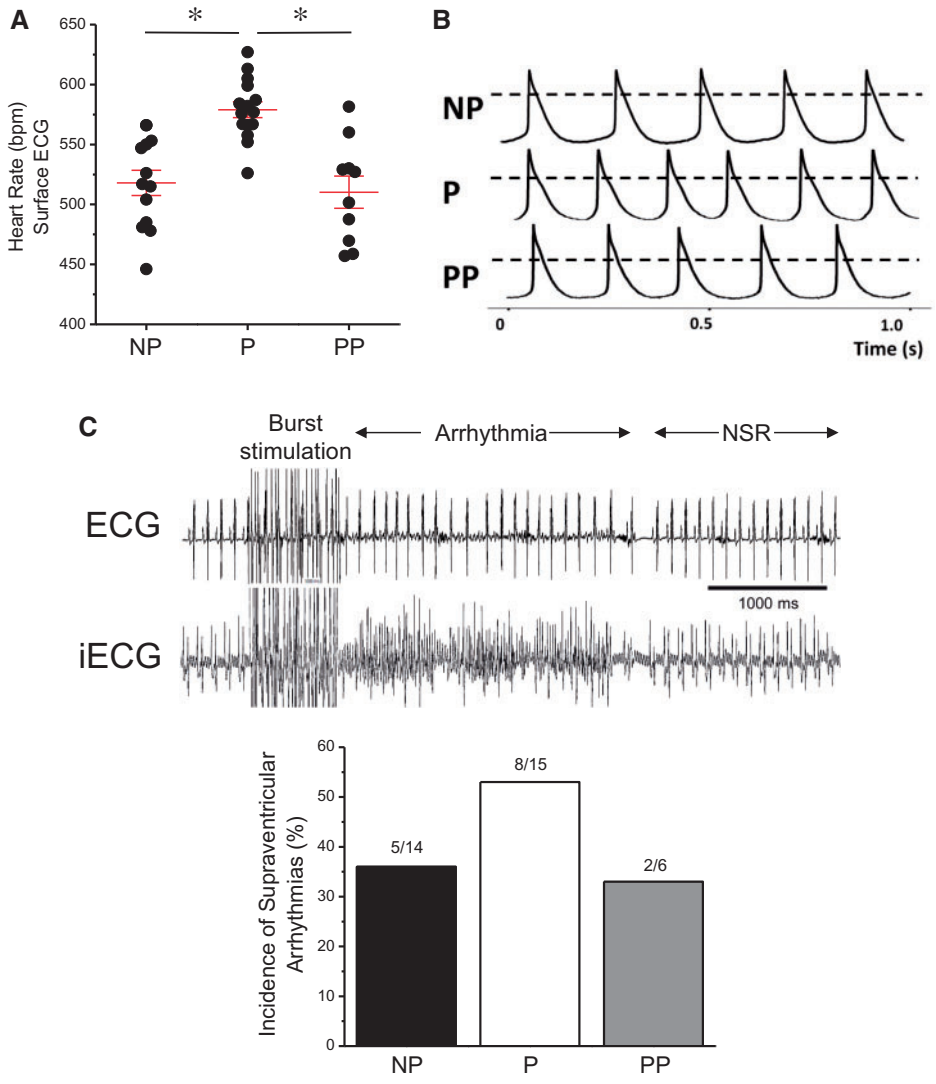

Figure 6 *In vivo* electrophysiological studies and arrhythmia susceptibility in NP, P, and PP mice. (*A*) Scatter plot data showing that HR was significantly higher in P compared with NP mice, consistent with our previous study.[20] These ECG data also reveal that 24–48 h following delivery (PP), the HR returned to baseline values and was significantly lower than P mice (NP, $N = 13$; P, $N = 15$; PP, $N = 10$, $^*P = 0.0001$ vs. NP, $^*P = 0.00008$ vs. PP, one-way ANOVA with Tukey *post hoc* test). (*B*). Typical examples of spontaneous AP recordings obtained from SAN cells of NP, P, and PP mice reveal a similar rate between PP and NP, compared with P. (*C*) Top: representative surface ECG and intra-cardiac electrophysiological recordings on a pregnant mouse reveals that the use of the programmed electrical stimulation sequences (burst stimulation) triggered supra-ventricular arrhythmia followed by restoration of normal sinus rhythm. Bottom: Arrhythmia incidence data show that the increased HR in the P group was associated with a higher susceptibility to arrhythmias that was normalized following delivery, as seen by comparable arrhythmic events between the NP and PP groups.

diastolic depolarization slope even further. The concerted actions of I_{CaL} through the voltage and Ca^{2+} clock pacemaking mechanisms support the concept that cardiac automaticity rely on the coordinated activity of voltage-dependent ion channels and intra-cellular Ca^{2+} cycling.

