

## Reduced aerobic capacity causes leaky ryanodine receptors that trigger arrhythmia in a rat strain artificially selected and bred for low aerobic running capacity

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### Abstract

**Aim:** Rats selectively bred for inborn low capacity of running (LCR) display a series of poor health indices, whereas rats selected for high capacity of running (HCR) display a healthy profile. We hypothesized that selection of low aerobic capacity over generations leads to a phenotype with increased diastolic Ca<sup>2+</sup> leak that trigger arrhythmia.

**Methods:** We used rats selected for HCR (*N* = 10) or LCR (*N* = 10) to determine the effect of inborn aerobic capacity on Ca<sup>2+</sup> leak and susceptibility of ventricular arrhythmia. We studied isolated Fura-2/AM-loaded cardiomyocytes to detect Ca<sup>2+</sup> handling and function on an inverted epifluorescence microscope. To determine arrhythmogenicity, we did a final experiment with electrical burst pacing in Langendorff-perfused hearts.

**Results:** Ca<sup>2+</sup> handling was impaired by reduced Ca<sup>2+</sup> amplitude, prolonged time to 50% Ca<sup>2+</sup> decay and reduced sarcoplasmic reticulum (SR) Ca<sup>2+</sup> content. Impaired Ca<sup>2+</sup> removal was influenced by reduced SR Ca<sup>2+</sup>ATP-ase 2a (SERCA2a) function and increased sodium/Ca<sup>2+</sup> exchanger (NCX) in LCR rats. Diastolic Ca<sup>2+</sup> leak was 87% higher in LCR rats. The leak was reduced by CaMKII inhibition. Expression levels of phosphorylated threonine 286 CaMKII levels and increased RyR2 phosphorylation at the serine 2814 site mechanistically support our findings of increased leak in LCR. LCR rats had significantly higher incidence of ventricular fibrillation.

**Conclusion:** Selection of inborn low aerobic capacity over generations leads to a phenotype with increased risk of ventricular fibrillation. Increased phosphorylation of CaMKII at serine 2814 at the cardiac ryanodine receptor appears as an important mechanism of impaired Ca<sup>2+</sup> handling and diastolic Ca<sup>2+</sup> leak that results in increased susceptibility to ventricular fibrillation.

**Keywords** aerobic capacity, arrhythmia, calcium.

The strong statistical association between low aerobic capacity and increased risk for development of complex diseases is suggestive of a causal link. In 1996,

Koch and Britton started testing for such linkage by selectively breeding to produce low and high strains of rats that contrast for inborn (i.e. untrained) aerobic

endurance treadmill running capacity (Koch & Britton 2001).

The hypothesis that rats selected for low capacity (low-capacity runners: LCR) would display negative health indices and the rats selected for high capacity (high-capacity runners: HCR) would have positive health indices. After 11 generations of selection, the LCR developed characteristics of the human condition known as metabolic syndrome relative to the HCR; the LCR rats have impaired cardiac function, elevated mean blood pressure, depressed endothelial function, insulin resistance, impaired glucose tolerance, increased visceral adiposity, higher plasma triglycerides and elevated plasma free fatty acids (Wisloff *et al.* 2005).

Consistent with a poor health indices, the LCR also demonstrate diminished longevity compared with the HCR. Generation 20 of the LCR group died at a median age of 24.3 months and HCR died at 34.7 months, representing a 45% (10.7 months) difference in longevity (Koch *et al.* 2011). Increased RyR2  $\text{Ca}^{2+}$  sensitivity that leads to increased frequency of spontaneously  $\text{Ca}^{2+}$  release from the SR during diastole seems to be a central feature of cardiac dysfunction and heart failure (Ai *et al.* 2005). This increase in spontaneous  $\text{Ca}^{2+}$  release may in turn trigger delayed after depolarization (DAD) (Pogwizd *et al.* 2001), ventricular arrhythmias and sudden cardiac arrests (Anderson 2004, Sag *et al.* 2009, van Oort *et al.* 2010).

Here, we tested the hypothesis that contrasting for low intrinsic aerobic capacity over generations leads to a phenotype with increased  $\text{Ca}^{2+}$  leak over the ryanodine receptors that causes a more arrhythmogenic heart. Indeed, hearts from LCR demonstrated marked susceptibility to induction of ventricular fibrillation relative to hearts from HCR. Furthermore, in heart failure, the activity of CaMKII is upregulated (Hoch *et al.* 1999, Kirchhefer *et al.* 1999), and several studies support that this may cause hyperphosphorylation of the ryanodine receptors 2 (RyR2) in the heart that causes spontaneously  $\text{Ca}^{2+}$  release. Our results support the notion that increased phosphorylation of RyR2 by CaMKII causes increased  $\text{Ca}^{2+}$  leak and susceptibility to ventricular arrhythmia in LCR rats.

## Materials and methods

The animals used in this study were senescent rats (2 years of age) artificially selected and bred for high aerobic running capacity over 21 generations (HCR,  $N = 10$ ) and low aerobic running capacity (LCRs,  $N = 10$ ). The selection started out from a founder population of the genetically heterogeneous N:NIH stock of rats obtained from the National Institutes of

Health (USA). The model system was previously described in detail (Koch & Britton 2001). The Norwegian council for Animal Research approved the study, which was in accordance with the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, revised 1996).

