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# The mechano-electric feedback mediates the dual effect of stretch in mouse sinoatrial tissue

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ABSTRACT

The sinoatrial node (SAN) is the primary heart pacemaker. The automaticity of SAN pacemaker cells is regulated by an integrated coupled-clock system. The beat interval (BI) of SAN, and its primary initiation location (inferior vs. superior) are determined by mutual entrainment among pacemaker cells and interaction with extrinsic effectors, including increased venous return which stretches the SAN. We aim to understand the mechanisms that link stretch to changes in BI and to heterogeneity of BI in the SAN.

Isolated SAN tissues of C57BL/6 mice were gradually stretched to different degrees [(low (5–10 % lengthening), medium (10–20 %), and high (20–40 %))] using motor controlled with a custom-made Arduino software. Recordings were acquired 30 s following each level of step. In 8/15 tissues, stretch led to a positive chronotropic response, while in 7/15 tissues, a negative chronotropic response was observed. In the positive chronotropic response group, BI was shortened in parallel to shortening of the local $Ca^{2+}$ release (LCR) period, a readout of the degree of clock coupling. In the negative chronotropic response group, in parallel to a prolongation of BI and LCR period, an unsynchronized firing rate was observed among the cells upon application of stretch. Eliminating the mechano-electrical feedback by addition of blebbistatin disabled the stretch-induced chronotropic effect. Reduction of the sarcoplasmic reticulum $Ca^{2+}$ levels, which mediates the mechano-electrical feedback, by addition of cyclopiazonic acid disabled the dual effect of stretch on SAN function and BI heterogeneity. Thus, the mechano-electric feedback mediates the dual effect of stretch in mouse SAN tissue.

## 1. Introduction

The sinoatrial node (SAN) is the primary heart pacemaker, comprised of pacemaker cells that are heterogenous in their morphology, electrophysiological characteristics, $Ca^{2+}$ dynamics and protein structure [1–4]. The classic theory is related to the initiation of electrical activity in the leading pacemaker, where pathophysiological conditions and drug perturbations can cause an anatomical shift in the leading pacemaker site [5,6]. A recent research showed distinct superior (located near the superior vena cava) and inferior (located near the inferior vena cava) domains in the SAN [7], each of which takes the lead under different circumstances [8].

At the single pacemaker cell level, beat interval (BI) is controlled by a coupled-clock function of surface membrane ion channels and exchangers together with intracellular $Ca^{2+}$ cycling [9]. The coupled clock system is mutually entrained by local $Ca^{2+}$ releases (LCRs) and through $Ca^{2+}$ that binds to calmodulin to activate both adenyl cyclase (AC)-cAMP-PKA and CaMKII phosphorylation cascades [10]. In vivo, SAN automaticity, and, subsequently, cardiac BI, is determined by mutual entrainment among pacemaker cells and interaction with extrinsic effectors, including increased venous return, that stretch the SAN [11].

The SAN can adapt its rate and rhythm to the body's demand for oxygen through the Bainbridge response [12]. In cases of high demand, the venous return to the heart increases, which affects right atrial filling and distends the atrial wall where the SAN is located. A positive chronotropic response to stretch has been shown in dog hearts [12,13], isolated rabbit [14] and cats [15] SAN tissue and isolated rabbit SAN cells [16]. Recently, Macdonald et al. showed variable responses to mechanical stretch of mouse SAN tissues [11]. However, the identity of the intracellular mechanisms responsible for this association remains unclear. Moreover, it is not known if and how stretch affects BI heterogeneity among pacemaker cells residing in the SAN tissue.

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The electrical activity of cardiac cells directly affects cell contraction and is affected by mechanical load through the mechanoelectrical feedback [17]. The immediate electrophysiological response to these mechanical changes has been investigated and linked to mechano-sensitive mechanisms in cardiac cells, including stretch-activated ion channels in cardiac myocytes [18–22], changes in $Ca^{2+}$ handling [23–25], and interactions with other mechano-sensitive cells [26]. It is not known if the mechano-electrical feedback is involved in the chronotropic response of SAN to stretch or in the variability of mouse SAN tissue response to mechanical stretch. The role of stretch-activated channels [11] as mediators of the mechano-electrical feedback on the chronotropic effect in SAN was rejected. It was postulated that $Ca^{2+}$ that can bind to and be released from the myofilament may affect SANC electrical activity through $Ca^{2+}$-activated channels and exchanger [27].

To investigate the mechanism that mediates between stretch and chronotropic response in the mouse SAN, the following three hypotheses were tested: (i) the nature of the chronotropic response to stretch is associated with heterogeneity of pacemaker cells residing in the SAN tissue, (ii) distinct patterns of changes in LCR characteristics are associated with positive vs. negative chronotropic responses, and (iii) the mechano-electric feedback triggered by $Ca^{2+}$ mediates the dual effect of stretch in mouse SAN tissue.

To test these hypotheses, $Ca^{2+}$ transients were measured with a confocal microscope during gradual stretching of isolated mouse SAN tissues. To identify the specific mechanisms involved in the stretch-induced response, the mechano-electrical feedback was inhibited and $Ca^{2+}$, PKA or CaMKII activity was disabled by specific pharmacological drugs.

## 2. Materials and methods

### 2.1. Animal use

All experimental procedures were approved by the Animal Care and Use Committee of the Technion-IIT (Ethics number: IL-002-01-19).

### 2.2. SAN isolation

Adult (12–14 weeks, 25–30 g) male C57BL/6JOlaHsd mice were anesthetized by injection of sodium pentobarbital (50 mg/kg, i.p.) diluted with heparin. After loss of reflexes, the hearts were quickly removed and placed in a 37 °C Tyrode solution containing (mM): 140 NaCl, 1 $MgCl_2$, 5.4 KCl, 1.8 $CaCl_2$, 5 HEPES, and 5 glucose, titrated to pH 7.4 with NaOH. The SANs and atria were isolated from the ventricles and pinned down, with the endocardium facing up, in a custom-made silicone-covered chamber. Blood vessels and fat tissue were carefully cut out. After exposing the SAN, the preparations were moved to a ring-shaped, silicone-covered Petri dish. The preparations were placed inside the ring and were flattened against the glass cover with small stainless-steel pins, to enable clear optimal imaging. The crista terminalis was clamped with the left pin and the interatrial septum was clamped with the right pin (Fig. 1A). The left atrium was clamped with an extra pin, if needed. The preparations were kept unstretched.

### 2.3. $Ca^{2+}$ imaging

Following SAN isolation and fixation, the preparations were loaded with 30 μM Fluo-4-AM for 1 h, at 37 °C and 60 RPM and then washed twice with Tyrode solution before being placed in an optical chamber at 37 °C as described before [28]. $Ca^{2+}$ fluorescence was imaged with an