4.3 Oestrogen-ERα regulate cardiac automaticity during pregnancy

There are substantial fluctuations in the levels of several hormones during pregnancy. Importantly, during pregnancy and the early post-partum period, the fluctuations coincide with important changes in cardiac automaticity. For instance, pre-pregnancy HR values are restored within the first 2 weeks following delivery in women which concur with a sharp drop in oestrogen and progesterone levels.[30] In this study, we also report a novel finding that HR in pregnant mice also reverts to control values rapidly following delivery. The reversal in HR was observed at the cellular and molecular level of the SAN as well. Specifically, a return of I_{CaL} density to control levels and a normalization of transient rate, $Ca_V1.3$ and RyR2 expression levels. Of note, in the PP group time to 90% decay of the Ca^{2+} transients remained faster suggesting that maybe NCX1, SERCA2 or PLB activity remains high. Since transcriptionally, these Ca^{2+} handling parameters were not affected, it is possible that their functional modulation requires more than 24–48 h to revert to

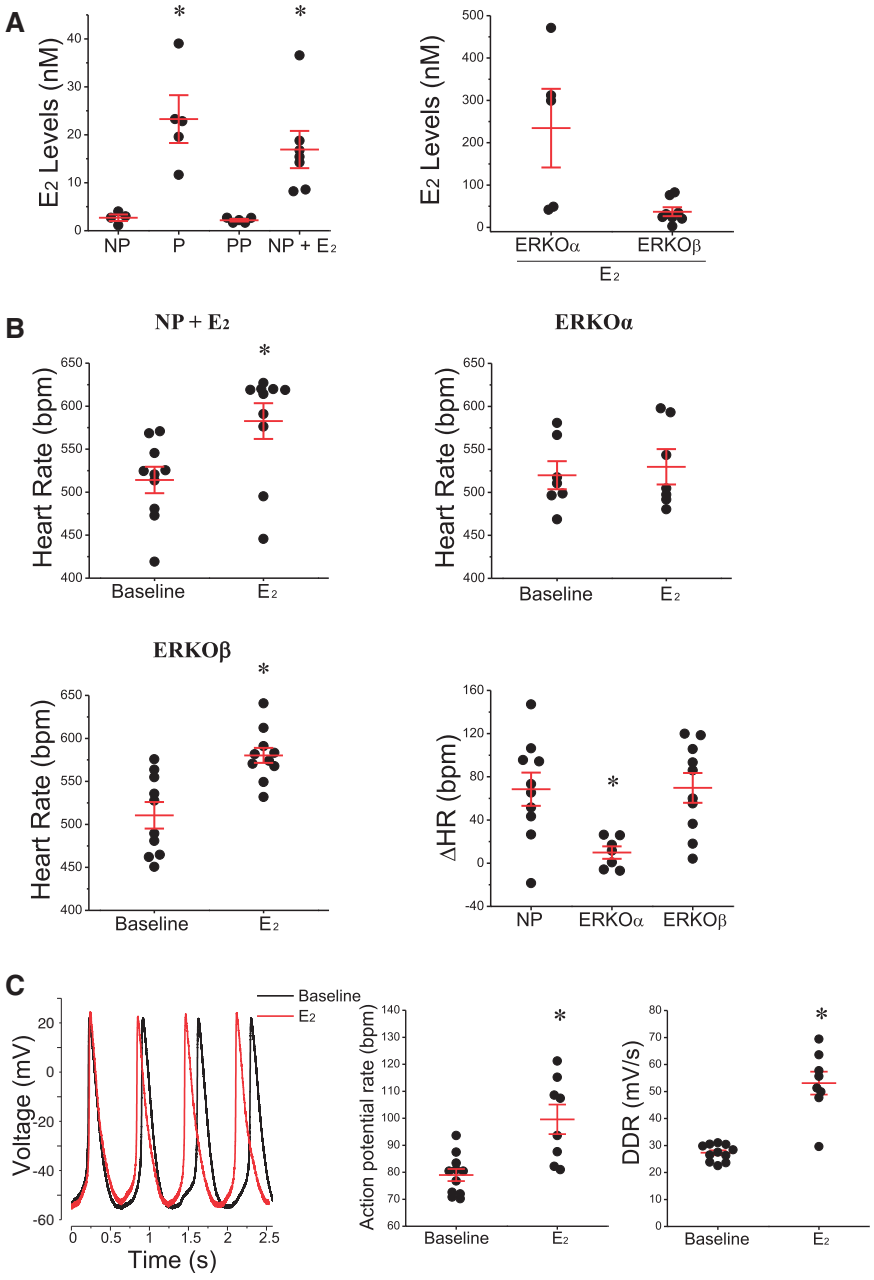

Figure 7 E_2 accelerates HR *in vivo* and AP rate in nodal-hiPSC-CM in an ERα-dependent manner. (*A*) Scatter plots present plasma E_2 concentrations in NP (4), P (5), PP (5), and E_2-treated NP (NP + E_2) (7) mice, whereas E_2 concentrations in E_2-treated ERKOα (5) and ERKOβ (8) mice are shown in the right panel. Data illustrated in these graphs show a ~10-fold increase in E_2 concentrations during pregnancy (*$P < 0.05$ vs. NP and PP, one-way ANOVA with Tukey *post hoc* test) and a significant increase in E_2 levels in NP + E_2, ERKOα, and ERKOβ mice following E_2 treatment. NP + E_2 and ERKOβ+E_2 mice had E_2 levels similar to those of P mice although ERKOα+E_2 had much higher levels. (*B*) HR of NP ($N = 10$), ERKOα ($N = 7$), and ERKOβ ($N = 10$) mice were measured before and after E_2 treatment; NP and ERKOβ but not ERKOα E_2-treated mice had a significant increase in HR (*$P < 0.05$, paired Student's *t*-test). (*C*) Typical spontaneous AP obtained from nodal-hiPSC-CM with or without E_2 are shown (left). E_2 treatment (100 nM, 48–72 h) resulted in a significant increase in the AP rate and diastolic depolarization rate (Baseline, $n = 11$; E_2, $n = 8$, unpaired Student's *t*-test).

baseline function. Interestingly, we also examined the pacemaker current I_f in PP mice, and found that its density also reverted to pre-pregnancy values, becoming comparable to non-pregnant data previously obtained (Supplementary material online, *Figure S4*).[20] This rapid reversal of the electrophysiological and Ca^{2+} handling parameters to baseline levels would be consistent with a hormonal effect on ion channels during pregnancy. Although many hormones including thyroid, relaxin, and progesterone might have a rate-altering effect, our study shows that oestrogen is a major hormone implicated in increased HR during pregnancy. Indeed, others have previously shown that increased levels of E_2 can up-regulate various ion channels including L-type Ca^{2+} channels in the ventricle and other cell types.[31–33] Herein, we provide evidence showing that E_2 also regulates automaticity. First, we show that the reversal in electrophysiological changes and normalization of HR coincides with a

sharp drop in E_2. Moreover, administration of E_2 accelerates HR. Furthermore, the use of ERKO mice provided additional support to our observations and established ERα as a major pathway and upstream signalling mechanism that drives electrical remodelling of the SAN and HR control.

4.4 Potential clinical significance

