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Heart rate reduction after genetic ablation of L-type $Ca_v1.3$ channels induces cardioprotection against ischemia-reperfusion injury

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Background: Acute myocardial infarction (AMI) is the major cause of cardiovascular mortality worldwide. Most ischemic episodes are triggered by an increase in heart rate, which induces an imbalance between myocardial oxygen delivery and consumption. Developing drugs that selectively reduce heart rate by inhibiting ion channels involved in heart rate control could provide more clinical benefits. The $Ca_v1.3$ -mediated L-type Ca^{2+} current ($I_{Ca_v1.3}$) play important roles in the generation of heart rate. Therefore, they can constitute relevant targets for selective control of heart rate and cardioprotection during AMI.

Objective: We aimed to investigate the relationship between heart rate and infarct size using mouse strains knockout for $Ca_v1.3$ ($Ca_v1.3^{-/-}$) L-type calcium channel and of the cardiac G protein gated potassium channel ($Girk4^{-/-}$) in association with the funny (f)-channel inhibitor ivabradine.

Methods: Wild-type (WT), $Ca_v1.3^{+/-}$, $Ca_v1.3^{-/-}$ and $Girk4^{-/-}$ mice were used as models of respectively normal heart rate, moderate heart rate reduction, bradycardia, and mild tachycardia, respectively. Mice underwent a surgical protocol of myocardial IR (40 min ischemia and 60 min reperfusion). Heart rate was recorded by one-lead surface ECG recording, and infarct size measured by triphenyl tetrazolium chloride staining. In addition, $Ca_v1.3^{-/-}$ and WT hearts perfused on a Langendorff system were subjected to the same ischemia-reperfusion protocol *ex vivo*, without or with atrial pacing, and the coronary flow was recorded.

Results: $Ca_v1.3^{-/-}$ mice presented reduced infarct size (−29%), while $Girk4^{-/-}$ displayed increased infarct size (+30%) compared to WT mice. Consistently, heart rate reduction in $Ca_v1.3^{+/-}$ or by the f-channel blocker ivabradine was associated with significant decrease in infarct size (−27% and −32%, respectively) in comparison to WT mice.

Conclusion: Our results show that decreasing heart rate allows to protect the myocardium against IR injury *in vivo* and reveal a close relationship between basal heart rate and IR injury. In addition, this study suggests that targeting $Ca_v1.3$ channels could constitute a relevant target for reducing infarct size, since maximal heart rate dependent cardioprotective effect is already observed in $Ca_v1.3^{+/-}$ mice.

KEYWORDS

heart rate, myocardial infarction, cardioprotection, ischemia-reperfusion injury, heart rate reduction, $cav1.3$ calcium channel, genetic model

1. Introduction

Cardiovascular diseases are the leading cause of morbidity and mortality worldwide in 2023. Among them, Acute Myocardial Infarction (AMI) is the number one killer and is responsible for a substantial fraction of fatalities of cardiovascular origin (1). Reperfusion is the only treatment available to reduce infarct size (2–4). There is an association between infarct size and the rate of mortality (5), making early reperfusion a critical therapeutic factor (“time is muscle”) (2). However, reperfusion therapy also induces detrimental effects referred to as lethal reperfusion injury (6–8). Reperfusion injury specifically induces cardiac cell death and significantly contributes to final infarct size as a second component of tissue damage besides ischemic damage (6, 7). To date, no pharmacologic or genetic therapy targeting ischemia-reperfusion (IR) injury and limiting the extent of the infarcted area is available (9). Clinical studies revealed that several factors, including aging and diabetes, are prone to change the integrity of survival pathways, impairing the effectiveness of recently proposed cardioprotective strategies (10, 11). Heart rate is another important determinant of cardiac pathology and mortality (2, 12, 13). Most ischemic episodes are triggered by an increase in heart rate that induces an imbalance between myocardial oxygen delivery and consumption (14). During cardiac ischemia, heart rate reduction prolongs the ventricular diastole augmenting the coronary perfusion, which improves oxygenation of the myocardium at rest and during exercise (15, 16). Development of new therapeutic strategies to reduce IR injury would constitute an important step towards reducing mortality and morbidity following AMI (17). In this context, appropriate heart rate reduction may limit the vulnerability of the myocardium during IR episodes (14).

Heart rate is generated in the sinoatrial node (SAN) by ion channels of the plasma membrane and ryanodine receptor-mediated intracellular Ca^{2+} release (18). Among ion channels, hyperpolarization-activated f-(HCN) channels underlying the “funny” current (I_f) (19, 20), L-type $Ca_v1.3$ (21) underlying L-type $I_{Cav1.3}$ Ca^{2+} current, are important determinants of heart rate (18, 22). Indeed, mice in which $Ca_v1.3$ channels have been genetically ablated display slow SAN rhythm and ventricular bradycardia (21, 23). Besides ion channels potentiating SAN pacemaking, G-protein gated (GIRK4) K^+ channels underlying cardiac acetylcholine-activated K^+ current (I_{KACH}) constitute an

important mechanism to slow heart rate upon parasympathetic activity (24, 25). Mice lacking GIRK4 channels ($Girk4^{-/-}$) display slight basal tachycardia and blunt cholinergic regulation of SAN pacemaking (25). The f-channel blocker ivabradine, which reduces heart rate was reported to decrease infarct size during an IR protocol through heart rate-dependent and independent mechanisms (26, 27). We hypothesized that, because of their implication in controlling heart automaticity, $Ca_v1.3$ channels, beside f-channels, are potential pharmacologic targets for heart rate regulation during cardiac ischemia to limit reperfusion injury. We thus investigated the impact of heart rate modulation by inactivation of SAN $Ca_v1.3$ or GIRK4 channels on IR injury in mice. Then, ivabradine was used as a heart rate reducing agent during IR injury and its effect compared to those mediated by genetic inactivation of $Ca_v1.3$ or GIRK4.

2. Materials and methods

2.1. Animals

Wild-type (WT, Charles River laboratory) and transgenic mice harboring $Ca_v1.3^{-/-}$, $Ca_v1.3^{+/-}$, or $Girk4^{-/-}$ genotype on C57BL/6J genetic background were used in this study. Experiments were performed in accordance with the European Communities Council directive of November 1986 and conformed to the Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication 8th Edition, 2011). All mice were maintained under environmentally controlled conditions ($22 \pm 2^\circ\text{C}$, 12 h light/12 h dark cycle) in the animal facility of the Institute. The surgical protocols were approved by the “Comité d’Ethique pour l’Expérimentation Animale Languedoc-Roussillon” (CEEA-LR) with the authorization number CE-LR-0814. For *ex vivo* experiments, the protocol “Study of physiological parameters and cardioprotection *ex vivo*” was approved by the SBEA (Structure Bien-être Animal) committee from the Institute according to the European directive 2010/63/EU and the French ministerial regulation of February 01, 2013. The “*n*” number of mice included in the study for each experiment is the “*n*” authorized by the ethics committee as being the minimal “*n*” number allowing a valid statistical analysis while respecting the 3R rule (Replacement, Reduction, Refinement) established in Europe.