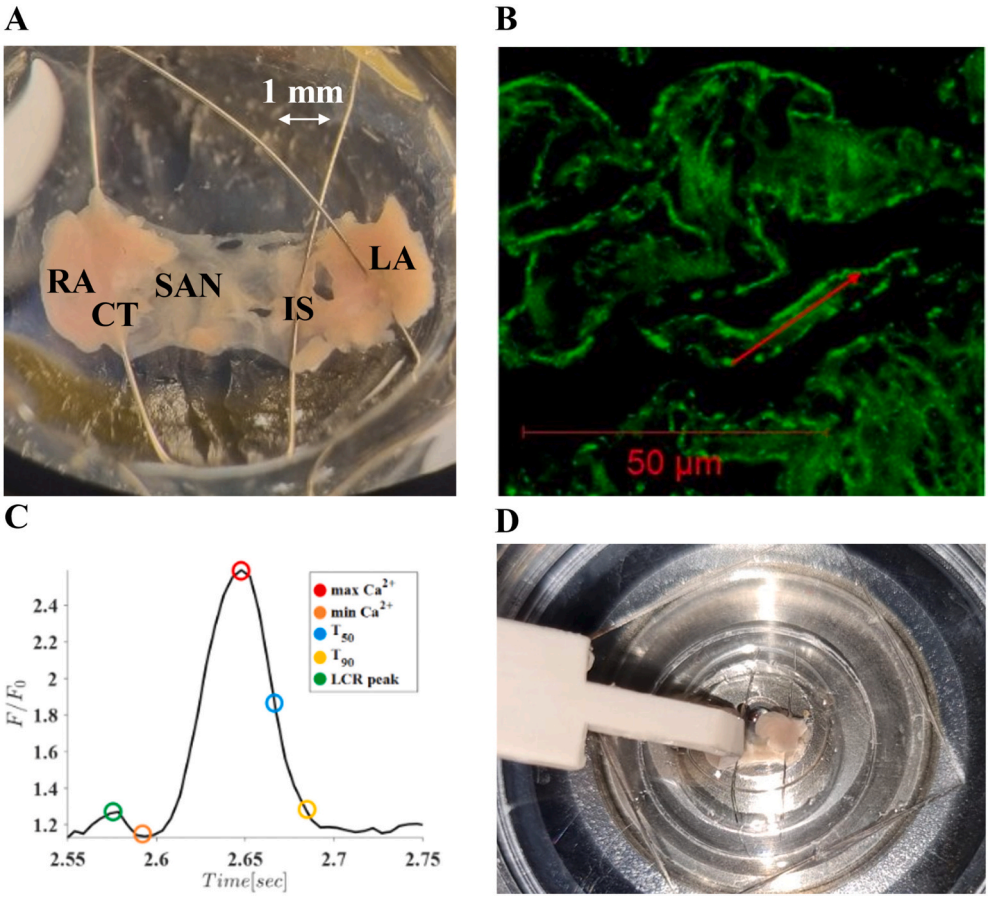


**Fig. 1.** (A) SAN preparation comprised of the right and left atria (RA, LA, respectively) flattened down by pins clamping the crista terminalis (CT) and the interatrial septum (IS). (B). Line-scan confocal image captured along a pacemaker cell within the SAN tissue. (C) Identification of the maximal $Ca^{2+}$ transient amplitude (max $Ca^{2+}$), diastolic $Ca^{2+}$ (min $Ca^{2+}$), 50 % relaxation time ($T_{50}$), 90 % relaxation time ($T_{90}$) and local $Ca^{2+}$ release (LCR) peak using "Sparkalyzer". (D) A motor-controlled hook was attached to the CT pin and operated to gradual stretch the SAN tissue.

LSM880 confocal microscope using a 40×/1.2 water immersion lens. Tissues were excited with a 488 nm Argon laser and emission was collected with LP 505 nm. Images were acquired in line scan mode (1.22 ms per scan; pixel size, 0.01 μm) along the pacemaker cells within the primary pacemaker tissue (Fig. 1B). The cells were identified by their spontaneous beating under imaging mode. Baseline recordings were acquired from multiple regions in the tissue to ensure a uniform BI among cells residing in the SAN tissue. Tissues with a BI longer than 500 ms were not included in the analysis due to more the risk of damaged preparations [28].

### 2.4. $Ca^{2+}$ analysis

$Ca^{2+}$ analysis was performed using a modified version of the software "Sparkalyzer" [29]. The fluorescence signal (F) was divided by the minimal value between beats ($F_0$). $Ca^{2+}$ transients were semi-automatically detected and $Ca^{2+}$ sparks were manually marked. BI of each cell residing in the SAN tissue was calculated as the average time between $Ca^{2+}$ transient peaks, and the BI variability was calculated as its coefficient of variance (CV). $Ca^{2+}$ transient amplitude, time to peak, 50 % and 90 % relaxation time ($T_{50}$ and $T_{90}$, respectively) and LCR parameters were automatically calculated by the software as described

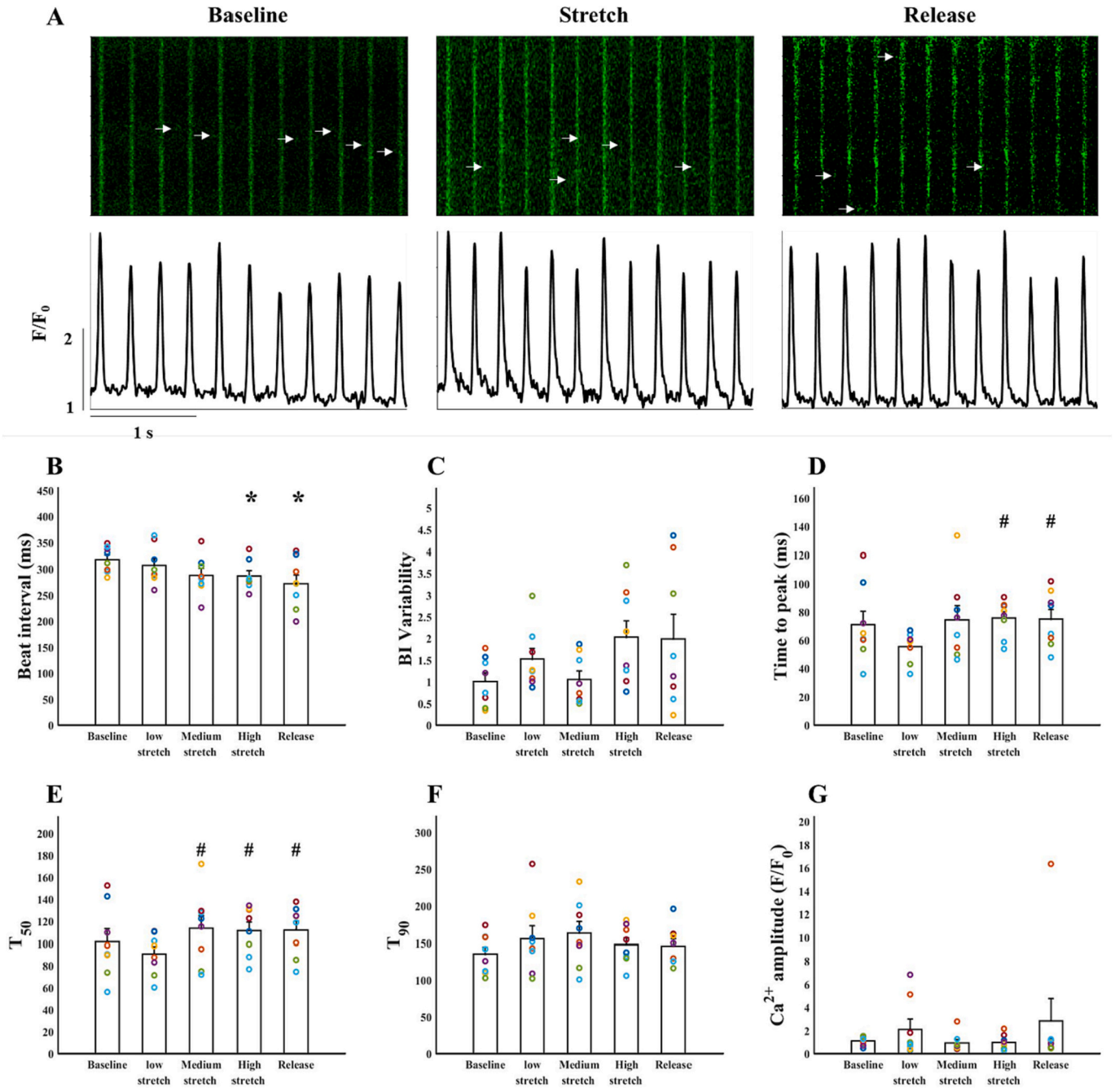


**Fig. 2.** Stretch leads to a positive chronotropic response in mouse sinoatrial node tissue. (A) Representative time course of $Ca^{2+}$ transients in pacemaker cells residing in the SAN before (Baseline), during (high Stretch), and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The effect of gradual stretch and its release on (B) beat interval (C) beat interval variability, (D) $Ca^{2+}$ transient time to peak, (E) 50 % $Ca^{2+}$ transient relaxation time, (F) 90 % $Ca^{2+}$ transient relaxation time and (G) $Ca^{2+}$ transient amplitude. $*p < 0.05$ compared to baseline, (number of cells = 8).

before [29] (Fig. 1C).