Understanding the mechanisms underlying the electrophysiological changes during pregnancy is becoming increasingly important. Indeed, as the average maternal age and number of pregnant women suffering from comorbidities including diabetes, hypertension and various cardiovascular conditions increase,[34,35] pregnancy-related complications, and mortality are becoming a complex issue requiring fundamental understanding of their underlying mechanisms. For instance, as previously noted, the increase in HR is a known risk factor for arrhythmias and during pregnancy there is a significant increase in the risk of electrical disturbances leading to supra-ventricular and sinus node arrhythmias, which comprise more than 70% of total arrhythmia occurrences in pregnancy.[36] While changes in sympathetic tone, hormones, and stress have been thought to contribute to the increased arrhythmia susceptibility,[35] little mechanistic evidence has been proposed. In this study and previous report, we showed that the HR during pregnancy increases due to major electrophysiological remodelling of the SAN. Furthermore, we showed that the mouse model of pregnancy was also associated with an increased risk of arrhythmias and although it was previously demonstrated that reversal of structural changes is relatively slow,[1] the modifications in electrophysiological parameters we observed were rapidly reversed following delivery, similarly to women.[1] These observations are consistent with the idea that electrophysiological changes due to direct regulation of ion channels and not cardiac structural remodelling are a major cause of arrhythmic events. Furthermore, it was previously reported that the increase in HR during pregnancy is protective in women with long QT syndrome and is associated with lower arrhythmia risk. However, in post-partum women, with the rapid reduction of HR comes a loss of the protective effect of pregnancy and a significant increase in ventricular arrhythmia susceptibility and torsades de pointes.[37]

Findings reported here address an important topic in women's health; as it provide novel and functional insight into the mechanisms of pregnancy-induced elevated HR and the basic mechanisms of action of oestrogens on cardiac pacemaker activity. Awareness of pregnancy and/or sex hormone-based differences in cardiac automaticity may help better understand the mechanisms of arrhythmias and may lead to specific therapeutic approaches. Furthermore, this knowledge could prove to be of major importance to women's health during different phases of a women's life cycle; for instance, in menopaused women (whether they are on hormonal replacement therapy or not) where age-dependent remodelling of the heart provides a substrate for the development of arrhythmias and may amplify the pro-arrhythmic effects of female hormones.

5. Conclusion

In conclusion, our study highlights an important role for pregnancy in regulating SAN function and pacemaking. Specifically, we demonstrated a major role for Ca^{2+} homeostasis, as well as pacemaker current, in the increased HR during pregnancy. This illustrates a notable case where key players in the SAN Ca^{2+} and voltage clocks are mutually entrained and regulated. These findings also portray the SAN as a dynamic pacemaker that can intrinsically and rapidly adjust to changing physiological requirements as witnessed by the rapid reversal of pacemaking function following delivery and significant response to E_2 treatment, through its receptor alpha. Understanding the cellular and molecular mechanisms that drive the SAN, HR control and arrhythmias related to female hormones and pregnancy is essential not only for expanding our knowledge of the regulatory pathways within the SAN but also for appropriate management of arrhythmias that correlate with female hormone fluctuation and/or pregnancy-induced arrhythmias.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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