Upregulation of the Hyperpolarization Activated Current Increases Pacemaker Activity of the Sino-Atrial Node and Heart Rate during Pregnancy in Mice

Running title: El Khoury et al.; Increased heart rate in pregnant mice

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Abstract:

**Background**—Pregnancy is associated with a faster heart rate (HR), which is a risk factor for arrhythmias. However, the underlying mechanisms for this increased HR are poorly understood. Therefore, this study was performed to gain mechanistic insight into the pregnancy-induced increase in HR.

**Methods and Results**—Using surface ECG we observed that pregnant (P) mice have faster HR (531±14bpm) compared to non-pregnant (NP) mice (470±27bpm, p<0.03). Results obtained with Langendorff-perfused hearts showed that this difference persisted in the absence of autonomic nervous innervation (NP: 327±16bpm, P: 385±18bpm, p<0.02). Spontaneous action potentials of sino-atrial node (SAN) cells from pregnant mice exhibited higher automaticity (NP: 292±13bpm, P: 330±12bpm, p=0.047) and steeper diastolic depolarization (NP: 0.20±0.03V/s, P: 0.40±0.06V/s, p=0.004). Pregnancy increased the density of the hyperpolarization-activated current (Ih) (at -90mV, NP: -15.2±1.0pA/pF, P: -28.6±2.9pA/pF, p=0.0002) in SAN cells. Voltage-dependence of the Ih activation curve and the intracellular cAMP levels were unchanged in SAN cells of pregnant mice. However, there was a significant increase in HCN2 channel protein expression with no change in HCN4 expression. Maximal depolarizing shift of the Ih activation curve induced by isoproterenol was attenuated in pregnancy. This reduced response to isoproterenol may be attributable to the lower cAMP sensitivity of HCN2 isoform compared to that of HCN4.

**Conclusions**—This study shows that an increase in Ih current density contributes to the acceleration of SAN automaticity and explains, in part, the higher HR observed in pregnancy.

**Key words:** heart rate, pregnancy, pacemaker, sinoatrial node, ion channel
Introduction

In women, cardiovascular function is strongly influenced by pregnancy and undergoes significant changes such as alterations in cardiac electrical function and higher vulnerability to arrhythmias.\textsuperscript{1, 2} During pregnancy, there is an increased risk of onset of new supraventricular tachycardia or exacerbation of pre-existing arrhythmia, which may compromise the wellbeing of the mother and the fetus.\textsuperscript{3-5} Furthermore, pregnancy is also associated with a higher resting heart rate (HR), which is a known risk factor for arrhythmias.\textsuperscript{4, 6, 7} This increase in HR suggests an influence of pregnancy on cardiac autonomic regulation and/or the automaticity of the heart.

Changes in cardiac autonomic modulation play a central role in the adaptation of the cardiovascular system to various hemodynamic changes required for a normal pregnancy.\textsuperscript{8-10} The heart is innervated by both the sympathetic and parasympathetic divisions of the autonomic nervous system. Stimulation of the sympathetic nerve fibers results in the release of norepinephrine that increases the automaticity of the sino-atrial node (SAN), the cardiac pacemaker. This is reflected on the electrocardiogram (ECG) by a faster HR. In contrast, parasympathetic stimulation by means of the vagus nerve releases acetylcholine and slows the pacemaker activity of the SAN.\textsuperscript{11}

The automaticity of the SAN is responsible for initiating heart rhythm. The diastolic depolarization phase of the spontaneous action potential (AP) generated in SAN cells is a major determinant of cardiac automaticity. Multiple mechanisms are involved in the diastolic depolarization of SAN cells. These include a hyperpolarization-activated current (\(I_f\)), T- and L-type calcium currents (\(I_{\text{CaT}}, I_{\text{CaL}}\)), the delayed rectifier K\(^+\) current (\(I_{\text{Kr}}\)), Na\(^+\)-Ca\(^{2+}\) exchanger current (\(I_{\text{NCX}}\)) and spontaneous Ca\(^{2+}\) release from the sarcoplasmic reticulum.\textsuperscript{12-14} While intracellular Ca\(^{2+}\) signaling is important in the generation of the late diastolic depolarization,\textsuperscript{15} \(I_f\)
plays a crucial role in the early phase of the diastolic depolarization of SAN cells.\textsuperscript{15} In mouse SAN, the hyperpolarization-activated cyclic nucleotide-gated (HCN) channel isoforms, HCN1, HCN2 and particularly HCN4, contribute to the native $I_f$ current.\textsuperscript{16, 17} Regulation of HCN by sympathetic and parasympathetic stimulation represents one of the main mechanisms by which the autonomic nervous system controls HR.\textsuperscript{18} A direct interaction of cyclic AMP (cAMP) with an intracellular cyclic nucleotide binding domain is known to regulate the voltage dependence of HCN channels. Indeed, β-adrenergic agonists increase the levels of cAMP, which positively shifts the HCN voltage dependence. The positive shift in the $I_f$ activation curve results in an increase in current density. In contrast, muscarinic stimulation decreases cAMP levels and has the opposite effect on $I_f$ voltage dependence.\textsuperscript{19}

Elevated HR can increase the susceptibility to arrhythmias; therefore, a better understanding of the mechanisms responsible for the increased cardiac automaticity associated with pregnancy is clinically important. As mentioned above, several mechanisms are involved in the automaticity of the heart. Since $I_f$ initiates the first part of the diastolic depolarization, the present study focused on the regulation of this ionic current during pregnancy. We therefore hypothesized that the increased physiological demands during pregnancy may lead to changes in $I_f$ channel expression and/or current density as well as $I_f$ response to stimulation by the autonomic nervous system. In turn, these modifications could increase the automaticity of the SAN and, in part, explain the mechanisms of elevated HR associated with pregnancy. Accordingly, the objectives of the present study were (1) to investigate the effect of pregnancy on HR in mice under control conditions and in the absence of autonomic nervous tone, and (2) to examine the effects of pregnancy on the automaticity of single SAN cells, and (3) to explore the mechanisms underlying the HR changes and how they relate to $I_f$. 
Materials and Methods

Animals
All experiments were performed on adult female CD1 mice (2-3 months old) obtained from Charles River (St-Constant, QC, Canada). Non-pregnant (NP) and late pregnant (P: 18-19 gestation days, gd) mice were used. All experiments were performed in accordance to the guidelines of the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No 85-23, revised 1996). Experiments were also approved by the Montreal Heart Institute Animal Care Committee (approval reference number 2009-80-02).