### 2.5. SAN mechanical stretch

The baseline tissue length was measured as the initial distance between the right and left pins (3 ± 0.3 mm). After stretch the tissue returned to its initial length. Following baseline recording, a linear motor was connected to the left pin, which clamped the crista terminalis, through a custom-made hook, while the right pin, where the interatrial septum is located, remained fixed (Fig. 1D), and was activated by a custom-made Arduino software to gradually stretch the tissue to the following levels: low stretch (5–10 % lengthening), medium stretch (10–20 %) and high stretch (20–40 %). Higher levels of stretch led to complete cessation of the spontaneous beats. Recordings were acquired 30 s after each level. Due to tissue movement, different cells residing in the SAN tissue were recorded in each step. After the maximal stretch was applied, the hook was raised, and the tissue was released back to its initial length. In some cases, high stretch led to pauses in or unstable beating rate, which were resolved as soon as the tissue was released.

### 2.6. Drugs

H-89 dihydrochloride hydrate (10 μM), KN-93 (3 μM), KN-92 (3 μM), cyclopiazonic acid (CPA; 5 μM) and blebbistatin (BLB; 5–10 μM) were

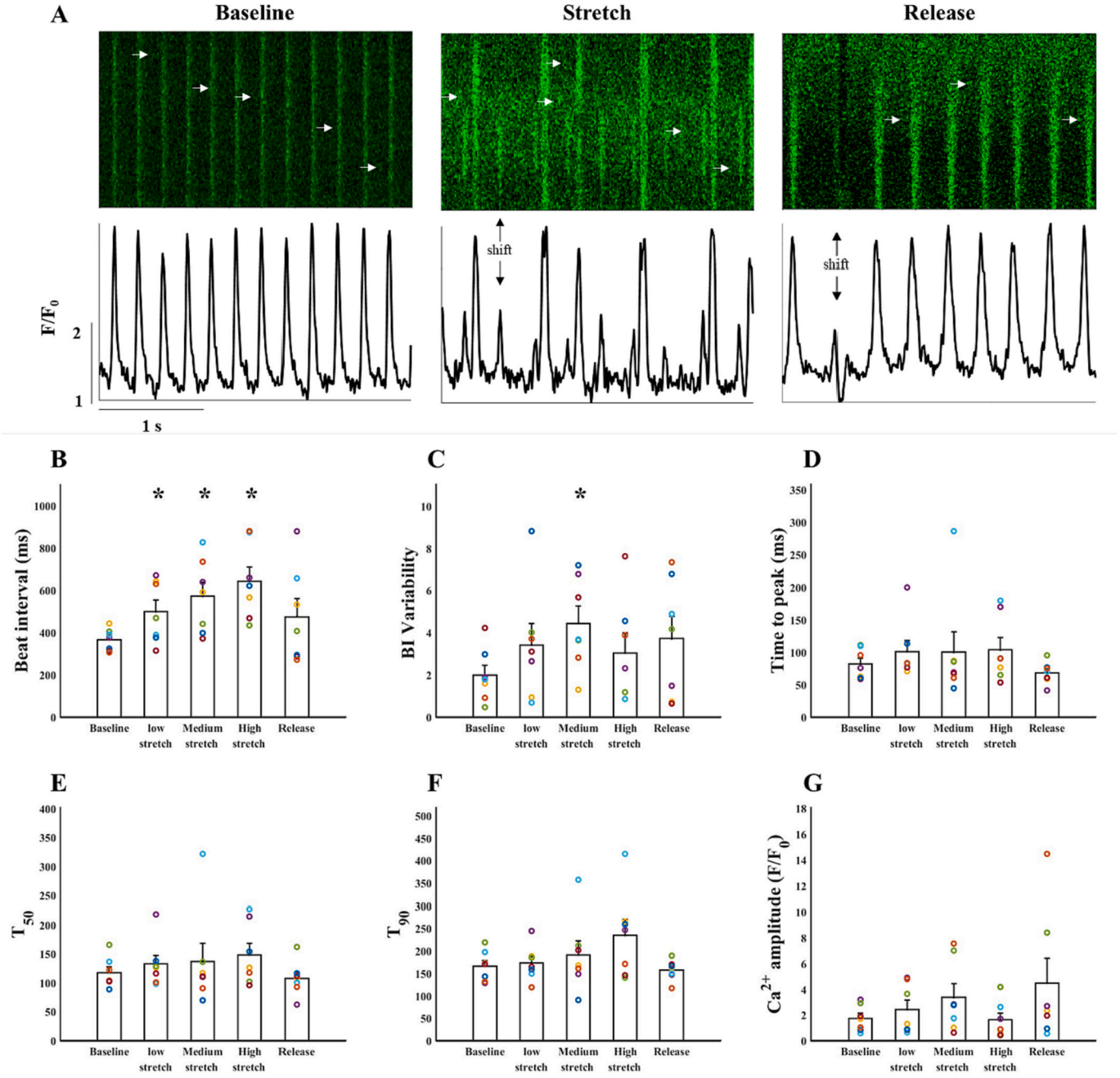


**Fig. 3.** Stretch leads to a negative chronotropic response in mouse sinoatrial node tissue. (A) Representative time course of $Ca^{2+}$ transients in pacemaker cells residing in the SAN before (Baseline), during (high Stretch), and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The shift shows the shift in beat interval. The effect of gradual stretch and its release on (B) beat interval (C) beat interval variability, (D) $Ca^{2+}$ transient time to peak, (E) 50 % $Ca^{2+}$ transient relaxation time, (F) 90 % $Ca^{2+}$ transient relaxation time and (G) $Ca^{2+}$ transient amplitude. *$p < 0.05$ compared to baseline, (number of cells = 7).

purchased from Sigma Aldrich. Fluo-4 AM (30 μM) was purchased from Thermo Fisher Scientific. All drugs were dissolved in DMSO as stock solution, stored at −20 °C and diluted in Tyrode solution before being applied to the SAN tissue.

### 2.7. Statistics

Experimental results are presented as mean ± SEM. Statistical comparisons were performed with paired or unpaired Student's *t*-tests and one-way ANOVA. Differences were indicated as statistically significant at $p$ value $< 0.05$.

## 3. Results

### 3.1. Stretch of SAN tissue has a dual effect on the spontaneous firing rate

SAN tissues were gradually stretched to three levels of strain. In 8/15 samples, the stretch led to a positive chronotropic response, while in 7/15 tissues, a negative chronotropic response was observed. Fig. 2A shows a representative example of $Ca^{2+}$ transients measured in a SAN tissue with a positive chronotropic response to high stretch. In this group of SAN tissues, a gradual increase in stretch led to a gradual shortening of the BI, which became significant at high level of stretch (Fig. 2B). Upon release, BI did not return to its basal state, but, rather, to a shorter BI. Although the average BI was shortened in response to stretch, no change in BI variability was measured (Fig. 2C). SAN tissues with a positive chronotropic response to stretch showed no changes in time to peak (Fig. 2D), $T_{50}$ (Fig. 2E), $T_{90}$ (Fig. 2F) or $Ca^{2+}$ amplitude (Fig. 2G), in response to stretch or after its release.

