Beneficial normalization of cardiac repolarization by carnitine in transgenic SQT1 rabbit models

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Abstract

Aims: Short-QT-syndrome type 1 (SQT1) is a genetic channelopathy caused by gain-of-function variants in HERG underlying the rapid delayed-rectifier K^+ current (I_{Kr}), leading to QT-shortening, ventricular arrhythmias, and sudden cardiac death. Data on efficient pharmaco-therapy for SQT1 are scarce. In patients with primary carnitine-deficiency, acquired-SQTS has been observed and rescued by carnitine-supplementation. Here, we assessed whether carnitine exerts direct beneficial (prolonging) effects on cardiac repolarization in genetic SQTS.

Methods and Results: Adult wild-type (WT) and transgenic SQT1 rabbits (HERG-N588K, gain of I_{Kr}) were used. *In vivo* ECGs, *ex vivo* monophasic action potentials (APs) in Langendorff-perfused hearts, and cellular ventricular APs and ion currents were assessed at baseline and during L-Carnitine/C16-Carnitine-perfusion. 2D computer simulations were performed to assess reentry-based VT-inducibility.

L-Carnitine/C16-Carnitine prolonged QT intervals in WT and SQT1, leading to QT-normalization in SQT1. Similarly, monophasic and cellular AP duration (APD) was prolonged by L-Carnitine/C16-Carnitine in WT and SQT1. As underlying mechanisms, we identified acute effects on the main repolarizing ion currents: I_{Kr}-steady, which is pathologically increased in SQT1, was reduced by L-Carnitine/C16-Carnitine and deactivation kinetics were accelerated. Moreover, L-Carnitine/C16-Carnitine decreased I_{Ks}-steady and I_{K1}. *In silico* modelling identified I_{Kr}-changes as main factor for L-Carnitine/C16-Carnitine-induced APD-prolongation. 2D-simulations revealed increased sustained reentry-based arrhythmia formation in SQT1 compared to WT, which was decreased to the WT-level when adding carnitine-induced ion current changes.

Conclusion: L-Carnitine/C16-Carnitine prolong/normalize QT and whole heart/cellular APD in SQT1 rabbits. These beneficial effects are mediated by acute effects on I_{Kr}. L-Carnitine may serve as potential future QT-normalizing, anti-arrhythmic therapy in SQT1.

Translational Perspective (100):

Available therapeutic strategies (ICD and/or quinidine) in SQTS are limited, not effective in each SQTS patient and carry side effects. Carnitine might be an alternative pharmacological therapy. In this study we demonstrate that carnitine can normalize QT/APD in transgenic SQT1 rabbits. These beneficial effects are mediated by alterations in I_{Kr}-steady and I_{Kr} deactivation kinetics. 2D computer simulations indicate anti-arrhythmic effects of these ionic changes. We expect similar effects in SQT1 patients, warranting confirmatory studies on beneficial QT-normalizing / anti-arrhythmic effects of carnitine in SQTS patients. As carnitine is well-tolerated and commonly used in primary carnitine-deficiency and food supplements, it could be readily used clinically.

Introduction

Short QT syndrome (SQTS) is a genetic cardiac channelopathy[1] with a high risk for ventricular arrhythmias and sudden cardiac death (SCD).[2] To date, eight subtypes have been described.[3] In the most frequent subtype, SQTS type 1, gain-of-function mutations in *KCNH2*/HERG (N588K) lead to an increased rapid delayed-rectifier K^+ current (I_{Kr}) and a consecutive shortening of action potential (AP) duration (APD) and QT duration.[4]

Current therapeutic strategies for SQTS patients are limited.[5] An ICD is recommended to prevent SCD[6] – especially after survived cardiac arrest – as there is a high risk of recurrence.[5] As pharmacological therapy, (hydro)quinidine has the best evidence to prolong QT and reduce arrhythmia burden[7,8] – but it carries a high risk for gastro-intestinal side effect that may decrease a patient's compliance. In addition, a study has shown that quinidine might not be effective in all SQTS-variants, highlighting a possible variant-specific effect.[9] Therefore, there is an unmet need for novel, efficient therapies in SQTS.