2.2. *In vivo* surgical model of myocardial ischemia and reperfusion

Acute myocardial ischemia and reperfusion were induced in $Ca_v1.3^{-/-}$, $Ca_v1.3^{+/-}$ and $Girk4^{-/-}$, as well as in WT controls as previously described (28). Adult male mice (22–32 g) were anesthetized with an intramuscular injection of ketamine (25 mg/kg; Imalgène 500, Merial, France), xylazine (10 mg/kg; Rompum 2%, Bayer, France), and chlorpromazine (1.25 mg/kg; Largactil 5 mg/ml, Sanofi-Aventis, France). Mice were ventilated via tracheal intubation with a Harvard rodent respirator (tidal volume, 7.2 μ l/g body mass; respiratory rate, 200 breaths per min). Body temperature was maintained between 36.8°C and 37°C via a thermoregulated surgical table connected to a rectal probe. One-lead surface electrocardiograms (ECG) were recorded by Ag/AgCl gel-coated electrodes (Unomedical) attached to the superior right and both inferior limbs of mice and connected to a standard one-lead ECG amplifier module (EMKA Technologies, Paris, France). The recordings were carried out from 10 min before ischemia (baseline) until the end of the experience. Mice were randomly enrolled in the control group [NaCl 0.9%, intraperitoneal (IP) injection] or ivabradine-injected group (6 mg/kg, IP, 30 min before ischemia). Then, after a second injection of ketamine (25 mg/kg) and xylazine (10 mg/kg), the chest was opened by a left lateral thoracotomy, and a reversible coronary artery snare occluder was placed around the left coronary artery. All animals underwent 40 min of ischemia followed by 60 min of reperfusion by loosening of the knot. At the beginning of reperfusion, a third administration of ketamine/xylazine was performed (same dose). At the end of reperfusion, the artery was reoccluded, phthalocyanine blue was injected into the left ventricular (LV) cavity and allowed to perfuse the non-ischemic portions of the myocardium, and the heart was removed for infarct size (IS) analysis.

2.3. *Ex vivo* model of regional ischemia-reperfusion

Adult male mice were anesthetized with an intraperitoneal dose of ketamine (100 mg/kg) and xylazine (10 mg/kg), followed by sodium pentobarbital (45 mg/kg; Ceva Santé Animale, France). An IP injection of heparin (7,500 IU/kg; Sanofi Aventis, France) was administered to avoid thrombus formation. A thoracotomy was performed and beating hearts were quickly removed and excised into ice-cold perfusion fluid. The aorta was cannulated under magnification and hearts were quickly mounted and perfused by the Langendorff method (29) on an isolated heart system apparatus (Isolated heart system, EMKA Technologies, France). The coronary circulation was perfused at 70 mmHg over a 1–4 ml/min flow range with modified Krebs-Henseleit buffer containing (mM): NaCl 116; KCl 5; MgSO₄ 1.1; NaH₂P0₄ 0.35; NaHCO₃ 27; CaCl₂ 1.8 and glucose 10. The buffer was gassed with 95% O₂ and 5% CO₂, giving a pH of 7.4 at 37°C. Before use, the buffer was initially passed through a 0.2 μ m filter

(Sarstedt, USA), and all perfusate delivered to hearts was filtered via an in-line 0.45 μ m filter for removing microparticulates.

During the stabilization phase, a 7.0 prolene ligature (Johnson & Johnson Ethicon, Belgium) was placed around the left coronary artery 1–2 mm distal from where it emerges beneath the left atrium. The ends of the ligature were passed through an occluder tube, initially without tension. ECG was continuously recorded by Ag/AgCl electrodes placed on the right atrium and near the apex. Heart temperature was continuously assessed by a probe positioned on the base of the heart and recorded by using a TH-5 monitoring thermometer (Phymep, USA). Perfused hearts were then immersed in a water-jacketed bath and continuously maintained at 37°C. After a stabilization phase of 15 min (baseline), regional normothermic ischemia was then induced by tightening the suture with the occluder tube for 40 min. After ischemia, reperfusion was allowed by loosening the ligature and perfusing the heart for 60 min. For atrial pacing, a bipolar stimulating electrode was placed on the right atrium and pacing was started during the stabilization phase.

At the end of reperfusion, the artery was reoccluded, the hearts were removed from the perfusion system and phthalocyanine blue was injected through the aortic cannula to perfuse the non-ischemic portions of the myocardium, allowing the area at risk to be delineated.

2.4. Recording of coronary blood flow

During the whole *ex vivo* IR protocol, the coronary flow from the isolated heart system apparatus was continuously digitized at 0.1 Hz and recorded using an IOX data acquisition system (EMKA Technologies, France). Excel software (Microsoft, USA) was used to perform offline analysis of the data recorded. For each mouse, a mean value of maximal coronary flow (MaxF) was calculated every 10 min. Values during ischemia and reperfusion were normalized with respect to the pre-ischemia phase (baseline) and were expressed in percentages.

2.5. ECG recordings and analysis

For both *in vivo* and *ex vivo* models, signals were digitized continuously at 1 kHz and recorded using an IOX data acquisition system (EMKA Technologies, France). EcgAuto software (EMKA Technologies, France) was used to perform offline analysis of the data recorded. For each mouse the mean heart rate (HR) value during baseline, ischemia and reperfusion was calculated.

2.6. Infarct size assessment

Infarct sizes were measured using the technique developed by Fishbein et al. (30). Hearts from *in vivo* and *ex vivo* experiments were dissected and the LVs were sliced transversally into 1 mm-thick sections and incubated in a 1% (w/v) solution of 2,3,5-

triphenyltetrazolium chloride (TTC; Sigma-Aldrich, St Louis, Mo) for 15 min at 37°C. After fixation in a 4% paraformaldehyde-phosphate-buffered solution, the slices were weighed, and each side was photographed with an Olympus camera. The ischemic risk area (unstained in blue) and the infarcted area (unstained by TTC) were outlined on each photograph and measured by planimetry using Image J (Scion corp., Frederick, MD). The area of each region was averaged from the photographs of each side for each slice, and multiplied by the weight of that tissue section. Infarct size was expressed as a percentage of the ischemic risk area (AR). Mice with an AR of less than 40 (in % of LV mass) and those greater than 60 were excluded from the study to ensure that infarct size values were compared in a pool of animals with the same infarct severity.