Fig. 3A shows a representative example of $Ca^{2+}$ transients measured in a SAN tissue with a negative chronotropic response to stretch. Upon application of stretch, firing rates were unsynchronized between cells residing in the SAN tissue. Additionally, a gradual increase in stretch level led to a gradual prolongation of the BI, which was significant even at low stretch levels (Fig. 3B). While BI returned to its basal state in most SAN tissues after stretch release, it remained high and unsynchronized between pacemaker cells residing in the SAN tissue in some tissues. In parallel to prolongation of the average BI, the BI variability increased and became significant at mid-stretch (Fig. 3C), while it returned to basal at a higher stretch level and after its release. However, no change was observed in time to peak (Fig. 3D), $T_{50}$ (Fig. 3E), $T_{90}$ (Fig. 3F) or $Ca^{2+}$ amplitude (Fig. 3G) in SAN tissues with a negative chronotropic response to stretch.

Note that under basal conditions, there was a marginal difference in both BI ($p = 0.04$) and BI variability ($p = 0.05$) between SAN tissues with a positive versus a negative chronotropic response to stretch.

### 3.2. Stretch of SAN tissue leads to a dual effect on the local $Ca^{2+}$ release parameters

Because LCRs are the main mechanism that couples between the clocks controlling BI [9], and LCR period correlates with the degree of clock coupling [30], LCR parameters in response to stretch were measured and compared between both SAN tissues with a positive and SAN tissues with a negative chronotropic response. In SAN tissues with a positive chronotropic response to stretch, increases in the level of stretch gradually shortened the LCR period, even at low levels of stretch (Fig. 4A). Upon release, the LCR period was shorter than the basal LCR period. No changes in the normalized amplitude (Fig. 4B), LCR length (Fig. 4C) or 50 % LCR duration (Fig. 4D) were observed. In SAN tissues with a negative chronotropic response to stretch, increases in the level of stretch gradually prolonged LCR period, with significant changes at high stretch only (Fig. 4E). Upon release, the LCR period returned to the basal LCR period. No changes in the normalized amplitude (Fig. 4F) or LCR length (Fig. 4G) were observed. However, at a high stretch level, 50 % LCR duration (Fig. 4H) was prolonged.

Note that under basal conditions, there was a difference in LCR period ($p = 0.025$) between SAN tissues with a positive versus SAN tissues with a negative chronotropic response to stretch.

### 3.3. Eliminating the mechano-electrical feedback eliminates the dual effect of stretch on SAN function

Because the mechano-electrical feedback was suggested as a mechanism that synchronized the cells residing in the SAN tissue, 10 μM blebbistatin was applied to inhibit SAN pacemaker cell contractions. Note that 5 μM blebbistatin did not eliminate SAN contractions. Fig. 5A shows a representative example of $Ca^{2+}$ transients in a stretched SAN tissue treated with blebbistatin. In all 7 tested tissues, stretch at all levels had no effect on the average BI (Fig. 5B) or BI variability (Fig. 5C). Moreover, $Ca^{2+}$ transient time to peak (Fig. 5D), $T_{50}$ (Fig. 5E) or $Ca^{2+}$ amplitude (Fig. 5F) were not affected. However, $T_{90}$ was prolonged at low and high levels of stretch (Fig. 5G). After release, BI or global $Ca^{2+}$ parameters returned to their basal values. Finally, no change in LCR period (Fig. 5H), LCR normalized amplitude (Fig. 5I), LCR length (Fig. 5J) or 50 % LCR duration (Fig. 5K) were observed.

### 3.4. Disabling $Ca^{2+}$ cycling affects the mechano-electrical feedback

Because $Ca^{2+}$ regulates the myofilament activity, and the sarcoplasmic reticulum (SR) is the main internal $Ca^{2+}$ store in the cell, the SR $Ca^{2+}$ ATPase (SERCA) was inhibited by addition of 5 μM CPA before stretch. This treatment prolonged the BI (662 ± 59 ms compared to 418 ± 25 ms). In 7/12 tissues tested, stretch under these conditions led to a positive chronotropic response, while in 5/12 tissues, no significant chronotropic response was observed.

Fig. 6A shows a representative example of $Ca^{2+}$ transients measured in a CPA-treated SAN tissue which showed a positive chronotropic response to stretch. In this group of tissues, a gradual increase in stretch led to gradual shortening of BI, which became significant already at medium stretch level (Fig. 6B) in contrast to the significant shortening of BI only at a high level of stretch in the absence of CPA. Note that upon release, BI returned to its pre-stretch level, in contrast to the return to a shorter BI in the control group. Although the average BI was shortened in response to stretch, no changes in BI variability (Fig. 6C), time to peak (Fig. 6D), $T_{50}$ (Fig. 6E), $T_{90}$ (Fig. 6F) or $Ca^{2+}$ amplitude (Fig. 6G) were observed in response to stretch application or after its release. CPA prolonged the LCR period (Fig. 6H), while medium and high levels of stretch led to its shortening. This was in contrast to the significant shortening reached at low level in no-drug state. Upon release, the LCR period returned to its pre-stretch state. LCR normalized amplitude (Fig. 6I), LCR length (Fig. 6J) or 50 % LCR duration (Fig. 6K) were maintained under the three tested stretch levels and upon their release.

Fig. 7A shows a representative example of $Ca^{2+}$ transients measured in a CPA-treated SAN tissue which showed no chronotropic response to stretch. Few tissues in this group exhibited an unsynchronized firing rate among their cells residing in the SAN tissue upon application of stretch. In all sampled tissues, stretch at different levels had no effect on the average BI (Fig. 7B) or BI variability (Fig. 7C). Moreover, it had no impact on $Ca^{2+}$ transient time to peak (Fig. 7D), $T_{50}$ (Fig. 7E), $T_{90}$ (Fig. 7F) or $Ca^{2+}$ amplitude (Fig. 7G). CPA prolonged the LCR period (Fig. 7H), while later application of stretch had no further effect on the period. LCR normalized amplitude (Fig. 7I) and 50 % LCR duration (Fig. 7J) were maintained under all applied stretch conditions and their release. However, LCR length (Fig. 7K) increased upon stretch and after release.

### 3.5. PKA activity does not mediate the chronotropic response to stretch

Because myofilament activity is controlled by PKA activity, the effect of PKA inhibition with 10 μM H-89 was measured. Application of H-89 prolonged SAN tissue BI (361 ± 35 ms compared to 570 ± 61 ms). Upon