Surface electrocardiograms (ECG)
Lead I surface ECGs were conducted in anaesthetized NP and P mice for 5 minutes at a rate of 2kHz after baseline period. Additional surface ECG recordings were obtained on mice before and after intravenous (IV) injection of isoproterenol (0.1ng/g) through the right jugular vein. Further information is presented in the online supplemental material.

Langendorff-perfused heart
Experiments were performed as previously described. After rapid excision, the heart was hung on a modified Langendorff apparatus and retrogradely perfused through the aorta. ECG recordings were obtained after an equilibration perfusion period; ECG parameters were calculated as explained in the supplemental material.

Hemodynamic measurements
Mice were anaesthetized with isoflurane before a pressure catheter was inserted into the right carotid artery. Systolic, diastolic and mean arterial blood pressures were measured by a Millar Mikro-Tip Catheter Transducer (model SPR-671, 2F, Millar Instruments, TX, USA) and data
were analyzed with the program IOX, version 2.5.1.6 (EMKA Technologies).

**Catecholamine and cAMP measurements**

Plasma epinephrine and norepinephrine levels were measured using the 2-cat (A-N) Research ELISA kit (Rocky Mountain Diagnostics, CO, USA) following the manufacturer's instructions. SAN intracellular cAMP concentrations were measured using a LANCE® cAMP 384 ELISA kit (PerkinElmer, Waltham, MA, USA) following the manufacturer's instructions.

**Sino-atrial cell isolation**

Mice were anaesthetized by inhalation of isoflurane and then sacrificed by cervical dislocation. The SAN cells were isolated using an adaptation of the protocol previously described by Mangoni *et al.* Briefly, SANs were dissected in normal HEPES-buffered Tyrode’s and digested by enzymatic dissociation. Cells were then obtained by trituration and kept in “Kraft-Brühe” (KB) solution until use. Isolated mouse SAN cells were identified as small spindle-type cells spontaneously beating in normal Tyrode’s solution. Details on the digestion protocol are presented in the supplemental material.

**Electrophysiological data**

**Current- and voltage-clamp recordings**

For $I_f$ recordings, 1 mM BaCl$_2$ (Sigma) was added to the external solution to block the inward rectifier K$^+$ current, $I_{K1}$. All recordings were carried out at 35±1°C. Pipette resistance was between 3-5MΩ. The $I_f$ IV curve was obtained in whole-cell configuration while the action potential and activation curves were obtained using perforated patch-clamp technique with nystatin (350ng/ml). To account for differences in cell size, current amplitudes were normalized to cell capacitance and expressed as densities (pA/pF). Solution composition and protocols are available in the supplemental material.
Current- and voltage-clamp protocols and data analysis

Current-voltage relationships for the hyperpolarized current (I_t) were constructed using records elicited by 2-s voltage steps from -120 to -20mV in 10mV increments from a holding potential of -30mV at a frequency rate of 0.2Hz. The density of I_t was measured at the end of the hyperpolarizing steps. Activation curves were obtained using a protocol in which 2-s voltage steps from -35 to -130mV were applied from a holding potential of -35mV, followed by a 1.5-s step at -90mV and a 1-s step at +5mV. To obtain voltage dependent steady-state activation curves, tail currents were determined as the difference between the peak current and the steady-state current (tail current at -90mV). Tail currents were normalized by the maximal tail current (Imax) and plotted against test voltage. The normalized plot of tail currents versus test voltages were fitted with a Boltzmann equation: \( I/Imax = 1/(1+exp[V-V_{1/2}]/k) \), where \( V \) is the test voltage, \( V_{1/2} \) is the mid-activation voltage and \( k \) is the slope factor.\(^{23,23,24}\)

Real time RT-PCR

Total RNA extraction and real-time PCR (qPCR) for the HCN channels were conducted using previously published protocols,\(^{16,25}\) which are detailed in the online supplemental material.

Western blot

Protein samples were extracted from 3 SANs of non-pregnant and pregnant mice. Protocols used for protein isolation and Western blot analysis are described in the online supplemental material.

Statistical analysis

All data are presented as mean ± SEM. “n” refers to the number of experiments and “N” the number of mice. \( p<0.05 \) was considered significantly different. Statistical analyses are detailed in the online supplemental material.
Results

Surface ECG parameters

To determine whether pregnancy in mice is associated with an increase in HR as seen in humans, lead I surface ECG data were obtained from anaesthetized non-pregnant and pregnant female mice under baseline conditions. ECG parameters were compared between both groups and representative examples are illustrated in Figure 1A. Pregnant mice exhibit a significantly higher resting HR compared to non-pregnant mice (NP: 470±27bpm and P: 531±14bpm, p=0.03). HR was also assessed in pregnant mice at gd 12-13 to determine whether the increased HR observed in late pregnancy was present at an earlier stage of gestation. Interestingly, the HR was already increased in pregnant mice at gd 12-13 (514±15bpm, n=9). These findings are consistent with reports in humans which show that the change in resting HR begins in early pregnancy.\textsuperscript{5, 6, 9, 26, 27} Mean data for the PR, QT and QTc intervals are also summarized in Figure 1A. The PR interval was significantly shorter in pregnant mice without any change in P wave duration (data not shown). The QT interval was on average 10ms shorter in pregnant mice; however, after adjustment for the difference in HR, this trend was no longer present as illustrated by the comparable QTc intervals measured in the two groups.

Arterial blood pressure

Using a Millar catheter we compared arterial blood pressure in anaesthetized non-pregnant and pregnant mice to confirm that the increase in HR associated with pregnancy was not secondary to a reduction in blood pressure. Results summarized in Table 1 show that systolic, diastolic and mean arterial blood pressures were similar in both groups.