2.7. Statistical analysis

All values are expressed as mean \pm SD. For comparisons between two groups, the Mann-Whitney non-parametric test was used. Multiple comparisons between groups were assessed by the Kruskal-Wallis nonparametric test followed by the Dunn's *post hoc* test or ANOVA (when the Normality Shapiro-Wilk test passed) or by the three-way ANOVA when considering three different variables (time, mutation and pacing). Values of $p < 0.05$ were accepted as statistically significant. The p values, indicated in the text, were noted in the figures as: ns for $p > 0.05$, * for $p < 0.05$, ** for $p < 0.001$, *** $p < 0.01$ and **** $p < 0.0001$. All data were analyzed with GraphPad Prism (GraphPad Software, San Diego, California, USA). A linear relationship between HR and IS was fitted by linear regression model using the least squares method (GraphPad Prism software) based on the minimization of the sum of squares of the vertical distances of the points from the line.

3. Results

3.1. Pharmacological inhibition of f-channels by ivabradine reduces infarct size upon IR condition *in vivo*

The hyperpolarization-activated current (I_f) plays an important role in the generation of heart rate. Therefore, we first investigated the effects of the selective I_f inhibitor ivabradine on IR injury (Figure 1A). Anesthetized WT mice were subjected to an *in vivo* surgery protocol. ECG was continuously recorded along the surgery protocol (Figure 1A; Supplementary Figure S1). In comparison with control WT mice (WT; 0.9% NaCl, IP, $n = 6$), ivabradine treated mice (WT Iva; 6 mg/kg; IP injection, 30 min before coronary ligation; $n = 7$) showed a significant decrease in both basal heart rate (179 \pm 19 bpm vs. 240 \pm 40 bpm, ** $p = 0.0047$; 25%-reduction; Figure 1B) and infarct size (24.73% \pm 5.25% vs. 36.37% \pm 3.56%, ** $p = 0.0047$; 32%-reduction; Figure 1C). The area at risk of ischemia (AR/LV mass) was similar among groups ($p^{ns} = 0.9452$; Supplementary Figures S2A).

3.2. Genetic ablation of L-type $Ca_v1.3$ channels reduced infarct size upon IR *in vivo*

Because ivabradine was described to provide also heart rate-independent benefit in cardioprotection (31), we evaluated the cardioprotective effects of heart rate reduction obtained through genetic deletion of $Ca_v1.3$ channels, a major contributor in the generation of diastolic depolarization. To this aim, anesthetized $Ca_v1.3^{+/-}$ and $Ca_v1.3^{-/-}$ mice were both subjected to the *in vivo* surgical protocol presented in Figure 2A with a continuous ECG recording along the protocol (Supplementary Figure S1). Figure 2B shows that heterozygous $Ca_v1.3^{+/-}$ mice exhibit moderate heart rate reduction (22%) (187 \pm 22 bpm, $n = 6$ for $Ca_v1.3^{+/-}$ vs. 240 \pm 40 bpm, $n = 6$ for Ctrl; $p^{ns} = 0.3714$) that was associated with a 26.7%-decrease in infarct size (26.67% \pm 6.16%, $n = 6$ for $Ca_v1.3^{+/-}$ vs. 36.37% \pm 3.56%, $n = 6$ for WT; $p^* = 0.0267$). In homozygous $Ca_v1.3^{-/-}$ mice ($n = 7$), characterized by a marked reduction (54%) of the basal heart rate in comparison to WT counterparts ($n = 6$) (109 \pm 14 bpm, $n = 7$ for $Ca_v1.3^{-/-}$ vs. 240 \pm 40 bpm, $n = 6$ for Ctrl; $p^{***} = 0.0004$), a 28.8%-decrease in infarct size vs. WT was observed (25.90% \pm 3.28%, $n = 7$ for $Ca_v1.3^{-/-}$ vs. 36.37% \pm 3.56%, $n = 6$ for WT; $p^* = 0.0190$; Figure 2C). Area at risk of ischemia (AR/LV mass) was similar among groups ($p^{ns} = 0.0511$; Supplementary Figure S2B), indicating that all hearts were exposed *in vivo* to quantitatively similar ischemic challenge. These results suggest that moderate heart reduction induced by genetic ablation of one allele of $Ca_v1.3$ channels exerted potent cardioprotective effect against IR injury.

3.3. Heart rate and infarct size *in vivo* displayed linear correlation

In order to elucidate the correlation between heart rate and infarct size, we used genetically modified mice harboring different basal heart rates. We thus measured infarct size in $Ca_v1.3^{+/-}$, $Ca_v1.3^{-/-}$ and $Girk4^{-/-}$ mice subjected to the same IR protocol (Figure 3A) in control conditions (WT) and after ivabradine (WT Iva; 6 mg/kg) treatment. Figure 3B shows variation in basal heart rate according to the different genotypes, with heart rate of $Ca_v1.3^{-/-}$ being the lowest and that of $Girk4^{-/-}$ the highest among groups (Supplementary Figure S1). Mean heart rates of $Ca_v1.3^{-/-}$, $Ca_v1.3^{+/-}$ and WT Iva were lower than that of WT counterparts (109 \pm 14 bpm, $n = 6$; $p^{****} < 0.0001$ for $Ca_v1.3^{-/-}$; 187 \pm 22 bpm, $n = 6$; $p = 0.0282$ for $Ca_v1.3^{+/-}$ and 179 \pm 19 bpm, $n = 7$; $p^{**} = 0.0069$ vs. 240 \pm 40 bpm, $n = 6$, for WT). By contrast, mean heart rates from $Girk4^{-/-}$ and $Girk4^{-/-}$ Iva were higher compared to WT [360 \pm 54 bpm, $n = 5$; $p^{****} < 0.0001$ for $Girk4^{-/-}$ and 271 \pm 32 bpm, $n = 5$; $p^{ns} = 0.356$ for $Girk4^{-/-}$ Iva vs. WT (240 \pm 40 bpm, $n = 6$). Figure 3B]. Figure 3C shows infarct size analysis among the various genotypes and the clear correlation between infarct size and heart rate. $Girk4^{-/-}$ mutant mice exhibit the largest (47.26% \pm 4.67%, $n = 5$) and $Ca_v1.3^{-/-}$ the lowest (25.9% \pm 3.28%, $n = 7$)