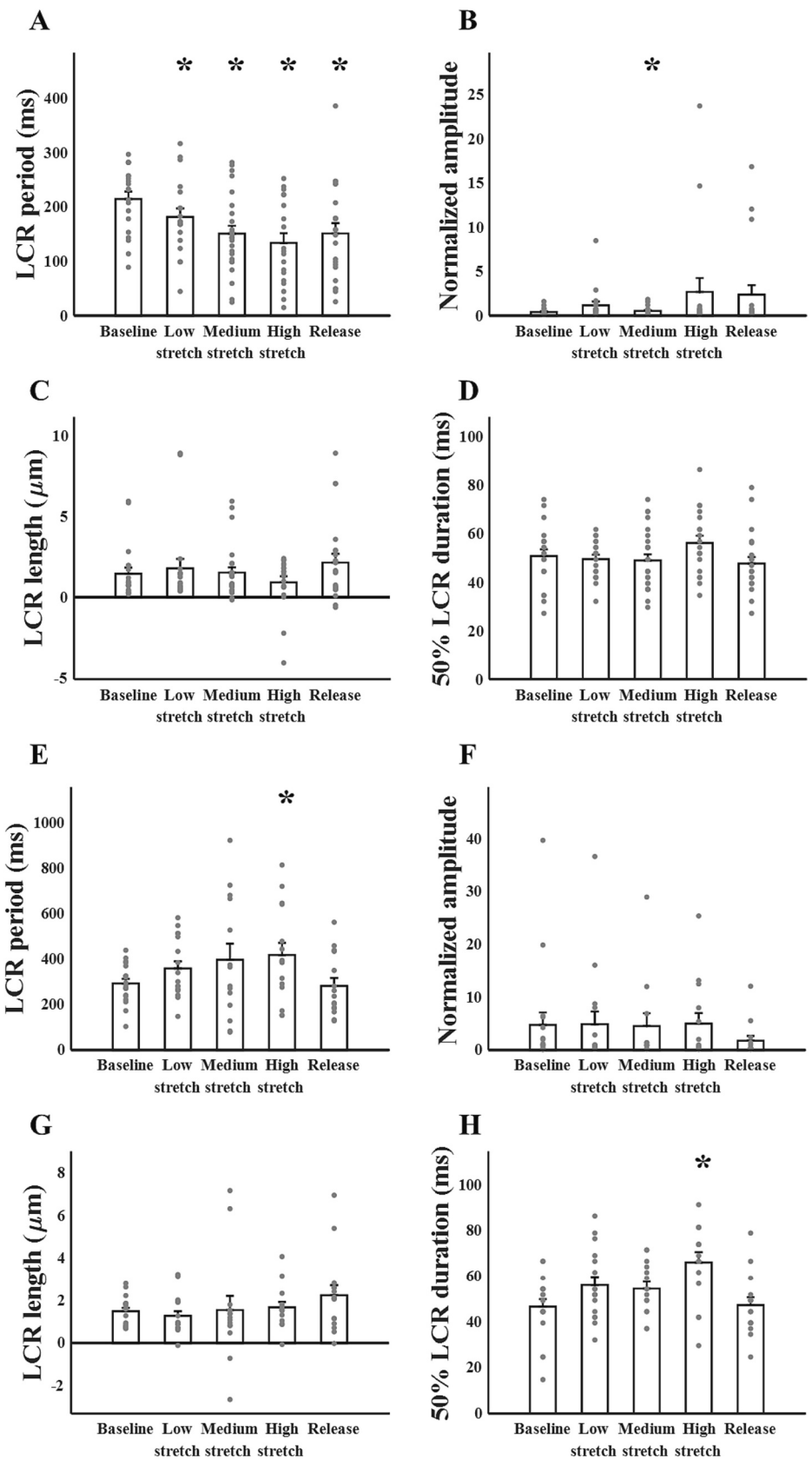


**Fig. 4.** Stretch leads to a change in local $Ca^{2+}$ release (LCR) in mouse sinoatrial node tissues. The effect of gradual stretch and its release on (A) LCR period, (B) LCR normalized amplitude, (C) LCR length and (D) 50 % LCR duration in tissues with a positive chronotropic response ($N = 8$). The effect of gradual stretch and its release on (E) LCR period, (F) LCR normalized amplitude, (G) LCR length and (H) 50 % LCR duration in tissues with a negative chronotropic response (number of cells = 7). *$p < 0.05$ compared to baseline.

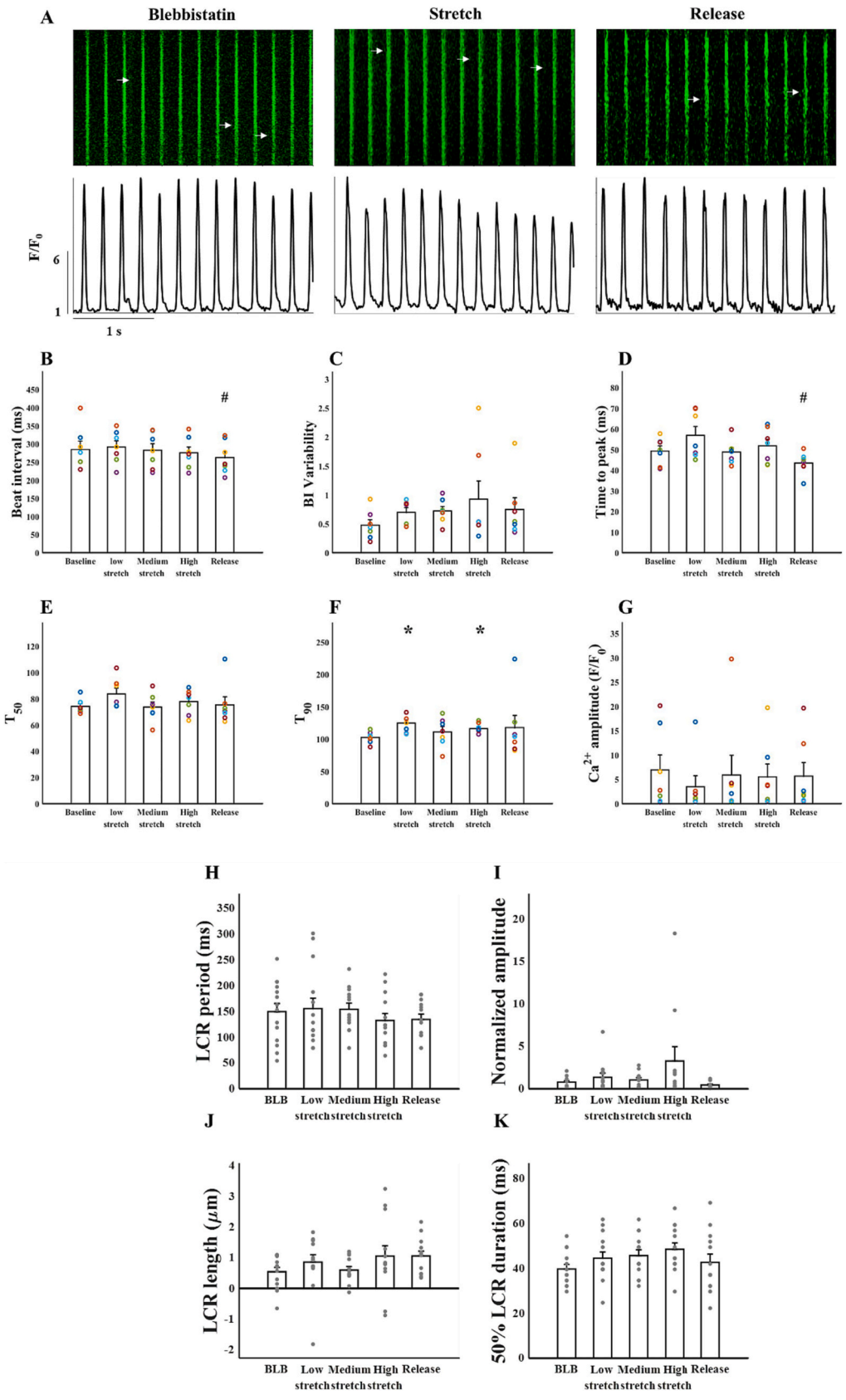


**Fig. 5.** Elimination of mechano-electrical feedback disabled the positive and negative chronotropic response in mouse sinoatrial node tissue. (A) Representative time course of $Ca^{2+}$ transients in blebbistatin-treated pacemaker cells residing in the SAN before (Baseline), during (high Stretch), and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The effect of gradual stretch and its release on (B) beat interval, (C) beat interval variability, (D) $Ca^{2+}$ transient time to peak, (E) 50 % $Ca^{2+}$ transient relaxation time, (F) 90 % $Ca2^{+}$ transient relaxation time, (G) $Ca^{2+}$ transient amplitude, (H) LCR period, (I) LCR normalized amplitude, (J) LCR length and (K) 50 % LCR duration. $^{*}p < 0.05$ compared to baseline, $^{\#}p < 0.05$ compared to blebbistatin, (number of cells = 7).