To determine the threshold for inducible arrhythmias, we performed *ex vivo* experiments using a standardized Langendorff perfusion. After obtaining stable recordings of ECG, we started electrical burst pacing of the left ventricle by a mini coaxial stimulation electrode with pacing lasting for 2 s with a 2 ms pulse width at a stimulation frequency of 20 Hz. The burst pacing was gradually increased from 0.2 mA in 1-mA steps every 20 s until VF threshold was reached, or to a maximal stimulation current of 32 mA. Isolated Fura-2/AM-loaded cardiomyocytes were stimulated by bipolar electric pulses for  $\text{Ca}^{2+}$  handling and cardiomyocyte function by epifluorescence microscopy. Specific CaMKII and protein kinase A inhibitors were used. Confocal imaging of cardiomyocytes loaded with Fluo-3/AM was used to determine  $\text{Ca}^{2+}$  release synchrony and frequency of  $\text{Ca}^{2+}$  waves, and transverse (T)-tubules were studied using the Di-8-ANEPPS (Stolen *et al.* 2009). Quantitative measurements of diastolic  $\text{Ca}^{2+}$  leak from the SR were performed on an inverted epifluorescence microscope by the method established by Shannon *et al.* (Shannon *et al.* 2002). To bring the cellular  $\text{Ca}^{2+}$  content to a steady state, we stimulated the cardiomyocytes electrically at 1 Hz in normal HEPES based 1.8 mM  $\text{Ca}^{2+}$  concentration solution for 30–60 s. After the last electric stimulus, we rapidly switched the perfusion to a  $0\text{Na}^+/0\text{Ca}^{2+}$  containing solution and measured diastolic  $\text{Ca}^{2+}$  concentration in quiescent non-stimulated cardiomyocytes and thereafter repeated the recordings in  $0\text{Na}^+/0\text{Ca}^{2+}$  solution with tetracaine (1 mM). SR  $\text{Ca}^{2+}$  content was measured by application of caffeine (10 mM).

To assess SERCA2a and NCX functions, we used the rate constant of  $\text{Ca}^{2+}$  decline in three different solutions to quantify the contribution from each factor; a) rate constant of  $\text{Ca}^{2+}$  decline during electrical stimulation in normal HEPES 1.8 mM  $\text{Ca}^{2+}$  solution, b) addition of 10 mM caffeine in normal HEPES 1.8 mM  $\text{Ca}^{2+}$  solution and c) addition of 10 mM caffeine in a  $0\text{Na}^+/0\text{Ca}^{2+}$  solution. The rate constant of  $\text{Ca}^{2+}$  decline in each situation was controlled for total SR  $\text{Ca}^{2+}$  content.

Mitochondrial respiration was studied *in situ* in saproin-permeabilized fibres as described by Veksler and colleagues (Veksler *et al.* 1987). Maximal ADP-stimulated respiration was measured by addition of 2 mM ADP as phosphate acceptor, and the maximal respiration rate ( $V_{\text{max}}$ ) was calculated as ( $V_0 + V_{\text{ADP}}$ ). Western blot analyses were performed as previously described (Stolen

*et al.* 2009) for phosphorylated threonine 286 CaMKII, RyR2 (Affinity Bioreagents, Golden, CO, USA), phosphorylated pRyR2 serine 2808 and phosphorylated pRyR2 serine 2814. These antibodies were a kind gift from Dr. Xander Wehrens, Houston, Texas, and previously described in Respress *et al.*, (2012). The use of serine 2808 and serine 2814 in the current paper refer to the human phosphorylation sites at the RyR2; serine 2808 equates to serine 2798 in rat RyR2 and serine 2814 in human RyR2 equates to serine 2804 in rat RyR2 (GenBank ID RyR2 RAT: NM\_032078).

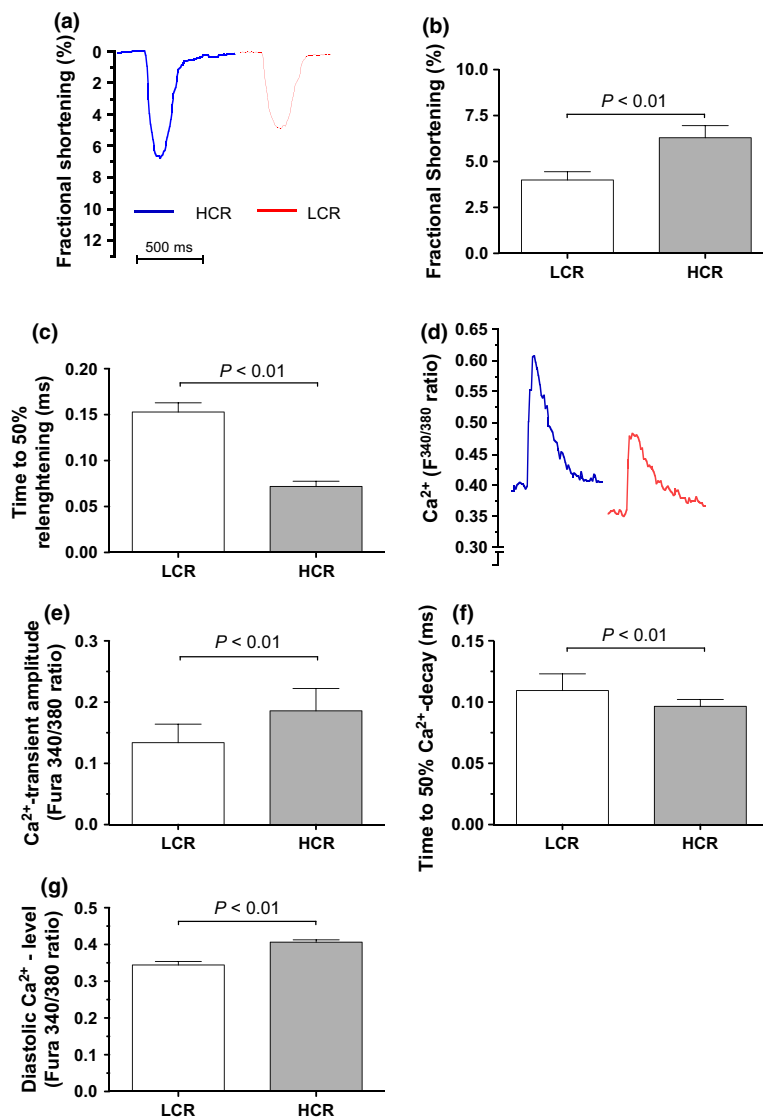
Independent samples t-test was used to determine the statistical differences between the groups. To test group differences in pacing-induced ventricular fibrillation, Fisher's exact test was used.  $P < 0.05$  was considered statistically significant. Data are presented as mean  $\pm$  SD unless otherwise stated. Detailed description of methods is presented in online supplements.