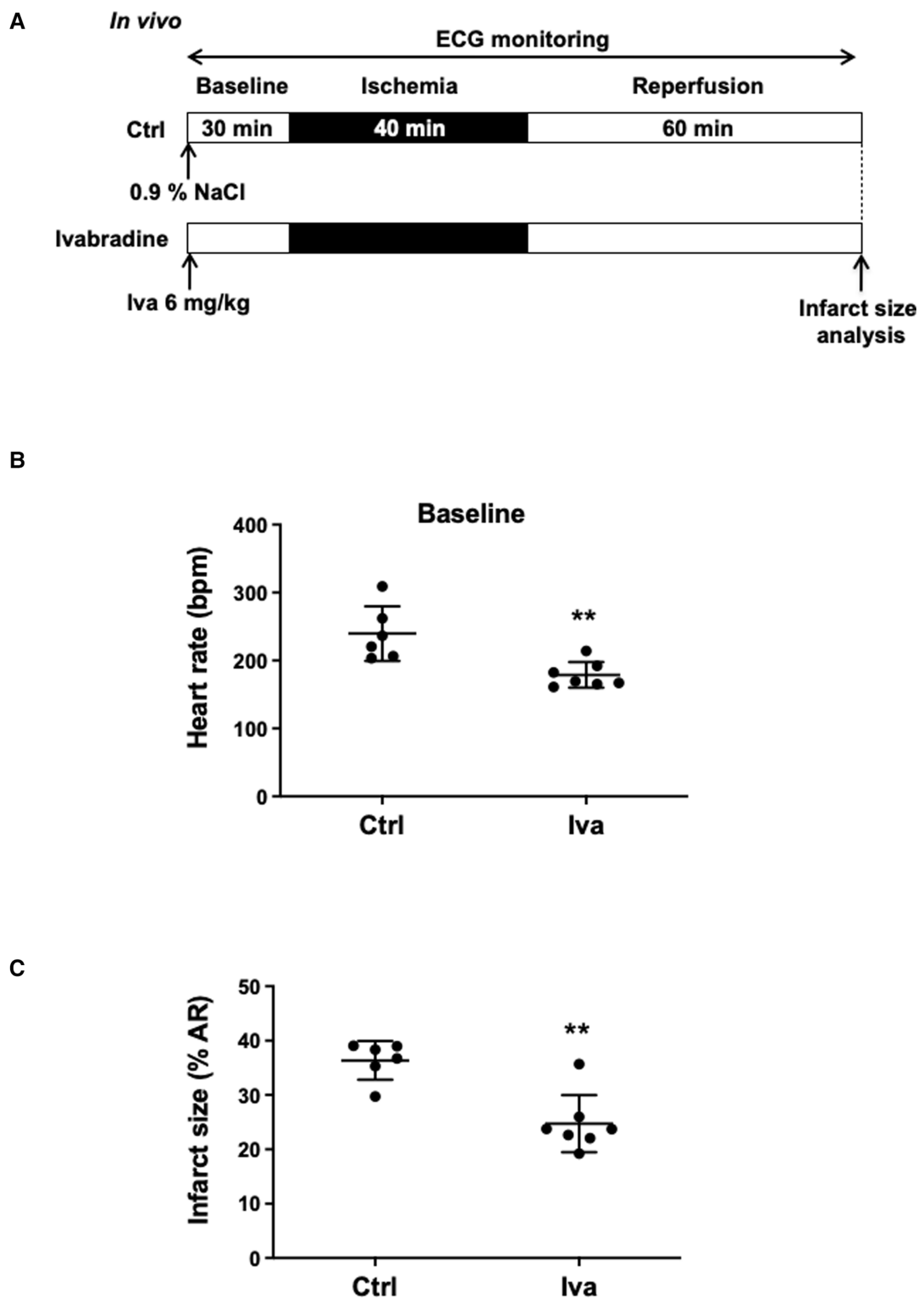


FIGURE 1

(A) *In vivo* experimental protocol applied to wild-type (WT) mice, consisting of 40 min of ischemia induced by ligation of the left coronary artery followed by 60 min of reperfusion. Mice were assigned to receive either 0.9% NaCl (Ctrl, $n = 6$) or Ivabradine (Iva, 6 mg/kg, $n = 7$), intraperitoneally 30 min before ischemia. ECG was recorded during the surgical protocol; (B) scatter dot plots and mean \pm SD were plotted for baseline HR measured before ischemia in control mice (NaCl 0.9%) ($n = 6$) or Iva pre-treated mice ($n = 7$); (C) scatter dot plots and mean \pm SD were plotted for infarct size (expressed as the percentage of the area at risk) and measured at the end of the surgical protocol in control (Ctrl, $n = 6$) and Iva pre-treated (Iva, $n = 7$) mice subjected to ischemia-reperfusion (IR) protocol; Statistical analysis was performed using the non-parametric Mann-Whitney test. Statistical significance is noted ** for $p = 0.0047$ for (B,C).