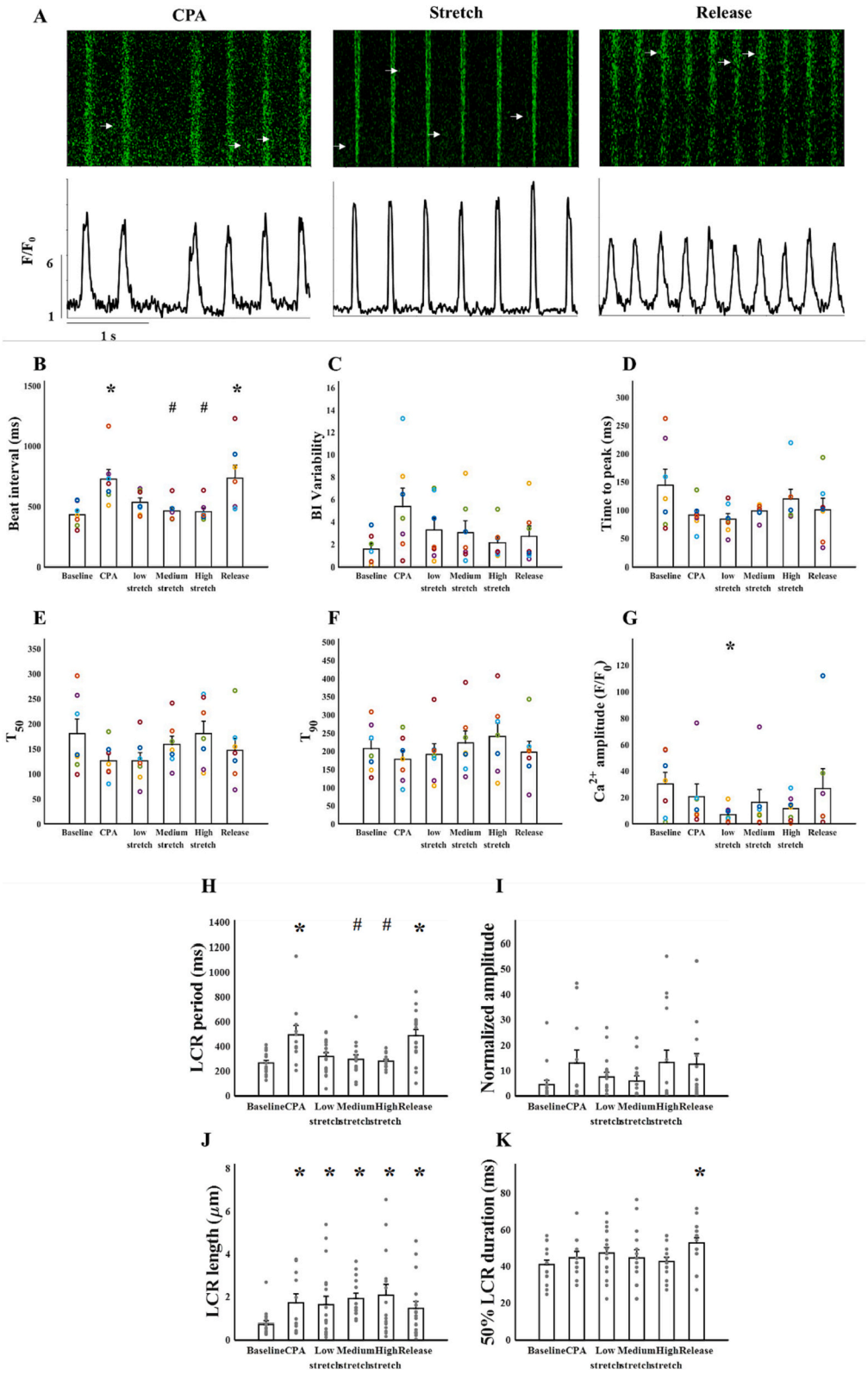


**Fig. 6.** Disabling $Ca^{2+}$ cycling does not affect the positive chronotropic response to stretch. (A) Representative time course of $Ca^{2+}$ transients in CPA-treated pacemaker cells residing in the SAN before (CPA), during (high Stretch) and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The effect of gradual stretch and its release on (B) beat interval, (C) beat interval variability, (D) $Ca^{2+}$ transient time to peak, (E) 50 % $Ca^{2+}$ transient relaxation time, (F) 90 % $Ca^{2+}$ transient relaxation time, (G) $Ca^{2+}$ transient amplitude, (H) LCR period, (I) LCR normalized amplitude, (J) LCR length and (K) 50 % LCR duration. $^{*}p < 0.05$ compared to baseline, $^{\#}p < 0.05$ compared to CPA, (number if cells = 7).