Primary carnitine deficiency (PCD) is a genetic metabolic disorder, in which mutations in the carnitine-transporter *OCTN2* cause a depletion of carnitine and carnitine long-chain fatty acids in the body.[10] The most important biological function of carnitine is the transport of fatty acid into the mitochondria for subsequent β-oxidation, a process which results in the esterification of L-Carnitine to form long-chain acylcarnitine derivatives, such as the C16-Carnitines.[11] The depletion of carnitine leads to impaired β-oxidation, and patients present with hypoglycaemia, steatosis, skeletal myopathy and/or cardiomyopathy.

Recent studies have provided an additional link between PCD and (acquired) SQTS. Roussel et al.[12] reported 3 PCD patients with associated symptomatic SQTS. A mouse

model confirmed the relationship between low plasma levels of carnitine and QT-shortening.[12] Similarly, Gélinas et al.[13] described a case of a young woman dying unexpected during sleep, in which postmortem genetic testing revealed a homozygous *SLC22A5* mutation leading to the diagnosis of PCD. Her brother was subsequently diagnosed with PCD and acquired SQTS after genetic testing.[13] In both publications, known SQTS-causing mutations were excluded, and carnitine supplementation normalized the previously shortened QT-interval, indicating that carnitine-deficiency may cause acquired SQTS.

As such, carnitine supplementation may similarly prolong QT-intervals in healthy subjects and in inherited SQTS, providing a novel "metabolic" treatment approach. Indeed, indirect evidence that carnitine may prolong QT stems from various studies on the role of energy drinks – which, in addition to caffeine, contain a substantial amount of carnitine – for cardiac arrhythmogenic events such as AF, VF or cardiac arrest.[14,15] After the consumption of energy drinks, longer QTc were observed as compared to simple caffeine consumption.[16]

The mechanisms underlying QT-prolonging effects of carnitine, however, are not well studied and no systematic assessment of carnitine on cardiac ion currents and its potential use for QT-normalization in SQTS has been performed to date. To investigate the effects of L-Carnitine and C16-Carnitine in SQT1, we used our recently established SQT1 rabbit model.[17] In contrast to the mouse heart, which differs in various aspects from the human heart – mainly in the AP shape[18] and in the underlying repolarizing ion currents[18,19] – the rabbit heart bears close resemblance to the human heart.[19] Our SQT1 rabbit model, which expresses the N588K gain-of-function mutation in *KCNH2* leading to

an impaired inactivation of I_{Kr} and therefore an increased I_{Kr} steady current, mimics the human disease phenotype with shortened QT, shortened APD, and an increase in VT/VF incidence and SCD.[17]

Methods

(A more detailed method section can be found in the online supplement)

Animals

All animal experiments were performed in compliance with EU legislation (directive 2010/63/EU) and the German animal welfare laws (TierSchG and TierSchVersV), after approval by the animal welfare committee of the local authorities (Regierungspräsidium Freiburg; approval number G17/57). All experiments were performed in female and male adult rabbits (aged 4-7 months for all experiments).

For *in vivo* experiments (ECG), rabbits were anesthetized with ketamine (Ketanest S® 25 mg/ml, Pfizer) and xylazine (Rompun® 2%, Bayer) (12.5 mg/kg / 3.75 mg/kg IM, followed by IV infusion). Beating hearts excision (for monophasic AP (MAP) recordings and patch clamping) was performed in ketamine/xylazine anesthetized rabbits after additional injection of 500 I.U. heparine (Heparin-sodium, 25000 I.U./ml, Braun) and euthanasia with 40 mg/kg thiopental-sodium (Thiopental-sodium 0.5 g, Inresa) IV.

Compounds

Palmitoyl-L-Carnitine (C16-Carnitine) and L-Carnitine were purchased from Tocris and Sigma. Stock solutions (30 µM) were prepared in water and stored at -20 Cº until use.