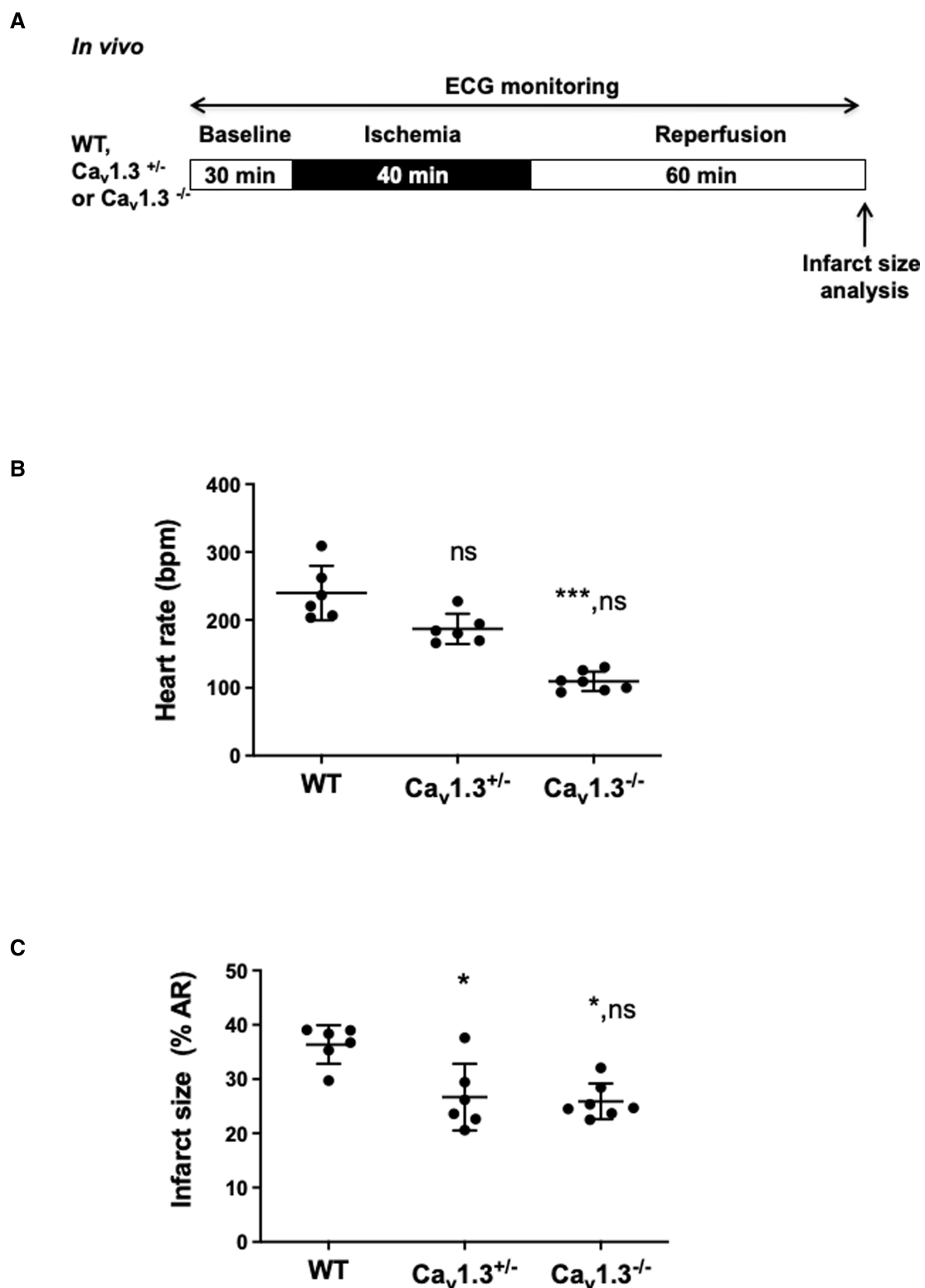


FIGURE 2
 (A) *In vivo* experimental protocol applied to wild-type (WT), Ca_v1.3^{+/-} and Ca_v1.3^{-/-} mice, consisting in 40 min ischemia, induced by ligation of the left coronary artery, followed by 60 min of reperfusion. ECG was recorded during the surgical protocol; (B) scatter dot blots and mean ± SD were plotted for baseline HR measured before ischemia for WT mice (NaCl 0.9%) (n = 6), Ca_v1.3^{+/-} (n = 6) and Ca_v1.3^{-/-} (n = 7); (C) scatter dot plots of infarct size and means ± SD were plotted for all mice subjected to IR protocol. Non parametric Kruskal–Wallis test followed by Dunn’s *post hoc* test was used to compare data among groups. ns is noted for p > 0.05, * for p < 0.05 and *** for p < 0.001 vs. control; ns in second place was noted for p > 0.05 vs. Ca_v1.3^{+/-}.

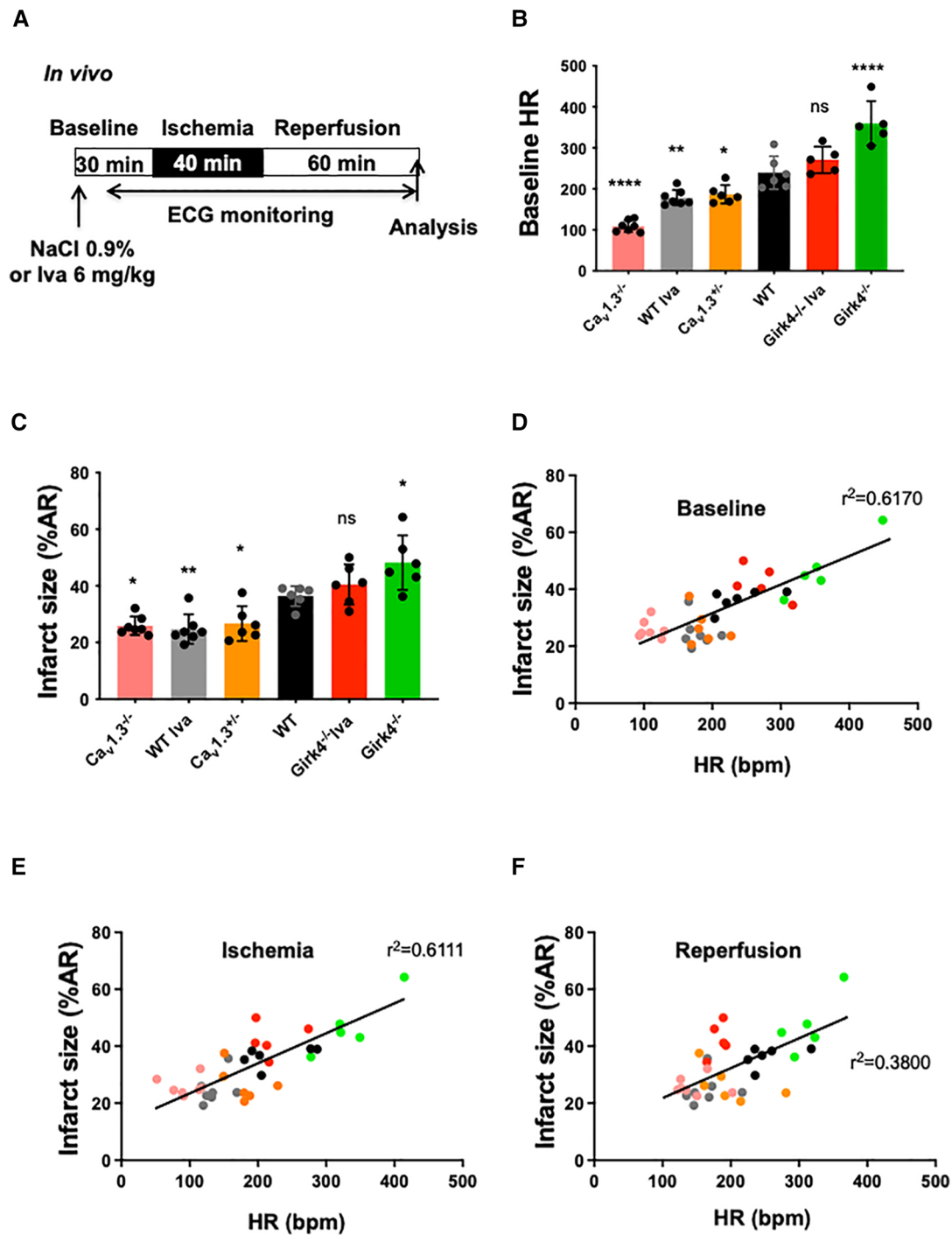


FIGURE 3

Correlation between infarct size and heart rate during ischemia-reperfusion in wild-type (WT) and mutant mice. (A) IR protocol was induced by reversible coronary ligation leading to 40 min ischemia followed by 60 min reperfusion. Ivabradine (6 mg/kg, IP route) was injected in WT (WT Iva, $n = 7$) and $Girk4^{-/-}$ ($Girk4^{-/-}$ Iva, $n = 5$) mice; (B) scatter dot plots and bars (mean \pm SD) are presented for heart rates recorded at baseline in WT ($n = 6$) and in $Ca_v1.3^{+/-}$ ($n = 6$), $Ca_v1.3^{+/-}$ ($n = 7$) and $Girk4^{-/-}$ ($n = 5$) mutant mice, or WT Iva and $Girk4^{-/-}$ Iva. Colors represent particular mouse genotype or ivabradine treatment. Data were compared using ANOVA (normality Shapiro-Wilk test passed) and p values are given vs. WT; * is noted for $p = 0.0282$, ** for $p = 0.0069$, **** for $p < 0.0001$ and ns for $p = 0.3557$. (C) scatter dot plots and bars (mean \pm SD) are presented for infarct size recorded at baseline in WT and mutant mice from different genotypes, or WT and $Girk4^{-/-}$ mice pre-treated with one bolus injection of ivabradine. Note that the lowest heart rate for $Ca_v1.3^{+/-}$ hearts is associated with reduced infarct size, while moderate tachycardia in $Girk4^{-/-}$ mice is associated to increased infarct size compared to WT mice. Data were compared using ANOVA (normality Shapiro-Wilk test passed) and p values are given vs. WT; ** is noted for $p = 0.0062$, * for $p = 0.0342$ ($Ca_v1.3^{+/-}$), $p = 0.0151$ ($Ca_v1.3^{+/-}$), $p = 0.0212$ ($Girk4^{-/-}$) and ns for $p = 0.3355$. A linear correlation between infarct size (in % of AR) and heart rate (bpm) during baseline (D) ischemia (E) and reperfusion (F) is shown. R^2 values are noted on each graph.