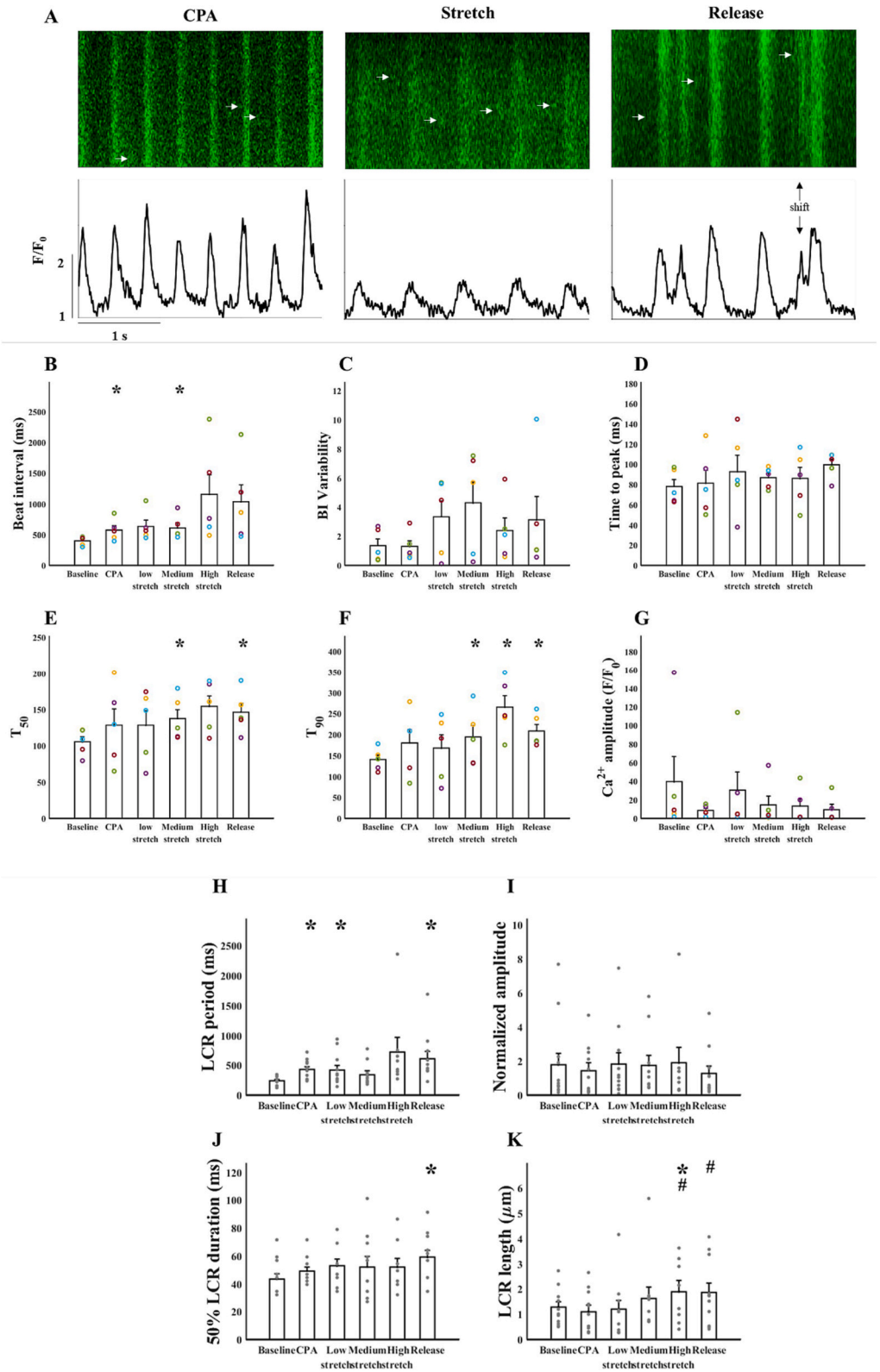


**Fig. 7.** Disabling $Ca^{2+}$ cycling eliminates the negative chronotropic response to stretch. (A) Representative time course of $Ca^{2+}$ transients in CPA-treated pacemaker cells residing in the SAN before (CPA), during (high Stretch), and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The shift shows the shift in beat interval. The effect of gradual stretch and its release on (B) beat interval, (C) beat interval variability, (D) $Ca^{2+}$ transient time to peak, (E) 50 % $Ca^{2+}$ transient relaxation time, (F) 90 % $Ca^{2+}$ transient relaxation time, (G) $Ca^{2+}$ transient amplitude, (H) LCR period, (I) LCR normalized amplitude, (J) LCR length and (K) 50 % LCR duration. *$p < 0.05$ compared to baseline, $^{\#}p < 0.05$ compared to CPA, (number of cells = 5).

application of stretch, 8/12 tissues showed a positive chronotropic response, while 4/12 tissues showed a negative chronotropic response.

Fig. 8A shows a representative example of $Ca^{2+}$ transients measured in a H-89-treated SAN tissue which showed a positive chronotropic response to stretch. In this group of tissues, a gradual increase in stretch level led to gradual shortening of BI, which became significant already at medium stretch level (Fig. 8B) in contrast to the high stretch level required in the absence of H-89. Note that upon release, the tissues returned to their pre-stretch BI, while control samples showed a shorter BI. In parallel to shortened average BI in response to stretch in this group, BI variability increased (Fig. 8C). Fig. 8D shows a representative example of $Ca^{2+}$ transients measured in a H-89-treated SAN tissue which showed a negative chronotropic response to stretch. In this group of tissues, a gradual increase in stretch level prolonged the BI and became significant at high level (Fig. 8E). BI variability (Fig. 8F) and the other $Ca^{2+}$ parameters were not affected by stretch.

### 3.6. CaMKII activity does not mediate the chronotropic response to stretch

Because excitation-contraction coupling is also controlled by CaMKII activity, the effect of its reduced activity was tested by addition of 3 μM KN-93. Application of KN-93 (without stretch) prolonged the BI (387 ± 24 ms compared to 671 ± 69 ms). In 5/12 tissues, the stretch led to a positive chronotropic response, while in 7/12 tissues, a negative chronotropic response was observed.

Fig. S1A shows a representative example of $Ca^{2+}$ transients measured in a KN-93-treated SAN tissue which showed a positive chronotropic response to stretch. In this group of tissues, a gradual increase in stretch led to a gradual shortening of BI, which became significant at a high stretch level (Fig. S1B), similar to the response of control tissues. Note that upon release, these tissues returned to their pre-stretch BI, in contrast to the shorter BI measured upon release of tissues in the no-drug group. Although the average BI was shortened in response to stretch, no change in BI variability was measured upon stretch (Fig. S1C). Fig. S1D shows a representative example of $Ca^{2+}$ transients measured in a KN-93-treated SAN tissue which showed a negative chronotropic response to stretch. In this group of tissues, a gradual increase in stretch led to BI prolongation which became significant at a medium stretch level (Fig. S1E), in contrast to the low-level stretch required to obtain significant BI prolongation in the absence of KN-93. While the average BI was affected by stretch, BI variability did not change (Fig. S1F), in contrast to the no-drug negative chronotropic group, that showed an increase in BI variability at a medium stretch level.

SAN tissues treated with KN-92 showed responses similar to those measured in no-drug controls (Fig. S2).

## 4. Discussion

The present study investigated the mechanisms that link stretch to changes in BI and to shifts in the leading pacemaker. Stretch was found to either shorten or prolong the BI and led to heterogeneity in BI in the pacemaker cells within SAN tissue. These observations support the first hypothesis that the nature of the chronotropic response to stretch depends on the heterogeneity among pacemaker cells residing in the SAN tissue. In addition, LCR characteristics were affected, even at low stretch levels, before significant changes were measured in BI. The pattern of changes in LCR characteristics was associated with the direction of the chronotropic response, as hypothesized. Inhibition of cell that residing in the SAN tissue contraction with blebbistatin, eliminated the effect of stretch on the spontaneous BI, supporting the third hypothesis that mechano-electric feedback mediates the dual effect of stretch in mouse SAN.

Stretch can either lead to positive or negative chronotropic responses. It was shown before that stretch of mouse SAN induces variable chronotropic responses [11]. Here, for the first time, the mechanisms that lead to these differences was explored by separately analyzing mouse SAN with a positive versus a negative chronotropic response. Multiple rhythms were recorded in SAN tissues with a negative response, suggesting shifts in the leading pacemaker site, and may have been the cause for the negative chronotropic response in some of the samples [31]. It is not known whether the heterogeneity of the SAN cell population is the cause or associated effect of the negative response. Note, before stretch, no heterogeneity was observed among cells residing in the SAN tissue.

LCR characteristics were affected even at low stretch level, conditions under which no significant change was observed in BI, and were associated with the direction of the chronotropic response. LCR period is an index of the coupled-clock coupling [30]. Shortening of the LCR period documented here in tissues with a positive chronotropic response suggest increased feedback between the clocks, that allows more $Ca^{2+}$ to activate the $Na^{+}$-$Ca^{2+}$ exchanger (NCX) [9] and other $Ca^{2+}$-dependent channels [32], both of which lead to a faster diastolic depolarization phase. Prolongation of the LCR period documented here in tissues with a negative chronotropic response leads to the opposite effect. The presence of multiple rhythms in tissues with a negative chronotropic response was suggested to be associated with non-heterogeneous LCR periods [4]. Taken together, the degree of clock coupling is non-heterogeneous in tissues with a negative chronotropic response. These changes in LCR period were induced at lower stretch levels than changes in BI, which suggests that changes in LCR determine the degree of clock coupling that determines the direction of the chronotropic response. In contrast to LCR period, no change in $Ca^{2+}$ parameters were obtained. Because $T_{90}$ is an indicator of SR load [33], it is possible that the SR load does not change in response to stretch and changes in LCR are by mediating between the myofilament, $Na^{+}$-$Ca^{2+}$ exchanger and other $Ca^{2+}$-dependent membrane channel. Future experiments are needed to prove this theory.

Eliminating the mechano-electrical feedback by inhibiting myofilament contraction disabled the chronotropic effect of stretch. Blebbistatin did not affect the BI; thus, under basal conditions, elimination of the mechano-electrical feedback on SAN function has a minor effect. In contrast, under stretch conditions, elimination of the mechano-electrical feedback disabled the negative chronotropic effect and the heterogeneity of BI, suggesting that force mediates cell-to-cell connections, which results in heterogeneity among cells residing in the SAN. Eliminating the mechano-electrical feedback also disabled the positive chronotropic effect, which implies that feedback between LCRs and myofilaments, the major buffers of $Ca^{2+}$ in the cell, regulates $Ca^{2+}$-dependent channels and exchangers.