Stimulation by the autonomic nervous system

Catecholamine levels
The contribution of the autonomic nervous system to the pregnancy-induced increase in HR was then explored. Data presented in Table 1 indicate that plasma concentrations of norepinephrine and epinephrine were not increased in pregnant mice.

**ECG parameters on Langendorff-perfused heart**

Additional ECG recordings were performed on Langendorff-perfused hearts isolated from non-pregnant and pregnant mice. As illustrated in Figure 1B, the HR measured in the Langendorff-perfused heart preparation was significantly slower than that obtained with surface ECGs. The slower HR measured in the Langendorff-perfused heart preparation reflects the intrinsic HR and is explained by the absence of sympathetic stimulation. Similar to what was seen in the surface ECG studies, the HR measured in the Langendorff experiments was significantly faster in pregnant (385±18bpm) compared to non-pregnant (327±16bpm, p=0.02) mice, indicating that the increased HR seen in pregnant mice was not due to alterations in the autonomic tone. The table in Figure 1B presents mean data for PR, QT and QTc intervals obtained from both groups. The PR interval was also significantly shorter in pregnant mice and this faster AV conduction time was still independent of atrial conduction since the P wave duration was unaltered (data not shown). Similar to the surface ECG data, the shorter QT interval in pregnant mice was also explained by a faster HR as there was no difference in QTc intervals between non-pregnant and pregnant mice. Results presented thus far indicate that HR is altered in pregnant mice and that this difference does not result from changes in blood pressure, circulating levels of catecholamines or autonomic nervous tone but rather reflects a change intrinsic to the heart.

**Influence of pregnancy on the spontaneous automaticity of the SAN cells**

Accordingly, the next series of experiments were performed to explore the cellular mechanism underlying the increased HR observed in pregnant mice. Spontaneous APs were recorded using
freshly isolated single SAN cells from non-pregnant and pregnant mice. The morphology of the
SAN cells was similar between both groups and had comparable cellular capacitance (NP:
30.2±1.5pF, n=42 and P: 29.4±1.1pF, n=46, p=0.66). Figure 2A compares spontaneous APs and
the corresponding first derivative of the AP (dV/dt) waveform recorded from SAN cells obtained
from a non-pregnant and pregnant mouse. Detailed analysis of the AP parameters is summarized
in Figure 2B. The frequency of spontaneous SAN AP was increased in cells from pregnant mice
(330±12bpm) compared to non-pregnant mice (292±13bpm, p=0.047). Diastolic depolarization
rate (DDR) was increased in cells from pregnant mice. As also reported in Figure 2B, pregnancy
was also associated with a more positive AP threshold (Eth). On the other hand, the maximum
diastolic potential (MDP), the maximal velocity of the AP upstroke (Vmax), the AP amplitude
(APA), the AP duration (APD) and the waveform of dV/dt during AP repolarization were not
affected by pregnancy.

**Influence of pregnancy on If density and its voltage-dependence of activation**

Considering the importance of If for the initiation of the diastolic depolarization of the SAN we
then compared the density of If in SAN cells from non-pregnant and pregnant mice. Data
reported in Figure 3 (A-B) show that the density of If was significantly higher in the SAN cells
of pregnant mice compared to non-pregnant mice from voltages of -40 to -120mV (at -90mV,
NP: -15.2±1.0, P: -28.6±2.9pA/pF, p=0.0002). Of note, If was recorded in Tyrode’s solution
containing BaCl2 to eliminate the inwardly rectifying K+ current, IK1 (see Methods). It is worth
mentioning that only a very small Ba2+-sensitive K+ current was activated in SAN cells from
non-pregnant mice (at -90 mV, -4.5±0.9pA/pF, n=7). Furthermore, there was no influence of
pregnancy on the density of IK1 (-4.8±1.0pA/pF, n=8, p=0.83) which is consistent with the
absence of differences in the MDP of the spontaneous SAN AP. We then compared the voltage-
dependence of the $I_f$ activation curve and found that neither the voltage at half-maximal activation ($V_{1/2}$) (NP: -102.8±1.5mV and P: -103.6±1.5mV, $p=0.69$) nor the slope factor (NP: 10.2±0.4 and P: 9.7±0.3, $p=0.33$) significantly differed between either group, suggesting that a change in the $I_f$ activation kinetics cannot explain the increased density of $I_f$.

**$I_f$ response to relaxin and SAN cAMP levels are unchanged in pregnancy**

In the next series of experiments, we compared the voltage-dependence of $I_f$ activation in SAN cells from non-pregnant and pregnant mice in the presence of relaxin (80nM), which has been reported to modulate other ionic currents by increasing intracellular cAMP. Data presented in **Figure 4A and B** show that relaxin shifted the activation curve to more depolarized potentials; however, the $I_f$ voltage-dependence of activation and the change ($\Delta V_{1/2} = \text{relaxin} V_{1/2} - \text{baseline} V_{1/2}$) was similar between the two groups (NP: $\Delta V_{1/2} = 2.8±0.6\text{mV}$ and P: $\Delta V_{1/2} = 2.3±0.3\text{mV}$, $p=0.46$). The effect of relaxin on the slope factor was also comparable (NP: $\Delta 1.3±0.3\text{mV}$ and P: $\Delta 1.4±0.1\text{mV}$, $p=0.73$). Thus, the data suggests that the basal cAMP level is not elevated in SAN from pregnant mice. These results are in agreement with the cAMP data reported in **Figure 4C**, where similar intracellular cAMP content was found in SANs isolated from non-pregnant and pregnant mice. Together these findings indicate that the increased $I_f$ density in SAN cells of pregnant mice can not be explained by a positive shift in the $I_f$ voltage-dependence due to higher intracellular cAMP levels.