## Results

### Cardiomyocyte function and $Ca^{2+}$ handling

Cardiomyocyte contractility measured as fractional shortening was reduced by 37% in LCR compared with HCR (Fig. 1a,b,  $P < 0.01$ ). Diastolic cardiomyocyte function measured as time to 50% relengthening was prolonged in cardiomyocytes from LCR rats (Fig. 1c,  $P < 0.01$ ).

We found a 28% lower  $Ca^{2+}$  transient amplitude (Fig. 1d,e) in cardiomyocytes from LCR rats compared with HCR, which is in line with the reduced cardiomyocyte fractional shortening. Furthermore, the time to 50%  $Ca^{2+}$  decay during diastole was significantly longer in LCR rats, which accompany the defective diastolic function in these rats (Fig. 1f,  $P < 0.01$ ). Despite the longer time to 50%  $Ca^{2+}$  decay, we were surprised to find lower end-diastolic  $Ca^{2+}$  concentration



**Figure 1** Cardiomyocyte function and  $Ca^{2+}$  handling properties. (a) Representative sample tracings of cardiomyocyte fractional shortening from LCR ( $N = 5$ ) and HCR rats ( $N = 5$ ). (b) Fractional shortening was significantly reduced in LCR rats. (c) Time to diastolic relengthening was longer in LCR rats. (d) Representative traces of Fura-2/AM ratio ( $F^{340/380}$ ). (e)  $Ca^{2+}$  transient amplitude (Fura-2/AM ratio  $F^{340/380}$ ) was lower in LCR rats. (f) Time to 50%  $Ca^{2+}$  decay was longer in LCR rats. (g) Diastolic  $Ca^{2+}$  level was lower in LCR rats.

level during twitch contractions in LCR (Fig. 1g,  $P < 0.01$ ), which may be caused by a  $\text{Ca}^{2+}$  efflux from the cell over the sarcolemma by the NCX.

### Diastolic SR $\text{Ca}^{2+}$ leak

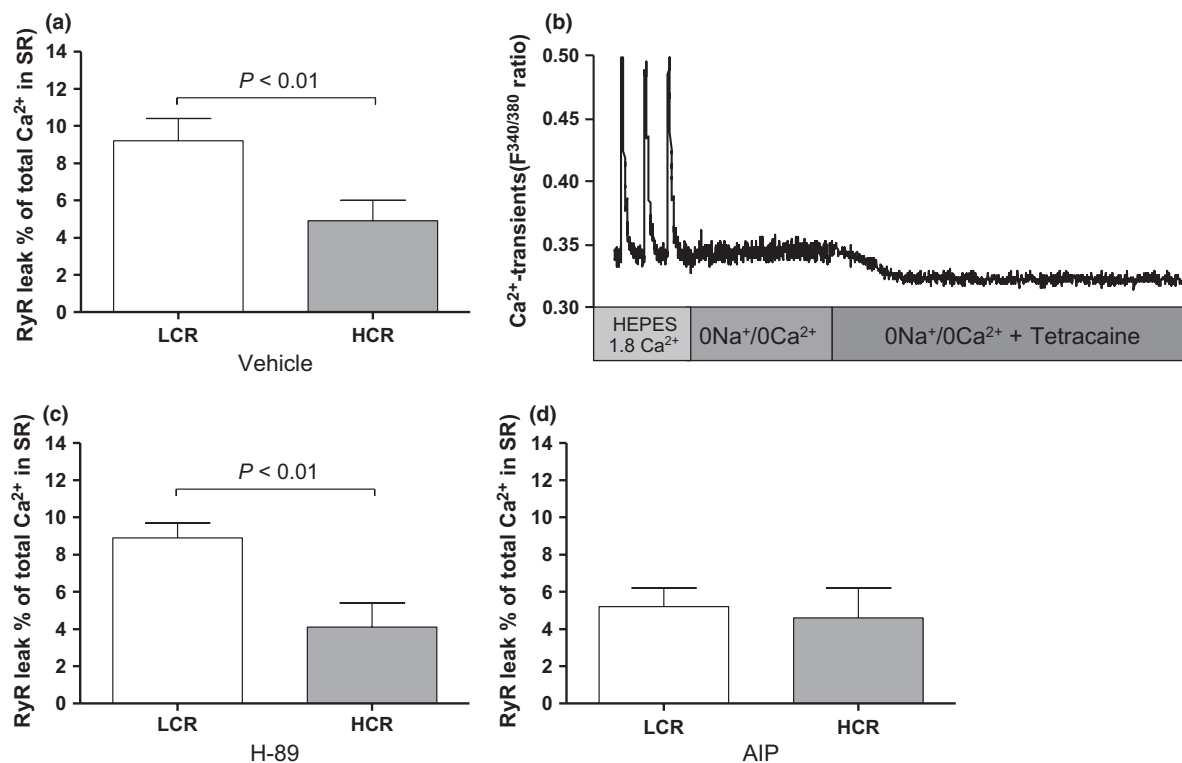
We detected 83% more SR  $\text{Ca}^{2+}$  leak during diastole in LCR rats compared with HCR (Fig. 2a,  $P < 0.01$  and Fig. 2b). Increased SR  $\text{Ca}^{2+}$  leak during diastole can trigger delayed after depolarizations (DADs) that also were found more frequent in cardiomyocytes from LCR rats (29% of LCR cells vs. none in HCR,  $P < 0.01$ ) (Figure S1 Online supplements). The increased SR  $\text{Ca}^{2+}$  leak probably reflects an increased RyR2  $\text{Ca}^{2+}$  sensitivity which earlier studies link to increased phosphorylation at serine 2808/9 by PKA or at serine 2814 by CaMKII. To examine the effect of PKA and CaMKII, we incubated separate cardiomyocytes with specific inhibitors. We did not find any effect of SR  $\text{Ca}^{2+}$  leak by the PKA inhibitor H-89, implying no involvement of this kinase (Fig. 2c). When the cells were incubated with the specific CaMKII inhibitor AIP, the SR  $\text{Ca}^{2+}$  leak was almost

abolished, implying that the RyR2 may be more sensitized by increased phosphorylation of CaMKII (Fig. 2d).