Inhibition of SERCA altered the mechano-electrical feedback and eliminated the negative chronotropic response to stretch. Under CPA conditions, the BI was already shortened at a medium level of stretch. A previous work showed that the negative chronotropic effect can be eliminated by blocking rapidly activating potassium currents [11]. Thus, it is possible that lengthening the plateau of the mouse SAN action potential may also eliminate the cell-to-cell connections that lead to heterogeneity among cells residing in the SAN. In contrast to the effect of CPA, inhibition of either PKA or CaMKII eliminated neither the negative nor the positive chronotropic response to stretch. Although both signaling cascades affect the myofilament activity [34,35], these observations suggest that their role in mechano-electrical feedback is minor.

Upon release, the BI in tissues with a positive chronotropic response did not immediately return to its initial value. In contrast, in most tissues with a negative chronotropic response, BI returned to its baseline values, but sometimes still showed heterogeneity. Such behavior suggests a memory effect in the system. It was shown before the coupled-clock system immediately responds to changes in the conditions of single SAN cells [36,37]. Thus, it is possible that other mechanisms, external to the pacemaker cell, are responsible for the memory phenomenon.

While the present work found only marginal differences in BI before stretch between the positive and negative chronotropic groups, distinct

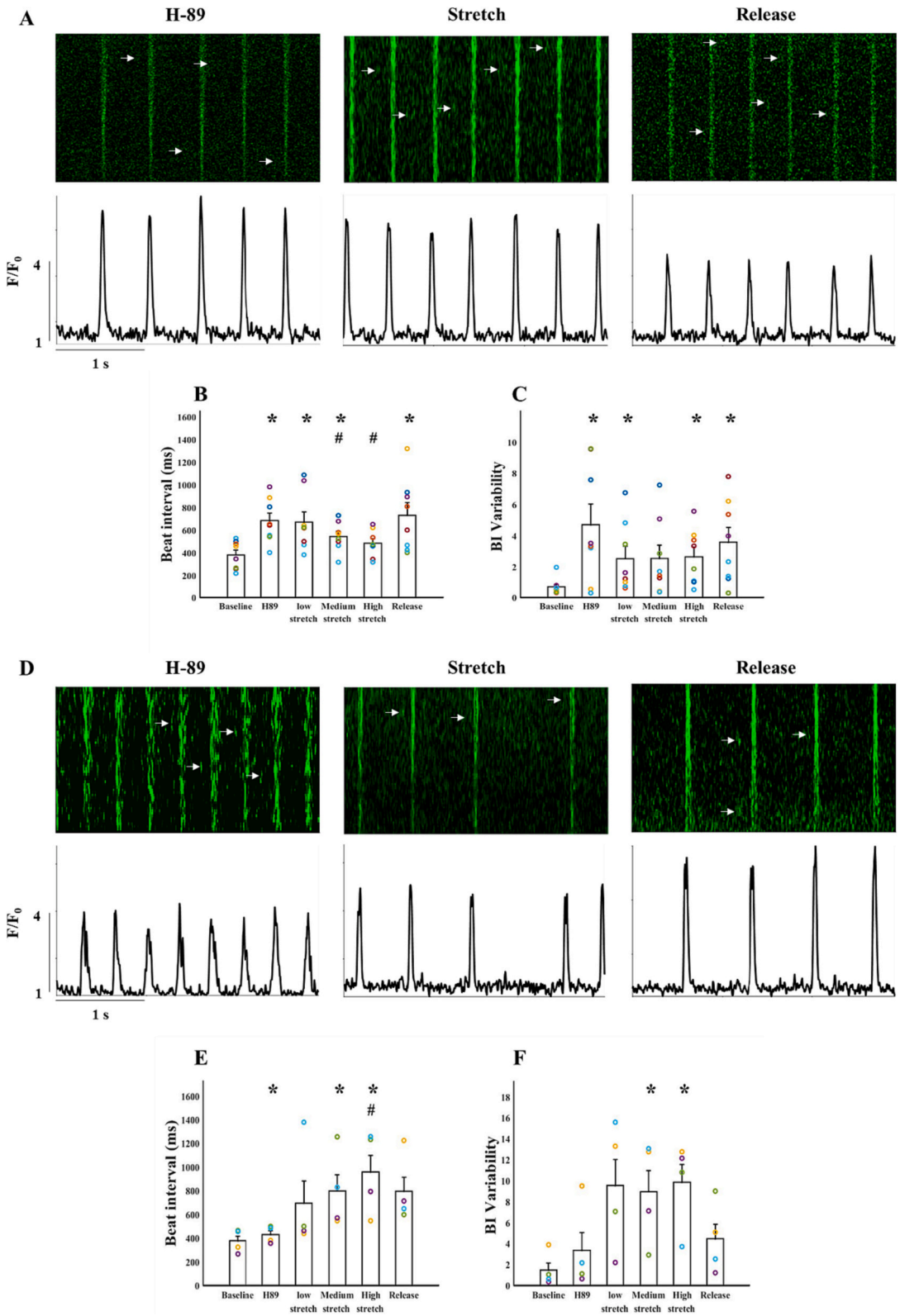


**Fig. 8.** Inhibition of PKA activity does not affect the chronotropic response to stretch. (A) Representative time course of $Ca^{2+}$ transients in H-89-treated pacemaker cells residing in the SAN before (H-89), during (high Stretch), and upon release and return to the baseline length of (Release) stretch application. Recordings were acquired 30 s after each level. The arrow shows the appearance of local $Ca^{2+}$ release. The effect of gradual stretch and its release on beat interval (B) and beat interval variability (C) in tissues with a positive chronotropic response to stretch (number of cells = 8). (D) Representative time course of $Ca^{2+}$ transients in pacemaker cells residing in the SAN before (H-89), during (Stretch (high level)) and after (Release) stretch application in a tissue with a negative chronotropic response to stretch. The effect of gradual stretch and its release on beat interval © and beat interval variability (F) in tissues with negative chronotropic response to stretch ($N = 4$). *$p < 0.05$ compared to baseline, $^{\#}p < 0.05$ compared to H-89.

intergroup differences were noted in the LCR period. These results imply that the degree of clock coupling, which is represented by the LCR [30], differs between the groups, and thus, theoretically, pre-classification can be performed to predict which tissue will show a positive versus a negative response.

Stretch resulting from increased venous return to the heart has been documented in heart failure patients [38]. The stretch affects right atrial filling, distending the atrial wall where the SAN is located. Thus, it is possible that stretch is involved in SAN dysfunction in heart failure. In line with this hypothesis, a preoperative analysis of right atrial pressure and SAN function in children scheduled to undergo a Fontan operation, showed that SAN dysfunction was absent in patients presenting normal right atrial pressure, but present in those with elevated right atrial pressure [39].

### 4.1. Limitations

We measured here the short-term memory effect of stretch on SAN tissues that had either a positive and negative chronotropic response. It is possible that long-term memory effects exist in negative chronotropic response. Because it is challenging to perform multiple scans on the same SAN tissue, future experiments with different techniques will be used to measure such potential effects on beat interval.

The multiple rhythms that appear in the tissues with a negative chronotropic response suggest shifts in the leading pacemaker site in part of the sample. Future experiments in which the electrical activity is measured in parallel, will be needed to prove this theory.

## 5. Summary

The mechano-electric feedback mediates the dual effect of stretch on mouse SAN tissue. Eliminating the $Ca^{2+}$ effect on the mechano-electrical feedback abolishes the negative chronotropic effect.

## Disclosures

None.

## Declaration of competing interest

None.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jmccpl.2023.100042.

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