infarct sizes. A moderate heart rate reduction in *Girk4*^{-/-} mice induced by a bolus injection of ivabradine (40.5% ± 7.09%, *n* = 7) yielded infarct sizes that were in between those recorded in WT and *Girk4*^{-/-} mice.

Of note, higher *r*² values of the linear regression were obtained for heart rate values recorded at baseline and during ischemia (*r*² = 0.6170 and *r*² = 0.6111, respectively; **Figures 3D,E**) than those recorded during reperfusion (*r*² = 0.3800; **Figure 3F**). Irrespective of heart rates recorded at baseline and the genotype of mice tested, the heart rate was significantly decreased during ischemia, while increased during reperfusion (**Figure 3C**; **Supplementary Figure S1**). The relationship between heart rate recorded at baseline and infarct size was preserved for heart rates recorded during ischemia (**Figures 3D,E**) and, to a lesser extent, during reperfusion (**Figure 3F**). AR/LV mass measured for each heart was similar among groups (*p*^{ns} = 0.0506) (**Supplementary Figure S3**).

3.4. Atrial pacing *ex vivo* negated the cardioprotective effect of heart rate modulation

Since the infarct size correlated with heart rate irrespective of the genotype *in vivo*, we investigated whether increase in heart rate prevented cardioprotection. To this aim, atrial pacing *ex vivo* was performed to increase heart rate in the absence of the vagus nerve influence (**Figure 4**). Hearts underwent the same protocol as *in vivo* through the implementation of a reversible ligation on the left coronary during 40 min ischemia followed by 60 min reperfusion (**Figure 4A**). In the atrial-paced group, hearts were placed at 475 bpm. Since *Ca_v1.3*^{-/-} hearts *ex vivo* exhibit complex atrioventricular conduction block (32), which could interfere in experiments of atrial pacing, we used hearts from *Ca_v1.3*^{+/-} associated with strong cardioprotection. *Ca_v1.3*^{+/-} hearts with a mean heart rate of 341 ± 20 bpm (*n* = 6) were paced at 475 bpm (*n* = 5), representing a 39%-increase. Infarct size in paced hearts was 44%-increased (74.22% ± 2.99%, *n* = 5 in paced vs. 51.52% ± 8.32%, *n* = 6 in un-paced *Ca_v1.3*^{+/-}; *p*^{**} = 0.0043; **Figure 4B**). Same experiments were performed in C57Bl6 WT mice in order to overcome the influence genetic modifications. Heart rate at baseline was 398 ± 50 bpm in the WT group (*n* = 9) and upon atrial pacing (20% increment in heart rate), infarct size was increased by 32% in those hearts (56.33% ± 7.45%, *n* = 7 vs. 42.78% ± 7.45%, *n* = 9; *p*^{**} = 0.0052; **Figure 4C**). Consistently, the relationship between infarct size and heart rate was maintained *ex vivo* by pacing WT hearts to lower heart rate values including 200, 300 and 400 bpm. **Figure 4D** shows a linear correlation established between infarct size and heart rate in the range of 200–475 bpm (**Supplementary Figure S5**).

Since heart rate reduction prolongs the ventricular diastole allowing to augment the coronary perfusion, we recorded the coronary flow during *ex vivo* experiments (**Figure 4E**; **Table 1**). Our results show that coronary flow dropped during the ischemic phase (≈50%) in WT and *Ca_v1.3*^{+/-} paced or un-paced hearts. However, during reperfusion, the coronary flow of un-

paced WT and *Ca_v1.3*^{+/-} hearts recovered and stabilized above 100% of the control baseline level, whereas the flows in paced hearts of both genotypes did not fully recover after stabilization and was reduced by 25% compared to the un-paced condition (*p*^{****} < 0.0001).

Taken together, these results demonstrate positive correlation between heart rate and infarct size *ex vivo* and suggest that the cardioprotective effect associated to a decreased heart rate might be related to a better coronary circulation during reperfusion.

4. Discussion

In this study we have investigated the relationship between heart rate and infarct size using a protocol of IR injury *in vivo*. We found that infarct size is closely correlated with heart rate recorded during the pre-ischemic and ischemic phases in WT and mutant mice tested. Genetic ablation of one allele of L-type *Ca_v1.3* channels led to moderate heart rate reduction and a reduced infarct size *in vivo* when compared to WT counterparts. Increased heart rate via atrial pacing *ex vivo* abolished both the cardioprotective effect of heart rate reduction induced by genetic ablation of *Ca_v1.3* and improvement of the post-ischemic coronary flow observed at a lower heart rate. Taken together, our data show, for the first time, that heart rate *per se* is a major predictor of IR injury: the lower is heart rate, the greater being cardioprotection. In conclusion, our results indicate positive correlation between heart rate and infarct size both *in vivo* and *ex vivo* in the absence of vagal stimulation. Furthermore, our data suggest that targeting of *Ca_v1.3* channels can constitute a new potential therapeutic strategy to reduce IR injury and infarct size.

Heart rate is a vital parameter assessed at admission in all patients with acute myocardial infarction. Several prognostic studies have shown that resting heart rate is predictive of poor outcome in patients and heart rate at discharge is predictive of long-term outcome in patients with coronary artery disease (33). In this context, β-blockers have shown beneficial effects in terms of reduction of mortality after AMI (34, 35). They are widely prescribed for cardiovascular disease because their heart rate reduction effect. These drugs are effective for restoration of contractility in left ventricular dysfunction (36). However, despite these beneficial effects, they can provide intolerance and exercise limitation in patients because of their negative inotropic effect. We have shown in a previous study that β-blocker treatment abolishes in human cells the high frequency-induced upregulation of human cardiac *Ca_v1.2*-driven calcium currents, a physiological mechanism involved in the Bowditch “staircase” allowing an adaptation to exercise and stress upon β-adrenergic regulation (37). In addition, β-blockers also exert α-adrenergic vasoconstriction, responsible for deleterious effects on myocardial coronary blood flow and contractile function (38). These findings highlight the importance of selective heart rate reduction through modulation of SAN automaticity in the absence of concomitant negative inotropism (14, 16). Pharmacological targeting of ion channels involved in pacemaker activity thus constitutes a