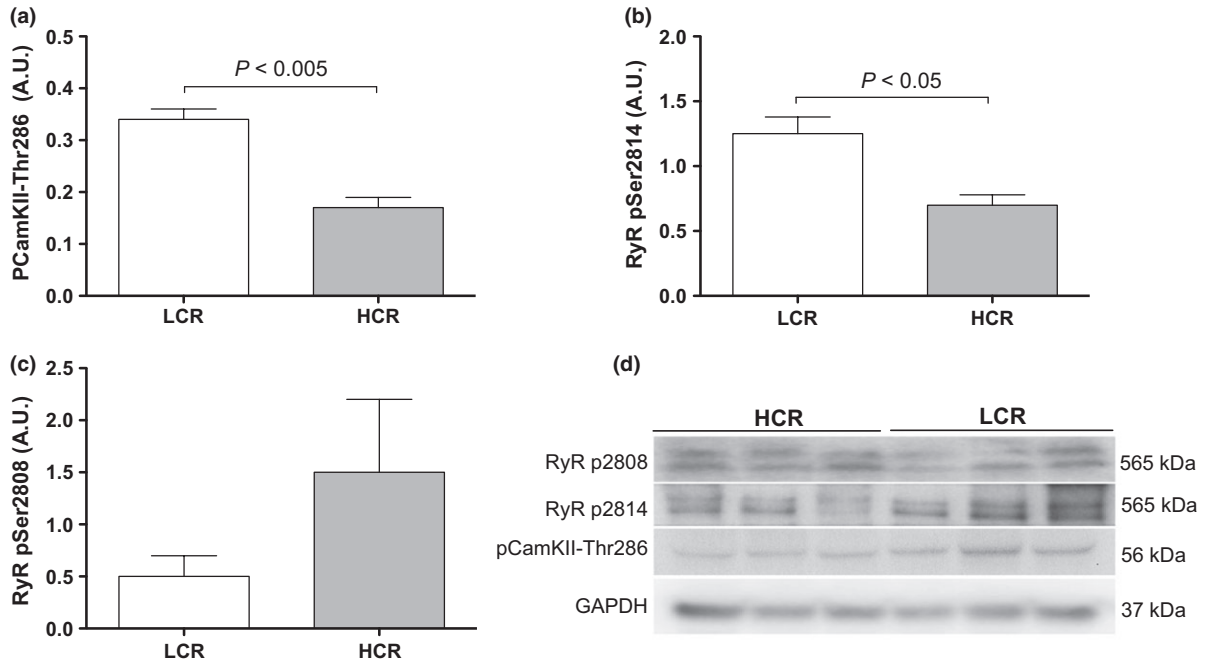
To follow up these findings, protein analyses showed a 136% increased phosphorylation at the auto activating pCaMKII threonine 286 site in the LCR rats (Fig. 3a,  $P < 0.05$ ) which imply increased activity of the kinase. Furthermore, the RyR2 was found significantly more phosphorylated at the CaMKII-specific phosphorylation site serine 2814 in the LCR (Fig. 3b,  $P < 0.05$ ). The PKA site serine 2808 at the RyR2 was not significantly different phosphorylated between LCR and HCR rats. (Fig. 3c, NS). Taken together, these data imply that phosphorylation of the RyR2 by CaMKII is causing the increased diastolic SR  $\text{Ca}^{2+}$  leak in the LCR rats. This increased SR  $\text{Ca}^{2+}$  leak may contribute to the 20% reduced SR  $\text{Ca}^{2+}$  content found in LCR rats (Fig. 4a,  $P < 0.01$ ).

### SERCA2a, NCX and mitochondrial functions

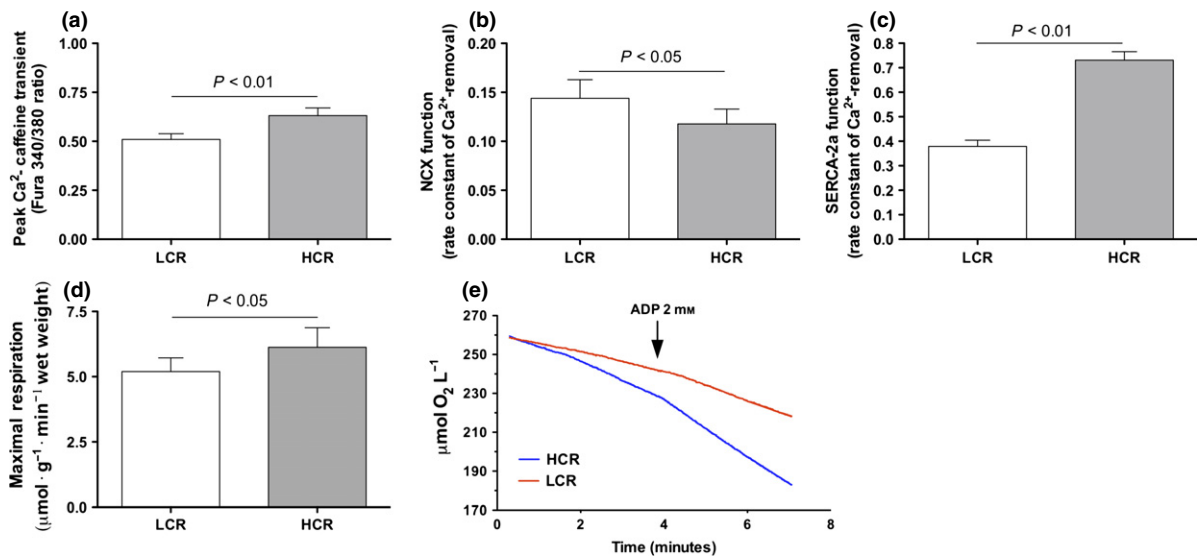
To more carefully determine the  $\text{Ca}^{2+}$  fluxes during diastole, we examined the rate constants of cytoplasmic