12-lead ECG

12-lead surface ECGs were recorded in anesthetized wild-type (WT) and SQT1 rabbits. ECGs were recorded at baseline and during perfusion with L-Carnitine (1 µmol/kg in total IV) or C16-Carnitine (0.1 µmol/kg in total IV) for up to 45 minutes, which results in L-Carnitine plasma levels of 16 µM in rabbits, as described in Roussel et al.[12] C16-Carnitine plasma levels reached 1.67 µM, similar to concentrations in normal myocardium.[20] Heart rate corrected QT index (QT_i) was calculated ($QT_i = QT_{measured}/QT_{expected}$; $QT_{expected} = 86 + 0.22*RR$)[17,21] at baseline and every five minutes after drug administrations. In addition, QT-dispersion (QTmax-QTmin) and short-term variability of the QT (STVQT) were assessed.

Monophasic action potential measurements

Rapidly excised rabbit hearts were Langendorff-perfused via the aorta with a modified Krebs-Henseleit solution warmed to body temperature. A balloon-tipped catheter was placed into the left ventricle (LV). Hearts were paced at a constant rate of 2 Hz and MAP were recorded at baseline and during perfusion with L-Carnitine (4 or 40 µM) or C16-Carnitine (3 µM) by four epicardial electrodes. MAP durations at 75% of repolarisation were measured using the ISOHEART Data Acquisition software.

Isolation of rabbit ventricular cardiomyocytes

Ventricular myocytes from the LV wall were obtained from the hearts of WT or SQT1 rabbits by standard collagenase digestion.[17] After euthanasia, hearts were rapidly excised and placed in ice cold Tyrode solution, mounted on a Langendorff apparatus, and perfused with Ca^{2+}-free solution supplemented with 0.8-1 mg/mL collagenase (Worthington type 2) and 33 µmol/L (µM) Ca^{2+} for 25-40 min. All perfusates were gassed with 100% O_2 and maintained at 37°C. At the end of the digestion, the LV was gently teased apart in Krafte-Brühe solution. Subsequently, the dissociated cells were filtered, washed, and centrifuged. The experiments were performed within 6-8 hours of isolation. Only quiescent, rod-shaped myocytes with clear cross striations and no evidence of membrane blebbing were selected for patch-clamp studies.

Electrophysiological recording in rabbit cardiomyocytes

Whole-cell currents and APs were recorded using an Axopatch 200B patch-clamp amplifier, digitized at a sampling frequency of 10 kHz with Digidata 1440A interface and acquired with pCLAMP software. APs were elicited by 5-ms stimulation pulses of ~1.5-2-times higher magnitude than threshold at 1 Hz stimulation frequency. APs were measured at steady state, defined as the last of a train of 15 beats at the same stimulation rate. All experiments were performed at room temperature. For studies of I_{Kr}, slow delayed-rectifier potassium current I_{Ks}, inward rectifier potassium current I_{K1} and transient-outward potassium current I_{to}, cardiomyocytes were superfused continuously at 1-2 mL/min with normal Tyrode. L-type calcium current ($I_{Ca,L}$), I_{Kr} and I_{Ks} were inhibited by 1 µM nisoldipine, 5 µM E-4031 and 30 µM chromanol 293B, respectively.

All currents were recorded at baseline as well as during superfusion with 10 µM L-Carnitine or C16-Carnitine after at least 90 seconds of superfusion once stable conditions were reached.

To record I_{Ks}, cells were depolarized from the holding potential of -40mV to +50mV for 1.5 s in 10-mV increments. I_{K1} was recorded as Ba^{2+}-sensitive current (2 mM $BaCl_2$) from a holding potential of -20 mV by 500-ms voltage steps from -120 mV to +50 mV in 10 mV increments every 5 s.[22] For I_{to} measurements, 300 µM $CdCl_2$ was added to block $I_{Ca,L}$ and to shift the *I-V* relationship of I_{to} and I_{Kr} to more positive potentials.[21] I_{to} was elicited from a holding potential of -80 mV by 400-ms voltage steps from -20 mV to +60 mV in 10 mV increments every 3 s. Standard *I-V* curves of $I_{Ca,L}$ were assessed with square voltage-clamp pulses (holding potential, V_H= -40 mV, 400-ms steps from -30 mV to +30 mV). Subsequently, only the peak current at +20 mV was recorded before and after drug application.

Individual currents were normalized to the membrane capacitance to control for differences in cell size and expressed as current density (pA/pF). pClamp 10.2 and Origin 8.2 software were used for data acquisition and analysis.