**The sensitivity of $I_f$ to isoproterenol is blunted during pregnancy**

To determine whether the responses of $I_f$ to $\beta$-adrenergic stimulation was modified by pregnancy, we measured the voltage-dependence of the $I_f$ activation curve in the presence of the $\beta$-adrenergic agonist, isoproterenol (100nM). We found that the maximal isoproterenol-induced shift to more positive voltages was attenuated in pregnancy (NP: $\Delta V_{1/2} = 4.8±0.6\text{mV}$ and P: $\Delta V_{1/2} = 4.8±0.6\text{mV}$).
2.8±0.5mV, p=0.02) (**Figure 5A-D**). Isoproterenol did not affect the slope factor in non-pregnant mice, (Δ0.1±0.5mV, p=0.75) but the slope was slightly increased in SAN cells from pregnant mice (from 9.6±0.6 to 10.9±0.6mV, Δ1.4±0.3mV, p=0.007) although this change did not reach statistical significance compared to the change (Δ0.1±0.5mV) in non-pregnant mice (p=0.06 NP vs P). In addition, another set of surface ECG experiments was performed on non-pregnant and pregnant mice before and after IV injection of isoproterenol (**Figure 5E**). Consistent with a lower If isoproterenol-induced response, data presented in **Figure 5F** indicates that although isoproterenol significantly accelerated the heart rate in both groups, the increase was lower in pregnant (Δ72±15bpm) compared to non-pregnant mice (Δ132±24bpm).

**If** response to muscarinic stimulation is unchanged during pregnancy

We then examined the effect of pregnancy on the voltage-dependence of the If activation curve after the application of acetylcholine (1μM). **Figure 6** shows that the hyperpolarizing shift in If activation caused by acetylcholine was similar in non-pregnant and pregnant mice (NP: ΔV1/2 -1.3±0.4mV, P: ΔV1/2 -1.7±0.3mV, p=0.52). No change in slope was found in the presence of acetylcholine in either group (NP: Δ -0.1±0.3mV, P: Δ -0.1±0.2mV, p=0.89).

**Influence of pregnancy on HCN channel expression**

To further explore the mechanisms for the increased If current density in pregnancy, we measured the mRNA levels of the three HCN channel isoforms expressed in mouse SAN (HCN1, HCN2 and HCN4) using qPCR analysis (**Figure 7A**). Results of these experiments reveal that the transcript levels of the three HCN isoforms did not differ between non-pregnant and pregnant animals. Additionally, we performed Western blot analysis to examine the protein expression of the HCN isoforms and found that although the protein level of HCN4 was not altered, the expression of HCN2 was significantly increased in pregnancy (**Figure 7B**).
together with the electrophysiological data described above, these expression studies indicate that an increase in HCN2 protein expression, with no change in the voltage-dependence of the If activation curve, contributes to the increased If density in pregnancy.

Discussion

Summary of main findings

In the present study, we provide the first mechanistic insight into the increase in HR associated with pregnancy. First, we show that in a mouse model of pregnancy the increased HR is not secondary to alterations in autonomic tone, arterial blood pressure or circulating catecholamine levels. We then demonstrate that the higher HR in pregnancy is associated with accelerated automaticity of SAN cells, steeper diastolic depolarization rate, and enhanced density of If, one of the major ionic currents modulating pacemaker activity of the heart. The increased If density could be explained by higher protein expression of HCN2 channels with no change in the voltage-dependence of the If activation curve. Together, these findings demonstrate that If upregulation is a major mechanism in the increase in HR during pregnancy.

Our mouse model of pregnancy reproduces the increased HR observed during pregnancy in humans. This effect is present even in the absence of autonomic nervous system input, suggesting that the pregnancy-induced increase in HR reflects changes intrinsic to the heart. Similar explanations have also been proposed for the increased HR observed in pregnant women.2,29 In addition, clinical studies have shown that women have a higher resting HR than men,2,30-33 and this difference is preserved after autonomic blockade.34 Interestingly, the faster HR in pregnant mice is accompanied by a shorter PR interval with no change in P wave duration. A shorter PR interval could be a direct consequence of the increased HR. However, since the
SAN and AV node share similar ion channels, it is possible that the AV node also undergoes remodelling mechanisms similar to the SAN. However, further studies will be required to assess the effects of pregnancy on AV node.

Elevated levels of cAMP lead to a positive shift in the If activation curve and hence increase its current density. However, this study rules against a cAMP-dependent regulation of If as a potential explanation for the enhanced If density in pregnancy. Indeed, there was no change in the voltage-dependence of the If activation curve or cAMP levels in SAN cells from pregnant mice. Results obtained with relaxin also support this conclusion. As mentioned above, relaxin has been shown to rapidly elevate cAMP content in various tissues.\textsuperscript{28, 35, 36} Therefore, if SAN cells from pregnant mice had a higher basal cAMP level, addition of relaxin should only minimally increase cAMP content. This would result in a much smaller shift in the If activation curve. However, the similar response of If to relaxin obtained in both groups does not support this hypothesis. Thus, it appears that mechanisms independent of cAMP participate in the upregulation of If in the SAN during pregnancy.

In the present study, the higher density of If is associated with an increased protein expression of HCN2 channel isoform. Previous studies have shown that HCN4 is the most highly expressed HCN isoform in mouse SAN whereas HCN2 and HCN1 are detected at moderate and low levels, respectively.\textsuperscript{37} HCN4 is considered to be the main component of If in murine SAN; however, evidence also supports a functional role, although to a lesser extent, for HCN2.\textsuperscript{37} Findings reported here show that HCN2 protein level is increased in SAN from pregnant mice suggesting that the contribution of this channel isoform to mouse SAN If would be enhanced during pregnancy. In support of this notion, the maximal isoproterenol-induced shift in the If activation curve was attenuated in pregnancy. This observation agrees with an increased
expression of HCN2 since this channel isoform is known to have a lower cAMP sensitivity than HCN4.\textsuperscript{37} Considering that HCN2 channels have faster activation kinetics compared to HCN4, it is tempting to speculate that an increase in HCN2 channels is essential in order to support the elevated HR needed to compensate for the greater physiological demand associated with pregnancy. Indeed, exponential fits of the $I_f$ activation trace at -60 mV reveal that the activation time constant was significantly reduced in pregnant mice (NP: 883±120 ms and P: 596±73 ms $p=0.04$). This is consistent with an increase in the HCN2 mole fraction in HCN heteromultimers of pregnant mice. As a result, a more rapid activation of the current near the diastolic potentials is observed. It is also worthwhile mentioning that the frequency of cells where $I_f$ was not activated at -60 mV was much higher in the non-pregnant mice. Although we were able to detect HCN1 transcripts, this isoform was not detectable at the protein level in SAN isolated from either experimental groups. This could be due to low sensitivity of the antibody used or reflects a low expression level of HCN1 in the mouse SAN as previously reported,\textsuperscript{37} suggesting that this isoform may not make an important contribution to $I_f$ in mouse SAN.