**Figure 2** Diastolic SR  $\text{Ca}^{2+}$  leak over the ryanodine receptor (RyR2). (a) Diastolic SR  $\text{Ca}^{2+}$  leak in normal HEPES 1.8  $\text{Ca}^{2+}$  concentration solution in LCR and HCR rats. (b) Exemplary recordings of diastolic SR  $\text{Ca}^{2+}$  leak in a LCR rat. (c) After incubation by AIP and (d) after inhibition by H-89. Note that LCR rats had significantly more diastolic SR  $\text{Ca}^{2+}$  leak than HCR rats in vehicle situation. In addition, inhibition of CaMKII by AIP reduced the leak to of the LCR to equal that of HCR rats, but AIP had no further effect on HCR rats. PKA inhibition by H-89 did not have any significant effect in either of the groups. Number of rats used for cell experiments: LCR = 5, HCR = 5.



**Figure 3** Protein expression from left ventricle tissue. Western blots from left ventricle tissue LCR and HCR rats. (a) The levels of phosphorylated CaMKII at the theorine 286 site were significantly higher in LCR rats than HCR. (b) Phosphorylation of the RyR2 at the serine 2814 site was increased in LCR rats. (c) Phosphorylation of the RyR2 at the serine 2808 site was lower, on average, in LCR but not significantly different from HCR. Data are presented as mean  $\pm$  SEM. (d) Exemplary images of western blot.



**Figure 4** Sarcoplasmic reticulum, sarcolemmal  $\text{Ca}^{2+}$  handling and mitochondrial function. (a) Total SR  $\text{Ca}^{2+}$  content was significant lower in LCR rats compared with HCR. (b) NCX function was increased in LCR rats assessed by the rate constant of  $\text{Ca}^{2+}$  removal (Fura-2/AM ratio<sup>340/380</sup>  $\text{s}^{-1}$ ), (c) SERCA2a function was reduced in LCR rats. (d) Mitochondrial respiration by ADP stimulation of respiration to maximal respiration in freshly dissected saponin-permeabilized left ventricle tissue was significantly lower in LCR rats compared with HCR (determined by  $\mu\text{mol O}_2 \text{ g}^{-1} \text{ min}^{-1}$  consumption). (e) Exemplary recordings from LCR (red) and HCR (blue). Number of rats used for experiments: LCR = 5, HCR = 5.

$\text{Ca}^{2+}$  removal from twitch-induced and caffeine-induced  $\text{Ca}^{2+}$  transients. We found that the  $\text{Ca}^{2+}$  removal rate of NCX was significantly higher in LCR rats (Fig. 4b,  $P < 0.05$ ), whereas SERCA2a  $\text{Ca}^{2+}$  removal rate was

increased in LCR compared with HCR (Fig. 4c,  $P < 0.01$ ).

As SERCA2a function is dependent upon the local ATP/ADP ratio in the pump's vicinity (Kuum *et al.*

2009), we analysed mitochondrial respiration in separate samples of tissue from the left ventricle to check for a potential effect of metabolism on  $\text{Ca}^{2+}$  removal. We found that LCR rats had a 23% lower maximal ADP-stimulated respiration than HCR rats (Fig. 4d,e,  $P < 0.05$ ).

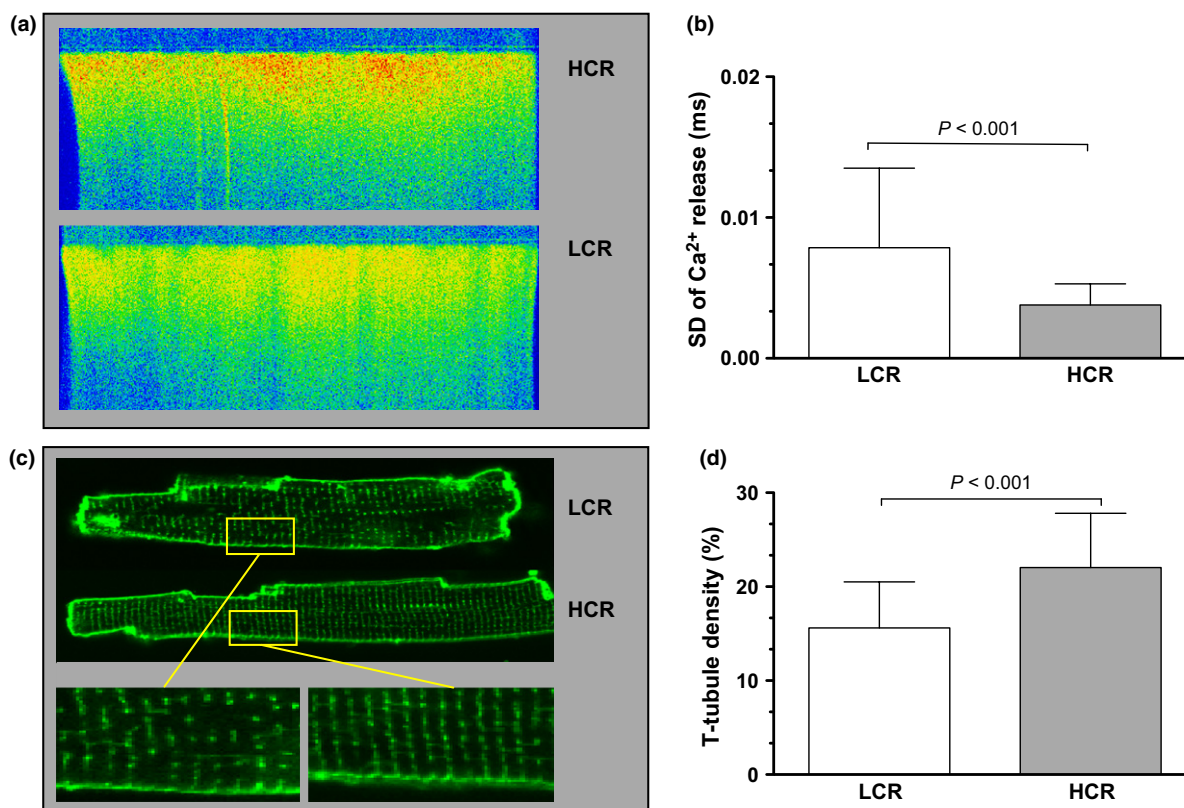
#### T-tubule density and $\text{Ca}^{2+}$ release synchrony