In silico modelling

The *in silico* KCNH2-p.(N588K) SQT1 and WT formulations of I_{Kr} from Loewe *et al.*[23] were embedded in the O'Hara-Rudy (ORd) human ventricular AP computational model[24] to simulate WT and SQT1 conditions in the absence or presence of L-Carnitine treatment at the cellular and 2D tissue levels (Suppl. Table 1). The experimental voltage-clamp protocol and intra/extracellular K^+ concentrations (120 mmol/L, 5.4 mmol/L) were mimicked *in silico*, whereas model temperature was set to 37 °C. In addition, I_{Ks} was

increased by 35% in the SQT1 model, as observed in SQT1 cardiomyocytes[17] to mimic the experimental phenotype, while the experimentally observed effects of L-Carnitine on I_{Ks} were simulated as a 25% and 35% reduction in WT and SQT1, respectively (Suppl. Figure 1, Suppl. Table 1). The effects of L-Carnitine on I_{K1} were similarly simulated by scaling down the inward-rectifying component of I_{K1} by 13% and 19% for SQT1 and WT, respectively based on experimentally observed effects (Suppl. Table 1).

The tissue simulations were performed using an S_1S_2 protocol applied to a homogenous piece of endocardial tissue of 9 x 9 cm (simulated with 600 x 600 cellular units) with an isotropic conduction velocity of ~60 cm/s. In addition, an apicobasal gradient was incorporated by scaling the background K^+ current (Suppl. Table 1) to phenotypically reproduce a ~28 ms APD difference from apex to base, similar to Sung et al.[25]. The tissue was initialized with single-cell steady state conditions obtained after 2000 s pre-pacing (1 Hz) followed by 10 s of tissue pre-pacing (1 Hz) with a planar wave from left to right. Subsequently, a stimulus (S_1) was applied to generate a regular excitation wave and a second stimulus (S_2) was applied to the upper-left quadrant of the tissue at varying coupling intervals. When the S_2 stimulus is timed correctly, the tissue is sufficiently recovered from the S_1 excitation to allow initiation of a new wave that may subsequently result in reentry. All the simulations were performed through Myokit and Python.[26] The model code, scripts and data can be found online at: https://github.com/HeijmanLab

Statistics

Data are presented as mean ± standard deviation for *in vivo* and *ex vivo* experiments. Patch clamp data are presented as mean ± standard error of the mean. Normal

distribution of all data was checked prior to statistical analyses. To analyse normally distributed data, Student's t-tests were used: paired t tests for comparison of parameters measured before vs. after drug administration and unpaired t tests to compare genotypes. For not normally distributed data, non-parametric tests were used: Wilcoxon rank-sum test for comparisons before and after treatment; Kruskal–Wallis test for genotype-specific comparisons. Statistical analyses were performed using Prism 8.0 (Graphpad, San Diego, USA). P-values <0.05, <0.01, and <0.001 were considered statistically significant and were indicated as *, ** and ***; respectively.

Results

Baseline differences between WT and SQT1 rabbits

SQT1 rabbits demonstrated shortened QT interval duration (Figure 1), shortened APD (Figures 2 and 3), and increased I_{Kr} steady current (Figure 4A) compared to WT, as previously described.[17]

Carnitine and C16-Carnitine effects on ECG *in vivo*

Carnitine

L-Carnitine prolonged the heart-rate corrected QTi in both, WT and SQT1 (Figure 1A; Suppl. Figure 2).

In **WT** rabbits, a significant prolongation of QTi was observed immediately (5 min) after L-Carnitine bolus (*p<0.01;* Suppl. Figure 2A), which lasted until the end of measurements. The average prolongation of QTi at 35 min was 5.2 ± 3.4 %.

In **SQT1** rabbits, a significant prolongation of heart-rate corrected QTi by L-Carnitine was also observed 5 minutes after carnitine application (*p<0.01;* Suppl. Figure 2B). The average prolongation of QTi at 35 min (ΔQTi) was 5.7 ± 3.4 % (Figure 1A). This effect lasted until the end of measurements.