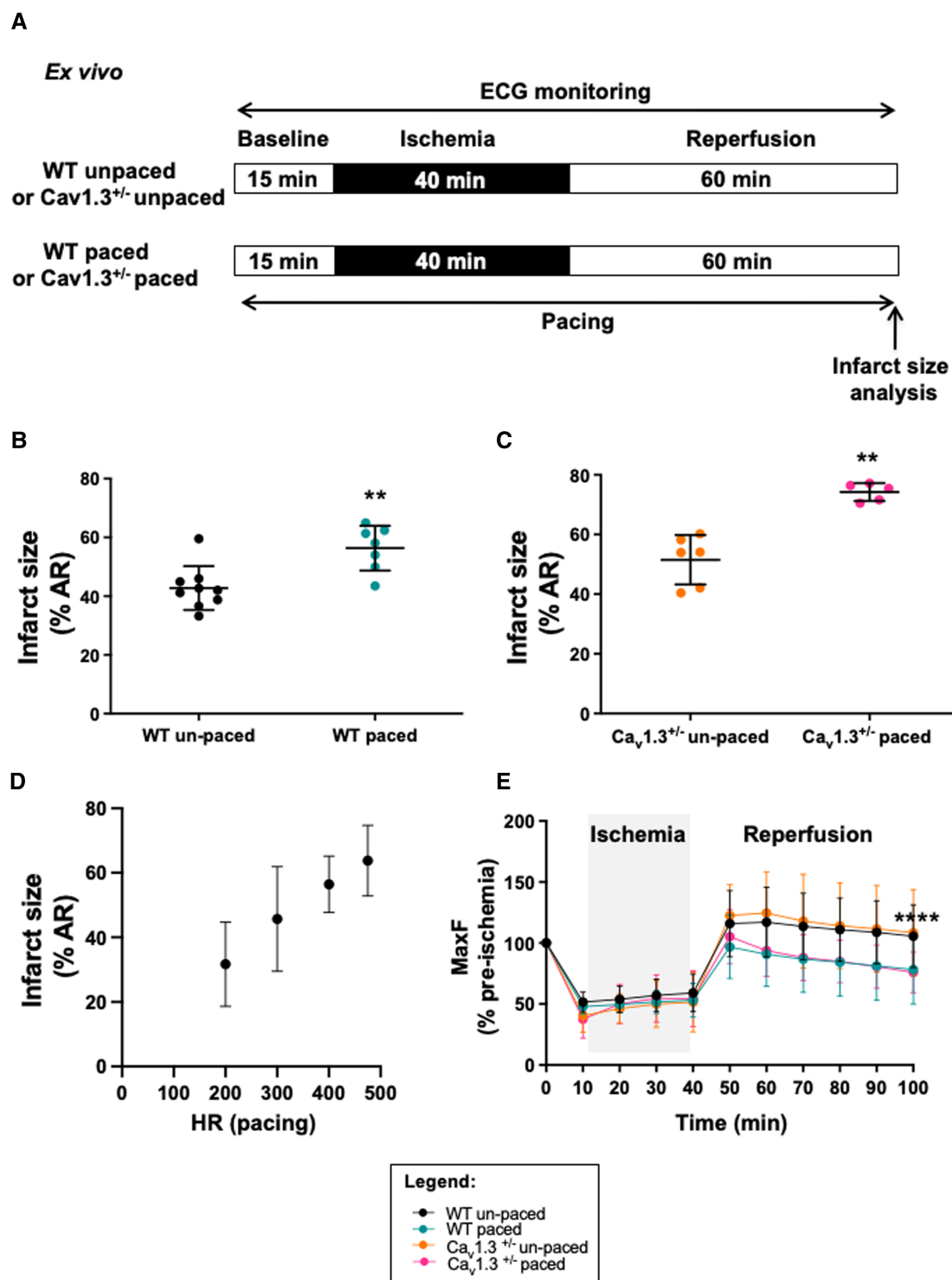


FIGURE 4

Infarct size and heart rate during *ex vivo* ischemia-reperfusion of Langendorff perfused WT and Cav1.3^{+/−} mouse hearts with or without arial pacing. Protocol of induction of ischemia-reperfusion injury and pacing is shown in (A). Scatter dot plot and mean ± SD are presented for infarct size values (expressed as the percentage of area at risk) for paced un-paced ($n = 6$) and paced ($n = 5$) Cav1.3^{+/−} hearts (B) as well as for paced ($n = 7$) and un-paced ($n = 9$) WT hearts (C). (D) relationship between infarct size and pacing frequency in WT hearts; (E) maximum coronary flow (MaxF) was expressed as a percentage of pre-ischemia value during *ex vivo* ischemia-reperfusion in un-paced WT (black symbol and line) and Cav1.3^{+/−} (orange color), paced WT (blue) and paced Cav1.3^{+/−} (pink) hearts. The grey area indicates the ischemic period. Statistical analysis was performed using the three-way ANOVA, considering the time, the pacing and the Cav1.3^{+/−} mutation as variables. Results show that variability was due to the time factor ($p^{****} < 0.0001$) and the pacing ($p^{****} < 0.0001$) with a significant interaction ($p^{**} = 0.0075$). However, the effect of the mutation of Cav1.3 was not significant ($p^{ns} = 0.9841$). If we consider only the period of reperfusion, only the pacing impacts the results ($p^{****} < 0.0001$).

TABLE 1 Mean, SD and *n* values are presented for the maximal coronary flow (maxF), calculated every 10 min and expressed as a percentage of the data recorded at baseline (stabilization phase). Data were obtained during *ex vivo* experiments in WT and $Ca_v1.3^{+/-}$ hearts, with or without pacing (475 bpm).

Phase	Time (min)	WT			WT pacing			$Ca_v1.3^{+/-}$			$Ca_v1.3^{+/-}$ pacing		
		Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>	Mean	SD	<i>n</i>
Stabilization	0	100	0	9	100	0	7	100	0	5	100	0	7
Ischemia	10	52	8	9	48	6	7	40	14	5	48	6	7
	20	54	11	9	50	6	7	46	12	5	50	6	7
	30	57	13	9	52	10	7	50	19	5	52	10	7
	40	59	15	9	53	14	7	52	24	5	53	14	7
Reperfusion	50	116	27	9	97	26	7	123	25	5	97	26	7
	60	117	28	9	91	26	7	125	34	5	91	26	7
	70	114	27	9	87	27	7	118	39	5	87	27	7
	80	111	26	9	84	28	7	114	35	5	84	28	7
	90	109	26	9	81	28	7	112	36	5	81	28	7
	100	106	26	9	78	28	7	108	35	5	78	28	7

promising avenue for protecting the myocardium from ischemic injury. In this respect, clinical trials have shown the beneficial effects of selective heart rate reduction by ivabradine, in the context of coronary artery disease and heart failure (39, 40). As suggested recently by Baka et al. cardioprotection by ivabradine could be beneficial in the management of COVID-19-related cardiovascular complications including myocarditis, acute coronary syndrome, cardiogenic shock or cardiac dysautonomia (41). However, clinical studies have shown that treatment with high doses of ivabradine involves an increased risk of cardiovascular mortality, infarction and atrial fibrillation (42, 43). These results underscore the need to investigate new potential ion channels as targets to develop new therapies aimed to selectively control heart rate without adverse effects on the myocardium and prognosis of patients.