Besides the increased HCN2 expression, other factors could also contribute to the increase in $I_f$ density. In fact, various factors are known to modulate $I_f$ in SAN cells, such as auxiliary subunits and cytoskeletal proteins, which functionally interact with HCN channels.\textsuperscript{15, 38, 39} Interestingly, the expression of HCN1 and HCN2 was upregulated when these isoforms were co-expressed with MinK-related protein 1 (MiRP1).\textsuperscript{38} Therefore, future studies are required to explore the contribution of these factors on the upregulation of $I_f$ in pregnancy.

It is plausible that the increase in HR itself may lead to an upregulation of $I_f$. Consistent with this idea, previous studies have shown that in response to HR acceleration, other cardiac ionic currents, such as L-type calcium currents ($I_{CaL}$) and ATP-sensitive K$^+$ current ($I_{KATP}$), are
also upregulated.\textsuperscript{40, 41} For instance, in human cardiac myocytes it has been reported that the density of I_{CaL} can be increased by high rates of cell stimulation. This increase was attributed to an incomplete inactivation of I_{CaL} at rapid pacing frequencies, which may also involve phosphorylation of Ca\textsuperscript{2+} channels. This process was thought to be crucial in the adaptation of the beating heart to stress and exercise.\textsuperscript{40} Pregnancy is also associated with accelerated HR as part of the adaptive response to the increased physiological requirements. Therefore, rate-dependent upregulation of I_f may also be mediated by similar mechanisms where an initial increase in the frequency of activation of I_{CaL}, as well as changes in I_{CaL} gating kinetics, (e.g., slowing of I_{CaL} decay) increases Ca\textsuperscript{2+} influx. The elevated intracellular Ca\textsuperscript{2+} levels could then trigger intracellular events upregulating I_f. In support of this hypothesis, a report in rat hippocampal CA1 cells showed that a Ca\textsuperscript{2+}-dependent modulation of the hyperpolarization-activated current (I_h, the neuronal equivalent of I_f) controls the synchronized thalamocortical rhythms.\textsuperscript{42} Thus, a rise in intracellular Ca\textsuperscript{2+} may promote the I_f-dependent pacemaker mechanisms and in addition modulate the Ca\textsuperscript{2+}-dependent pacemaker process referred to as the “Ca\textsuperscript{2+} clock,” in which the rhythmic SR Ca\textsuperscript{2+} release plays a major role in the SAN automaticity. These functional interactions between the two pacemaker mechanisms may provide additional means to accelerate HR as part of the adaptation to pregnancy.

Hormonal changes that occur during pregnancy are likely to be involved in the increased automaticity of the heart during pregnancy.\textsuperscript{28, 43, 44} It is also noteworthy that sex-specific hormonal fluctuations increase the inducibility of arrhythmias, which has been correlated with gestation, menopause, and even changes in the menstrual cycles.\textsuperscript{26, 45, 46} In other cell types, pregnancy or sex steroid hormones have been shown to regulate hyperpolarization-activated inward currents. For instance, during pregnancy, uterine smooth muscle cells also exhibit a
hyperpolarization-activated inward current similar to $I_f$ in SAN cells. This current is believed to
contribute to the spontaneous automaticity and enhanced myometrial contractility that occurs
near parturition.\textsuperscript{47} In the brain, $I_h$ also plays a critical role in many rhythmic neurons.
Specifically, 17β-estradiol regulates the density of $I_h$ in GnRH neurons. In these cells, rising
levels of 17β-estradiol also increase mRNA expression of multiple ion channels, such as HCN,
T- and L-type Ca\textsuperscript{2+} channels, which increase the overall excitability of GnRH neurons.\textsuperscript{48}
Additionally, ovariectomy was shown to reduce the number of action potentials in hippocampal
CA1 pyramidal neurons, supporting a role for female hormones on neuronal automaticity.\textsuperscript{49}

Taken together, these data support the notion that hormonal changes occurring during pregnancy
can contribute to the higher HCN2 channel expression and increased $I_f$ density.

Upregulation of $I_f$ is most likely not the only change associated with enhanced
automaticity of the heart during pregnancy. In fact, regulation of other ionic currents by
pregnancy-related hormonal changes has been previously reported.\textsuperscript{28} It is therefore plausible that
other mechanisms involved in the diastolic depolarization could also contribute to the steeper
diastolic depolarization. Furthermore, the fact that the AP threshold was more positive in
pregnant mice also suggests that additional mechanisms are involved in the AP remodelling of
the SAN during pregnancy. Conceivably, alterations in ionic mechanisms such as Ca\textsuperscript{2+} currents,
Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger and spontaneous SR Ca\textsuperscript{2+} release could also contribute to these changes.

Interestingly, Elzwiei F. \emph{et al} recently reported that the α1 isoform of the Na\textsuperscript{+}-K\textsuperscript{+} pump
expression and flux are reduced in pregnant rats. The consequences were an accumulation of Na\textsuperscript{+}
which drove the Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger into reverse mode, thereby increasing intracellular Ca\textsuperscript{2+}.\textsuperscript{50}
Thus, it is possible that a similar scenario occurs in the SAN where increases in intracellular Ca\textsuperscript{2+}
cycling would act in concert with an enhanced $I_f$ density in order to sustain the elevated HR.
during pregnancy. Future studies will be required to explore the contribution of these ionic and Ca$^{2+}$ mechanisms on the increased HR in pregnancy.