For detailed examination of  $\text{Ca}^{2+}$  release properties and synchrony of  $\text{Ca}^{2+}$  release along the cardiomyocyte length, we performed line-scan confocal imaging in Fluo-3 loaded electrically stimulated cells. We found that cardiomyocytes from LCR rats had a less synchronous  $\text{Ca}^{2+}$  release compared with HCR cardiomyocytes (Fig. 5a,b,  $P < 0.001$ ). Synchrony of  $\text{Ca}^{2+}$  release is closely linked to the density and organization of T-tubules in the cardiomyocyte (Brette & Orchard 2003, Lipp *et al.* 1996, Louch *et al.* 2004). In line with this, we found both reduced T-tubule density and distorted structure in LCR cardiomyocytes (Fig. 5c,d). Additionally, we measured the distance

between T-tubules to control for the possibility that the spacing between the T-tubules was altered. We found that the spacing between T-tubules (for intact T-tubules) tended to be longer in LCR, but did not reach statistical significance ( $1.84 \pm 0.18 \mu\text{m}$  vs.  $1.76 \pm 0.12 \mu\text{m}$ , in LCR and HCR, respectively,  $P = 0.055$ ). This indicates that the difference in T-tubule density was due to loss of T-tubules in small regions of the cell and not spacing between intact T-tubules.

#### Propensity for burst pacing-induced ventricular fibrillation

Finally, to assess whether altered  $\text{Ca}^{2+}$  handling properties and especially the increased RyR2  $\text{Ca}^{2+}$  leak observed had any effect upon the arrhythmogenicity of the heart, we performed experiments by burst pacing of the left ventricle in Langendorff-perfused hearts in sinus rhythm. We found that LCR rats were significantly more susceptible to ventricular fibrillation than HCR, with 5 of 5 hearts that experienced sus-



**Figure 5**  $\text{Ca}^{2+}$  release synchrony and T-tubule density. (a) Exemplary recordings of  $\text{Ca}^{2+}$  release synchrony in Fluo-3 loaded electrically stimulated cardiomyocytes. (b) Quantification of  $\text{Ca}^{2+}$  release synchrony measured as the standard deviation (SD) of  $\text{Ca}^{2+}$  release along the line-scanned whole length of the cardiomyocyte showed significant more variation in the synchrony in LCR rats. (c) Exemplary images of Di-8-ANEPPS stained cardiomyocytes as measurement for T-tubule density in LCR and HCR rats. (d) Quantified measures of T-tubule density in di-8-ANEPPS stained cardiomyocytes revealed significantly reduced density in LCR rats. Number of rats used for cell experiments: LCR = 5, HCR = 5.

tained ventricular fibrillations in LCR (threshold  $12.4 \pm 2.9$  mA) compared with only 1 of 5 in HCR (Fig. 6a–c,  $P < 0.01$ ).

## Discussion

The present study was initiated to test the hypothesis that long-term artificial selection for contrasting intrinsic aerobic capacities would lead to senescent phenotypes that diverge for diastolic  $\text{Ca}^{2+}$  control that may cause a more arrhythmogenic heart. The results demonstrate that a low aerobic capacity harbours numerous negative cardiac phenotypes including (i) depressed cardiomyocyte systolic and diastolic function, (ii) increased diastolic SR  $\text{Ca}^{2+}$  leak over the RyR2 associated with autophosphorylated CaMKII and increased RyR2 phosphorylation at the serine-2814 site, and (iii) increased propensity to ventricular arrhythmia.