$Ca_v1.3$ channels underlying $I_{Cav1.3}$ current constitute with I_f the predominant mechanisms in the generation of diastolic depolarization (21, 32, 44), while GIRK4 channels underlying I_{KACH} constitute both tonic and phasic brakes of diastolic depolarization and heart rate (25, 45). In addition, our previous study about heart rate determination by Cav1.3 and f-channels indicates that these channels possibly act independently in pacemaker activity because $Ca_v1.3$ knockout and ivabradine reduced heart rate by similar percentages in freely-moving mice (39). This is in line with our results in the present study. Indeed, f-channel blockade and $Ca_v1.3$ knockout lead to comparable infarct sizes, supporting our hypothesis that cardioprotection mediated by $Ca_v1.3$ targeting is primarily due to heart rate reduction, at least under our experimental conditions. Furthermore, $Ca_v1.3$ channels are not expressed in the adult ventricle (21) and are not involved in excitation-to-contraction coupling in the adult heart (46). $Ca_v1.3$ channels thus constitute an important candidate, in addition to I_f , to obtain slowing of heart rate without concomitant negative inotropism. Consequently, we have compared cardioprotection conferred by pharmacological inhibition of I_f to that obtained upon genetic ablation of $Ca_v1.3$ and GIRK4 channels.

Pharmacological heart rate reduction by ivabradine effectively reduced the heart rate and the infarct size in control WT mice (Figure 1), thus validating our *in vivo* mouse model of IR for

studying the effects of genetic ablation of ion channels involved in pacemaking. Our results show that genetic ablation of $Ca_v1.3$, or GIRK4 channels regulates the infarct size in close correlation with heart rate recorded in three phases considered: baseline, ischemia and reperfusion. Importantly, genetic targeting of $Ca_v1.3$ channels and pharmacologic inhibition of I_f reduced infarct size, similarly, suggesting comparable efficacy in controlling IR injury (Figure 3). Consequently, when the correlation between heart rate and infarct size is considered in the three phases, genetic ablation of $Ca_v1.3$ compares to ivabradine, suggesting that selective pharmacological targeting of $Ca_v1.3$ could induce at least as much cardioprotection as ivabradine. Consistently with heart rate being an important factor in determining cardioprotection against IR injury, similar heart rates obtained by ivabradine infusion, or genetic ablation of $Ca_v1.3$ channels yielded comparable infarct sizes. We noticed that despite $Ca_v1.3^{-/-}$ mice presented with lower heart rates than $Ca_v1.3^{+/-}$ mice, deletion of the two $Ca_v1.3$ alleles did not significantly reduce the infarct size in comparison to heterozygous $Ca_v1.3^{+/-}$ mice (Figure 2), possibly as a result of a maximal saturating protective effect of such mechanism in our model. Indeed, given the results obtained with ivabradine, we cannot exclude that infarct size value of 25% of the area at risk constitutes the lowermost limit in size that can be obtained by genetic or pharmacological targeting of ion channels involved in the generation of SAN diastolic depolarization. However, other hypotheses can be put forward to explain the equivalent infarct sizes between $Ca_v1.3^{+/-}$ heterozygous and $Ca_v1.3^{-/-}$ homozygous hearts have increased degree of remodeling secondary to sporadic atrioventricular block found in this genotype (32, 45). Such a remodeling may limit indirectly cardioprotection in relation to absolute heart rate; A second, non-exclusive, reason could reside in an intrinsic physiological limit of cardioprotection—around 29% in our model—that can be obtained by heart rate reduction during the ischemic phase. The additional effect of ivabradine on reducing infarct size (reaching 32%-reduction) could be explained by previously reported rate-independent cardioprotection effect (26, 27). In this regard, it is worth to note that the 50% reduction in heart rate observed in anesthetized $Ca_v1.3^{-/-}$ mice exceeds bradycardia observed *in vivo* in freely

moving *Cav1.3^{-/-}* mice. This could be related to the cardiodepressant effect of xylazine-ketamine-based anesthesia, which would tend to exacerbate the effects on heart rate of total genetic ablation of *Ca_v1.3* in cardiac pacemaker activity, an effect that would not translate into overt cardioprotection compared to *Ca_v1.3^{+/-}* hearts. Bradycardia during the acute phase of myocardial infarction is correlated to early cardiovascular mortality. In their study, Salwa et al. demonstrated a J-curve for the mortality as a function of heart rates suggesting that bradycardia was associated to worsen injury within the myocardial tissue (47). Future study using selective pharmacological *Ca_v1.3* blockade may help explain these discrepancies.

The cardioprotective effect observed in *Ca_v1.3^{+/-}* hearts was abolished when atrial pacing was applied to compensate the lowering effect of *Ca_v1.3* inactivation expressed in the pacemaker of *Ca_v1.3^{+/-}* mice, clearly showing that cardioprotection was related to heart rate reduction, since *Ca_v1.3* channels are not expressed in the adult ventricle (Figure 4). These observations reinforce our conclusion that heart rate is a major determinant of cardioprotection conferred by genetic ablation of *Ca_v1.3* channels. Our data indicate that, similar to that reported for ivabradine (14, 26), improvement of coronary flow at the reperfusion phase underlies rate-dependent cardioprotection (Figure 4F). In line with this hypothesis, rapid pacing abolished the improvement of coronary perfusion in both WT and *Ca_v1.3^{+/-}* hearts. In this context, previous work showed that part of the cardioprotective effect of ivabradine is not dependent on its heart rate reduction effect (26, 27). For *Ca_v1.3* mutant hearts, atrial pacing *ex vivo* negated the cardioprotective effect of the genetic ablation of one allele of the *Ca_v1.3* gene. These results obtained in isolated perfused hearts suggest that cardioprotection observed in *Ca_v1.3^{+/-}* was dependent on its heart rate reduction effect.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Comité d’Ethique pour l’Expérimentation Animale Languedoc-Roussillon” (CEEA-LR) with the authorization number CE-LR-0814, University of Montpellier.

Author contributions

MM and SB-L designed the whole project and the experiments, PM, AV, CP, JN, SK and KW contributed to design and interpreted

the data for the work. VD-B, KC, AC, LG, CB and IB performed the experiments and analysed the results. MM and SBL wrote the manuscript with the input of PM, JN and CP. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcvm.2023.1134503/full#supplementary-material>

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