**Conclusion**

In conclusion, our data provide evidence that the accelerated heart rate in pregnant mice is associated with higher HCN2 channel protein expression leading to increased If density and automaticity of SAN. Our results demonstrate novel and functionally important ionic mechanisms, which contribute to the well-known pregnancy-induced increase in HR. Additional work will be directed toward examining how these changes in If may predispose the heart to initiation of arrhythmias during pregnancy.

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**Conflict of Interest Disclosures:** None.

**References:**


Table 1. Arterial Blood Pressure and Catecholamine Levels in Non-Pregnant and Pregnant Mice.

<table>
<thead>
<tr>
<th></th>
<th>Non-Pregnant</th>
<th>Pregnant</th>
<th>p-value</th>
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<tr>
<td><strong>Arterial Blood Pressure</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Systolic pressure (mm Hg)</td>
<td>99.5 ± 1.4 (8)</td>
<td>100.8 ± 3.1 (7)</td>
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<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>70.0 ± 1.8 (8)</td>
<td>70.3 ± 3.0 (7)</td>
<td>0.95</td>
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<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>79.0 ± 1.5 (8)</td>
<td>79.7 ± 3.0 (7)</td>
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<tr>
<td><strong>Plasma Catecholamines</strong></td>
<td></td>
<td></td>
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<tr>
<td>Epinephrine level (ng/ml)</td>
<td>2.0 ± 0.5 (6)</td>
<td>2.8 ± 0.8 (5)</td>
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<td>Norepinephrine level (ng/ml)</td>
<td>9.8 ± 1.0 (6)</td>
<td>10.8 ± 1.3 (7)</td>
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Numbers in parenthesis represent numbers of mice.
Figure Legends:

Figure 1. Pregnancy is associated with increased HR and shortened PR interval in surface ECG and Langendorff-perfused heart experiments. (A) Typical examples of surface ECG traces from anaesthetized NP and P mice. Table comparing mean data for HR, PR, QT and QTc intervals in NP and P mice show that HR is significantly increased and PR interval shortened in P mice (NP; N=10 and P; N=15). (B) Representative epicardial ECG recordings from Langendorff-perfused hearts in NP and P mice. Table comparing mean ECG parameters reveals a faster HR and a shorter PR interval in P mice (NP; N=10 and P; N=11).

Figure 2. Pacemaker activity of isolated SAN cells is increased in pregnant mice. (A) Representative spontaneous APs (top) and their first derivative (bottom) recorded using perforated patch-clamp configuration in SAN cells isolated from NP (left) and P (right) mice. Arrows indicate diastolic depolarization while the hatched line indicates 0 mV in this and subsequent figures. (B) Measurements of the intrinsic cycle length (CL), diastolic depolarization rate (DDR), AP threshold (Eth), maximum diastolic potential (MDP), maximal velocity of AP upstroke (V_{max}), AP amplitude (APA) and AP duration (APD) of SAN cell APs were made as shown by the dotted lines and arrows on the AP (left). Summary table of AP parameters from NP (n=17, N=9) and P mice (n=12, N=8).

Figure 3. Electrophysiological properties of I_f in SAN cells of non-pregnant and pregnant mice. (A) Typical examples of I_f current recorded in whole-cell configuration in SAN cells isolated from NP and P mice. Insets depict recording protocols. (B) Mean data for the current-voltage (I-V)
V) relationship for $I_f$ in SAN cells of NP (n=26, N=18) and P (n=26, N=17) mice (*p<0.05). **(C)** Typical examples of $I_f$ current recorded in perforated patch configuration using a tail current protocol (*shown in inset*) in SAN cells isolated from NP and P mice. **(D)** Mean data for the steady-state $I_f$ activation curves of SAN cells in NP (n=18, N=12) and P (n=17, N=8) mice. Tail currents were determined by subtracting the peak current from the steady-state current (tail current at -90mV), normalized and plotted against test voltage. The relationships were fitted with the Boltzmann equation.

**Figure 4.** $I_f$ response to relaxin is similar in pregnant and non-pregnant mice. (A) Typical examples of $I_f$ current traces from NP mice (left) and corresponding steady-state $I_f$ activation curves (right) before and after application of relaxin (RLX, 80nM). (B) Typical examples of $I_f$ current traces from P mice (left) and corresponding steady-state $I_f$ activation curves (right). Similar results were obtained in n=5 SAN cells from N=3 mice for each group. (C) Bar graph summarizing cAMP assay results on SAN derived from NP and P mice (N=10 in both groups, p=0.07).

**Figure 5.** Effect of isoproterenol on $I_f$ is not affected by pregnancy. (A) Representative examples of $I_f$ recorded in SAN cells of a NP mouse in the absence or presence of isoproterenol (ISO, 100nM). (B) Corresponding $I_f$ activation curve. (C) Representative examples of $I_f$ recorded in SAN cells of a P mouse in absence or presence of isoproterenol (ISO, 100nM). (D) Corresponding $I_f$ activation curve. Similar results were obtained from n=5, N=4 in NP and n=6, N=3 in P groups. (E) Typical surface ECG recordings from anaesthetized NP (top) and P mice (bottom) in presence of ISO. (F) Bar graphs presenting the mean data for HR obtained on surface
ECGs from anaesthetized NP and P mice before and after ISO (NP: ΔHR +132±24, N=6 and P: ΔHR +72±15, N=7).

Figure 6. Response of $I_f$ to acetylcholine is similar in SAN cells of pregnant and non-pregnant mice. (A) Representative examples of $I_f$ recorded in SAN cells of a NP mouse in absence or presence of acetylcholine (ACH, 1μM). (B) Corresponding $I_f$ activation curve. Similar results were obtained in n=8 SAN cells from N=4 mice. (C) Representative examples of $I_f$ recorded in SAN cells of a P mouse in absence or presence of acetylcholine (ACH, 1μM). (D) Corresponding $I_f$ activation curve. Similar results were obtained in n=6 SAN cells from N=2 mice.