### Cardiomyocyte function and $\text{Ca}^{2+}$ handling properties

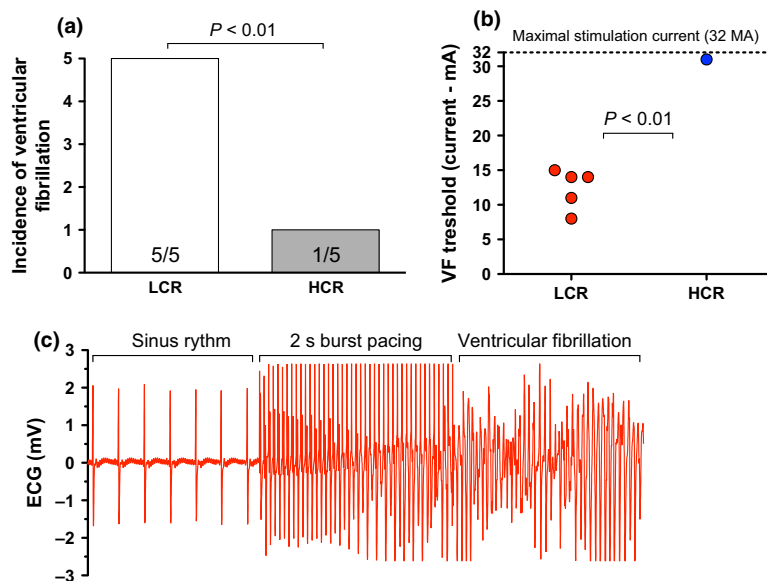
Impaired cardiomyocyte function in LCR rats was associated with deterioration of  $\text{Ca}^{2+}$  handling properties including (i) reduced systolic  $\text{Ca}^{2+}$  amplitude that could account for the reduced cardiomyocyte fractional shortening and (ii) the reduced removal of  $\text{Ca}^{2+}$  from cytosol during diastole that resulted in prolonged time to diastolic relengthening of the cardiomyocyte. Several factors may causatively explain this prolongation, but the main route of  $\text{Ca}^{2+}$  removal is the SERCA2a (Bers 2002), which was found significantly impaired in the LCR rats. The combination of reduced SERCA2a function and increased NCX function in LCR rats may further contribute to reduce SR  $\text{Ca}^{2+}$  content that was observed in LCR rats, as this earlier

have been reported to cause higher  $\text{Ca}^{2+}$  extrusion over the cell membrane (Hasenfuss *et al.* 1999, Litwin & Bridge 1997, Pogwizd *et al.* 2001, Sipido *et al.* 2000, Bers *et al.* 2006). Furthermore, loss of the T-tubular network causes disruption of the dyads and impairs EC coupling by dyssynchronous  $\text{Ca}^{2+}$  release as observed in the LCR. The lower T-tubule density in the LCR was mainly caused by loss of T-tubules in small areas within the cardiomyocyte as the T-tubule spacing did not differ. Reduced T-tubule density/organization and inefficient coupling are commonly observed in heart failure models and have been associated with loss of the structural proteins junctophilin-2 and bin-1 (Hong *et al.* 2010, Lee *et al.* 2002, van Oort *et al.* 2011). However, how these essential proteins are regulated is unknown.

### Increased diastolic SR $\text{Ca}^{2+}$ leak and ventricular fibrillation in LCR rats

The cardiomyocyte functional data and  $\text{Ca}^{2+}$  handling measurements clearly pointed to a malfunctioning cardiomyocyte with disordered  $\text{Ca}^{2+}$  homeostasis. Because RyR2 instability has been associated with spontaneous  $\text{Ca}^{2+}$  release during diastole that may trigger DADs, depolarization of the cardiomyocyte and subsequent ventricular arrhythmias (Pogwizd *et al.* 2001), we performed diastolic  $\text{Ca}^{2+}$  leak recordings in isolated cardiomyocytes.

In the presence of tetracaine, which desensitizes the RyR2, we were able to detect a significant increased  $\text{Ca}^{2+}$  leak in LCR rat compared with HCR rats. It has earlier been shown that increased RyR2  $\text{Ca}^{2+}$  leak is associated with triggering of DADs through activating forward mode of the NCX, leading to a net ion influx.



**Figure 6** Aerobic capacity and incidence of ventricular fibrillation. Recordings of ventricular fibrillation (VF) after burst pacing of the left ventricle. (a) LCR ( $N = 5$ ) rats were significantly more prone for sustained ventricular fibrillation than HCR rats ( $N = 5$ ). (b) LCR rats had a mean threshold for ventricular fibrillation of 12 mA, and in all measured hearts, we managed to induce sustained fibrillation. Only one HCR heart sustained fibrillation at a current of 31 mA. (c) Exemplary recordings of burst pacing-induced ventricular fibrillation in a LCR heart.

Based upon studies from heart failure models, it have been shown that increased activity of CaMKII may lead to increased phosphorylation of the RyR2 and hence increased  $\text{Ca}^{2+}$  sensitivity of the receptor making it more 'leaky'. To determine the role of CaMK as a potential mechanistic explanation to the increased SR  $\text{Ca}^{2+}$  leak observed in LCR rats, we used the specific CaMKII inhibitor AIP. The results displayed a significant reduction in  $\text{Ca}^{2+}$  leak by AIP inhibition of CaMKII. These data indicate an important role of CaMKII as a determinant of the increased leak in LCR rats in the present study. In 2000, Marks *et al.* proposed a new theory that PKA hyperphosphorylates the RyR2 at serine 2808 site causing FK506-binding protein 12.6 (FKBP 12.6) dissociation from the receptor and diastolic SR  $\text{Ca}^{2+}$  leak. These initial data were followed up by series of highly consistent and compelling data published in prominent journals that supported their hypothesis (Wehrens *et al.* 2003, 2006, 2004). The hypothesis has, however, been challenged in later years as several independent laboratories have been unable to reproduce the findings, rising a series of controversies and debate of its fundamental role in causing SR  $\text{Ca}^{2+}$  leakage (reviewed in e.g. Bers 2012, Eschenhagen 2010, Valdivia 2012). In the light of the controversies on the potential role of PKA-dependent influence on the RyR2 SR  $\text{Ca}^{2+}$  leak and the fact that various experimental animal models may display different mechanisms, we opened the possibility as an explanation for the increased SR  $\text{Ca}^{2+}$  leak observed in LCR rats. To determine the effect of PKA on reducing the higher SR  $\text{Ca}^{2+}$  leak in LCR rats, we therefore performed a set of experiments using the PKA inhibitor H-89. The PKA inhibition had no significant effect on the diastolic SR  $\text{Ca}^{2+}$  leak compared with vehicle solution. The data from our model of LCR rats are therefore in line with previous data from heart failure models that question the role of PKA for influencing RyR2  $\text{Ca}^{2+}$  leak and rather designate CaMKII as a key effector of the increased RyR2  $\text{Ca}^{2+}$  leak (reviewed in e.g. Bers 2012, Eschenhagen 2010, Valdivia 2012).