Figure 7. qPCR and Western blot analysis of HCN channel isoforms from pregnant and non-pregnant mouse SAN. (A) Mean qPCR data showing relative mRNA expression of HCN1, HCN2 and HCN4 in SAN of NP and P mice. Each bar graph represents mean data of 6 samples, each analyzed in triplicate. 10-12 SAN were pooled per sample (HCN1, $p=0.88$, HCN2, $p=0.55$, HCN4, $p=0.14$). (B) Western blot analysis for HCN2 and HCN4 on SAN from P and NP mice (top) and bar graph summarizing mean data for relative protein expression (bottom) (n=3 for both groups, 3 SANs were pooled per n, $*p=0.04$).
Figure 1

A Surface ECG

<table>
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<tr>
<th></th>
<th>NP</th>
<th>P</th>
<th>HR (bpm)</th>
<th>PR (ms)</th>
<th>QT (ms)</th>
<th>QTc (ms)</th>
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<tr>
<td>NP</td>
<td></td>
<td></td>
<td>470 ± 27</td>
<td>43.5 ± 1.4</td>
<td>56.5 ± 6.7</td>
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<tr>
<td>P</td>
<td>531 ± 14*</td>
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<td>34.8 ± 0.7*</td>
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<td>p-value</td>
<td>0.03</td>
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<td>0.14</td>
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B Langendorff-Perfused Heart

<table>
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<tr>
<th></th>
<th>NP</th>
<th>P</th>
<th>HR (bpm)</th>
<th>PR (ms)</th>
<th>QT (ms)</th>
<th>QTc (ms)</th>
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<tr>
<td>NP</td>
<td></td>
<td></td>
<td>327 ± 16</td>
<td>44.3 ± 1.8</td>
<td>98.2 ± 6.7</td>
<td>71.5 ± 4.5</td>
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<tr>
<td>P</td>
<td>385 ± 18*</td>
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<td>38.3 ± 2.3*</td>
<td>80.9 ± 7.6</td>
<td>62.4 ± 4.3</td>
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<tr>
<td>p-value</td>
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<td>0.052</td>
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Figure 2

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<tr>
<td>Pacing rate</td>
<td>292 ± 13</td>
<td>330 ± 12*</td>
<td>0.047</td>
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<td>DDR (V/s)</td>
<td>0.20 ± 0.03</td>
<td>0.40 ± 0.06*</td>
<td>0.004</td>
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<td>Eth (mV)</td>
<td>-45.8 ± 1.3</td>
<td>-41.5 ± 1.4*</td>
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<td>MDP (mV)</td>
<td>-58.7 ± 1.2</td>
<td>-56.8 ± 1.6</td>
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<td>Vmax (V/s)</td>
<td>44.3 ± 5.2</td>
<td>58.8 ± 7.0</td>
<td>0.10</td>
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<td>APA (mV)</td>
<td>97 ± 5</td>
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<td>ADP (ms)</td>
<td>119 ± 4</td>
<td>110 ± 3</td>
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Figure 3
Figure 4
Figure 6
Upregulation of the Hyperpolarization Activated Current Increases Pacemaker Activity of the Sino-Atrial Node and Heart Rate during Pregnancy in Mice
Nabil El Khoury, Sophie Mathieu, Laurine Marger, Jenna Ross, Gracia El Gebeily, Nathalie Ethier and Céline Fiset

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SUPPLEMENTAL MATERIAL

Upregulation of the hyperpolarization activated current increases pacemaker activity of the sinoatrial node and heart rate during pregnancy in mice

Corresponding author: C. Fiset

SUPPLEMENTAL METHODS

Surface electrocardiograms (ECG)

Mice were anaesthetized with isoflurane. Body temperature was maintained at 37°C using a heating pad. Platinum electrodes positioned subcutaneously were connected to a Biopac System MP100 (EMKA Technologies, France). Surface ECGs were recorded in lead I configuration at a rate of 2 kHz and performed for 5 minutes of continuous experimental recording at the end of a baseline period. The signal was filtered at 100Hz (low pass) and 60kHz (notch filter). Data were analyzed using ECG-auto 1.7 (EMKA Technologies). RR, PR, and QT intervals were calculated manually by a blinded observer from signal averaged ECG recordings (500-1000 cardiac cycles). QT intervals were corrected (QTc) for HR using the correction formula for mice (QTc=QT/(RR/100)^{1/2}). Additional surface ECG recordings were obtained on anaesthetized mice before and after intravenous (IV) injection of isoproterenol (0.1ng/g) through the right jugular vein. After the maximal isoproterenol effect occurred (within 1-2 minutes), ECG parameters were measured as described above.

Langendorff-perfused heart

Experiments were performed as previously described. Mice were heparinized (1 U/kg, IP) 20 minutes prior to sacrifice. They were then anaesthetized with isoflurane and sacrificed by cervical dislocation. The heart was rapidly excised and hung on a modified Langendorff
apparatus and retrogradely perfused through the aorta at a constant perfusion pressure of 75 mmHg with a Krebs-Henseleit buffer at 37°C and constantly gassed with 95% O\textsubscript{2}/ 5% CO\textsubscript{2} containing (mM) 11.2 glucose, 118.5 NaCl, 25 NaHCO\textsubscript{3}, 4.7 KCl, 2.45 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 1.2 CaCl\textsubscript{2}. Epicardial ECG recordings were obtained by placing one silver electrode at the base of the heart under the right atrium and another in the apex of the left ventricle. After an equilibration perfusion period, epicardial ECG measurements were recorded and ECG parameters were calculated as above.

**Sino-atrial cell isolation**

Mice were anaesthetized by inhalation of isoflurane and then sacrificed by cervical dislocation. The SAN cells were isolated using an adaptation of the protocol previously described by Mangoni et al.\textsuperscript{4} Briefly, SANs were dissected in normal HEPES-buffered Tyrode’s solution containing (mM): 140 NaCl, 5.4 KCl, 1.8 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 5 HEPES and 5.5 glucose (pH=7.4 with NaOH). SAN tissue was cut into small pieces and rinsed twice for 4 minutes in a Ca\textsuperscript{2+}-free Tyrode’s solution containing (mM) 0.5 MgCl\textsubscript{2}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, 50 taurine and 0.1% bovine serum albumin (BSA Fraction V, Sigma, St. Louis, MO, USA) (pH=6.9 with NaOH). The tissue strips were then digested at 36±1°C for 20-30 minutes with the same solution supplemented with collagenase type IV (229U/ml, Worthington, Lakewood, NJ, USA), elastase (1.9U/ml, Worthington), protease type XIV (0.9U/ml, Sigma) and 200µM CaCl\textsubscript{2}. The SAN tissue was then washed three times and transferred into “Kraft-Brühe” (KB) solution (mM): 100 K\textsuperscript{+}-glutamate, 10 K\textsuperscript{+}-Aspartate, 25 KCl, 10 KH\textsubscript{2}PO\textsubscript{4}, 2 MgSO\textsubscript{4}, 20 taurine, 5 creatine, 0.5 EGTA, 5 HEPES, 0.1% BSA, 20 glucose (pH=7.2 with KOH). The tissue was triturated gently with a Pasteur pipette until single SAN cells were obtained, usually within 2-3 minutes. Physiological extracellular Ca\textsuperscript{2+} concentration
was gradually restored. Isolated mouse SAN cells were identified as small spindle-type cells spontaneously beating in normal Tyrode’s solution.4

**Electrophysiological data**

*Current- and voltage-clamp recordings.* An aliquot of SAN cell solution was placed in a recording chamber (volume 200µl) mounted on the modified stage of an inverted microscope and superfused with a normal Tyrode’s solution containing (mM): 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES and 5.5 glucose (pH=7.4 with NaOH). For Iᵥ recordings, 1 mM BaCl₂ (Sigma) was added to the external solution to block the inward rectifier K⁺ current, Iᵥ₁. Bath was perfused with continuously gassed (100% O₂) at 35±1°C. Current- and voltage-clamp recordings were obtained using the Axopatch 200B patch-clamp amplifier (Molecular Devices, Sunnyvale, CA, USA). Pipettes were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL, USA), and had resistance of 3-6 MΩ when filled with the following solution (mM): 100 K⁺-aspartate, 30 KCl, 10 NaCl, 2 MgATP, 6.6 Na₂phosphocreatine, 0.1 Na₂GTP, 0.04 CaCl₂, 1 MgCl₂ and 5 HEPES (pH=7.2 with KOH). Voltage-clamp recordings were low-pass filtered at 10kHz (4-pole Bessel), digitized and analyzed using pCLAMP 10.2 software (Molecular Devices). Capacitive transients were elicited by a 10-ms voltage step of ±10 mV from a holding potential (HP) of -30mV. Cell capacitances were measured by integrating the area of the capacitive transients. Junction potential was not corrected.

**Real time RT-PCR**

Total RNA extraction and real-time PCR (qPCR) for the HCN channels were conducted using previously published protocols.5, 6 For each sample, 10-12 SANs were pooled and used to isolate total RNA from NP and P mice. Total RNA was isolated using TriReagent® and treated with DNase I as previously described.5 cDNA was then synthesized with SuperScript™ III Reverse
Transcriptase (Life Technologies). mRNA was transcribed using pdN6 random primers. Gene specific primers and conditions for PCR reactions were used for HCN1 and HCN4 as previously described. The following primers specific to HCN2 were designed (forward: GCTGGGTGTCCATCAACAAC, reverse: AGATGTCTGTCATGCTCTCG) and the PCR reactions were cycled using a 3-step procedure (denaturation at 95°C for 30s, annealing at 55°C for 60s, elongation at 72°C for 60s). The qPCR was performed with Platinum SYBR Green qPCR Supermix (Life Technologies) using a real-time PCR system (MX3005P QPCR system, Stratagene). Quantitative measurements were performed in triplicate and normalized to the average of three housekeeping genes (18S, lamin C, and succinate dehydrogenase complex subunit A (SDHA)).

**Western blot**

The protein samples were extracted from SAN tissues of non-pregnant and pregnant mice for Western blot analysis. For each sample, 3 SANs were pooled for protein isolation. SANs were pulverized in liquid nitrogen and were then resuspended in ice cold extraction buffer containing 1% Triton-X100 and a cocktail of protease and phosphates inhibitors and agitated 1 hour at 4°C. Samples were subsequently centrifuged at 12,000g and the supernatant containing the proteins was recovered, quantified using the Bradford assay and stored at -80°C for later use. Proteins (25 μg) were loaded and separated on 10% SDS-PAGE, blotted on a PVDF membrane (Millipore) and probed with anti-rabbit HCN2 and HCN4 specific antibody (1:1000) (Alomone Labs, Israel). After secondary antibody incubation, chemiluminescence analysis using an ECL system (PerkinElmer) was performed. All bands were normalized to GAPDH (Fitzgerald, Acton, MA, USA).
Drugs

Recombinant Human Relaxin-2 (H2 Relaxin; B-29/A24) was purchased from R&D Systems, Minneapolis, MN USA. All other compounds were obtained from Sigma. Isoproterenol and acetylcholine were prepared in water while relaxin was prepared in sterile PBS. Stock solutions of all drugs were then diluted in Tyrode’s solution to achieve their respective final concentration. Isoproterenol was protected from degradation by the addition of ascorbic acid (100µM) to the final solution.

Statistical analysis

All data are presented as mean ± SEM. “n” refers to the number of experiments and “N” the number of mice. Descriptive statistics were used to compare ECG parameters. A Mann-Whitney Test was used to either compare data between NP and P mice obtained with ECG recording methods under control conditions and after treatment with isoproterenol. A Wilcoxon Signed Ranks test was used to compare HR in the same group before and after isoproterenol (Fig. 1,5F). Unpaired t-tests were used to compare blood pressure, catecholamine and cAMP levels, cellular electrophysiology, mRNA and protein data between NP and P mice (Table 1, Fig. 2-4 and 7). Electrophysiological data in NP and P mice obtained before and after pharmacological manipulations were compared using two-way ANOVA for repeated measures, no adjustment was used for multiple comparisons (Fig. 3-6). Non parametric analyses were conducted using SAS9.2. T-tests and ANOVA were performed with Origin 8.0 (OriginLab, MA, USA). p<0.05 was considered significantly different.