Given the marked effect on reduction in  $\text{Ca}^{2+}$  leak by the inhibition of CaMKII, we hypothesized that the LCR rats had increased activity of CaMKII that indeed was confirmed by increased expression of threonine 286 phosphorylation of CaMKII (pCaMKII threonine 286) in LCR rats. pCaMKII threonine 286 increases the affinity of the CaMKII complex and traps CaMKII on the autophosphorylated subunit. As a result, the kinase retains close to fully activity as long as CaM is trapped, regardless of the  $\text{Ca}^{2+}$  concentration level (Meyer *et al.* 1992). The effect of increased activity of CaMKII was also found in the increased phosphorylation status at the CaMKII-spe-

cific site serine 2814 at the RyR2 in LCR. Hence, these data further substantiate that increased  $\text{Ca}^{2+}$  sensitivity of the RyR2 is attributed to augmented CaMKII activity as a key player underlying the increased diastolic SR  $\text{Ca}^{2+}$  leak in the LCR rats.

The increased CaMKII activity caused by pCaMKII threonine 286 autophosphorylation is commonly observed in conditions of heart failure (Maier & Bers 2007). We do not have any direct explanation to the increased CaMKII activation in the LCR rats in the present study, but several previous studies have shown that the LCR rat model display a series of characteristics indicative of numerous metabolic disease states including obesity, hyperlipidaemia, impaired glucose tolerance and insulin resistance (Bowman *et al.* 2010, DeMarco *et al.* 2012, Haram *et al.* 2009, Lessard *et al.* 2009, Wisloff *et al.* 2005). Recently, it was shown in an elegant study by Bers group that hyperglycaemia activates CaMKII by covalent modification of CaMKII by O-linked N-acetylglucosamine (O-GlcNAc). O-GlcNAc modification of CaMKII at serine 279 activated further CaMKII autonomously through increased phosphorylation of threonine 286 (Erickson *et al.* 2013). To relate these findings to the increased CaMKII activity in the present study on LCR rats, the recent findings showing that LCR rats have augmented cardiac O-GlcNAc protein modification (Johnsen *et al.* 2013) are interesting and highly relevant and suggestive for a possible mechanistic explanation. Further studies are, however, needed to establish this link directly. In addition, the contrasting phenotypic differences observed between the LCR and HCR lines have in a previous publication been linked to a range of differently expressed genes in the heart (1540 of 28 000 screened genes) that influence signalling processes that potentially activates CaMKII, including  $\text{Ca}^{2+}$  signalling and glucose metabolism pathways (Bye *et al.* 2008). The current experiment can, however, not in detail, quantify how much of the breakdown of  $\text{Ca}^{2+}$  handling properties in the LCR that are affected by the genetic differences either directly or remotely as influenced by enhanced disease processes caused by a multitude of metabolic and pathological processes of the senescence phase. A recent study showed, however, that the proportion of total running performance that is due to the additive effects of genes (i.e. narrow-sense heritability) is about 40% in each line (Ren *et al.* 2013). In addition, earlier studies have shown that the differences between HCR and LCR lines are significantly changed also in younger rats (Hoydal *et al.* 2007, Wisloff *et al.* 2005). This is consistent with the possibility that LCR-HCR phenotypic differences may be mediated via expression of contrasting allelic variants. Whether the cardiac differences are mediated directly via genetic variation or by remote disease influences, the models can still serve to evaluate interven-



tions that retrieve or enhance negative phenotypes in the LCR. In this regard, earlier studies have shown that exercise training in LCR rats is to improve cardiomyocyte function as well as Ca<sup>2+</sup> handling properties in LCR rats (Hoydal *et al.* 2007, Wisloff *et al.* 2005). It is therefore possible that preventive strategies, including exercise training, starting from young age and throughout life could induce a more advantageous health profile, prevent deterioration of cardiomyocyte properties and thereby improve longevity. This needs to be addressed in further studies.

Ventricular arrhythmias in non-ischaemic hearts are mainly initiated by non-re-entrant mechanisms that appears to be due to triggered activity primarily from DADs that arise from altered cellular Ca<sup>2+</sup> handling and ionic currents (Pogwizd & Bers 2004). As the measurements were recorded in isolated Langendorff-perfused hearts, intrinsic properties of the ventricle determined the VF threshold. Recent literature has attributed an altered VF threshold to imbalanced Ca<sup>2+</sup> homeostasis, increased diastolic SR Ca<sup>2+</sup> leak in combination with altered electrogenic balance via increased NCX activity and elevated resting membrane potential (Pogwizd & Bers 2004). It is therefore highly possible that the increased SR Ca<sup>2+</sup> leak, combined with the increased NCX activity, is an important player in the increased incidence of VF observed in the LCR rats.

In conclusion, during two-way selection for low and high aerobic running capacity increased susceptibility for disease risks segregated with low capacity. Here, we show that the low aerobic capacity line also presents with increased propensity for ventricular fibrillation that was underwritten by impaired Ca<sup>2+</sup> handling, increased diastolic SR Ca<sup>2+</sup> leak over the RyR2 trough, and increased CaMKII activation and RyR2 phosphorylation at serine 2814.

### Conflict of interest

None declared.

We acknowledge the expert care of the rat colony provided by Molly Kalahar and Lori Gilligan. Contact L.G.K. (lgkoch@med.umich.edu) or S.L.B. (brittons@umich.edu) for information on the LCR and HCR rats: these rat models are maintained as an international collaborative resource at the University of Michigan, Ann Arbor. The authors acknowledge Ragnhild Røshjølgen for technical assistance and isolation of cardiomyocytes.

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### Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Data S1.** Material and methods.

**Figure S1.** We found significant higher frequency of spontaneous Ca<sup>2+</sup> releases and contractions that may be triggered by delayed after depolarization (DAD) in LCR rats.