At baseline, there was a significant difference in QTi with shortened QTi in SQT1 compared to WT (*p<0.01*). These genotype-differences persisted during carnitine perfusion as the extent of QTi-prolongation was similar in WT and SQT1 at both dosages. When comparing the QTi of SQT1 rabbits treated with L-Carnitine to baseline QTi in WT, however, there was no significant difference (*p>0.05,* Suppl. Figure 3A), indicating that carnitine may normalize QTi to WT-values observed in healthy animals.

We further investigated whether L-Carnitine treatment had any (potentially harmful) effects on regional QT-dispersion or temporal short-term variability of the QT (STVQT). No differences were observed in QT-dispersion and STVQT between WT and SQT1 at baseline, and importantly, in both genotypes, L-Carnitine had no effect on QT-dispersion and on STVQT (Suppl. Figure 5).

C16-Carnitine

C16-Carnitine similarly prolonged the heart rate-corrected QTi in WT and SQT1-rabbits (Figure 1B, Suppl. Figure 4).

In **WT** and **SQT1** rabbits, a significant prolongation of QTi was observed starting at 5 min post iv-bolus application (*p<0.01*; Suppl. Figure 4A). This effect lasted consistently for the duration of the measurements; the average prolongation (ΔQTi) 35 min post bolus application was 4.1 ± 3.7 % for WT and 3.6 ± 3.8 % for SQT1 (Figure 1B).

Similar to Carnitine, genotype difference in QTi between SQT1 and WT persisted throughout the measurements with C16-Carnitine due to similar QTi-prolonging effects in both genotypes. When comparing the QTi in SQT1 rabbits treated with C16-Carnitine with WT rabbits at baseline, there was no significant difference in QTi (Suppl. Figure 3B), indicating a normalization of QTi of SQT1 animals treated with C16-Carnitine to WT-values.

Effects of Carnitine and C16-Carnitine on monophasic action potentials *ex vivo*

L-Carnitine

In line with the QTi changes *in vivo*, L-Carnitine significantly prolonged MAP durations (APD_{75}) in both WT and SQT1 rabbit hearts (Figure 2A) *ex vivo*. Two different dosages (4µM and 40µM) corresponding to low and high extremes of physiological plasma concentrations[10] were assessed.

In **WT** rabbit hearts, the L-Carnitine-induced prolongation of APD_{75} was not significant for low dose (4 µM) but was significant for the high dose (40 µM) of L-Carnitine (*p<0.001, Figure 2A*), resulting in a significant difference in the extent of APD-prolongation (ΔAPD) between low and high dose of L-Carnitine (*p<0.05;* Figure 2A).

By contrast, in **SQT1** rabbits, the L-Carnitine-induced APD_{75}-prolongation was already significant at low dose (4 µM) (*p<0.01;* Figure 2A) and further increased at high dose (40 µM) (*p<0.05*).

When comparing WT and SQT1 hearts, there were significant differences in APD_{75} both at baseline, (WT: 146.5 ± 9.8 ms vs. SQT1: 124.3 ± 2.6 ms, *p<0.001*) and in the presence of different L-Carnitine concentrations due to similar APD-prolonging effects of L-

Carnitine in both genotypes. In contrast to the observations *in vivo*, APD_{75} in L-Carnitine-treated SQT1 hearts remained shorter compared to WT baseline APD_{75} (WT baseline 146.5 ± 9.8 ms vs. SQT1 L-Carnitine 4 µM 130.5 ± 3.5 ms, $p<0.05$ vs. SQT1 L-Carnitine 40 µM 134.2 ± 6.8 ms, $p<0.05$).

We further investigated whether L-Carnitine had any effects on regional apico-basal APD heterogeneity in WT and SQT1 rabbit hearts during *ex vivo* MAP experiments. At baseline, there was no apico-basal APD heterogeneity in WT, while SQT1 hearts showed a non-significant trend (p=0.1) towards a slightly (+10 ms) longer APD in the LV base. Importantly, L-Carnitine did not induce any changes in the apico-basal APD heterogeneity in WT or SQT1 hearts (Suppl. Figure 6).

C16-Carnitine

The effect of C16-Carnitine on APD_{75} *ex vivo* was investigated at one concentration (3 µM), which is in the same range as previously investigated.[20]

C16-Carnitine significantly prolonged APD_{75} in **WT** ($p<0.001$) and in **SQT1** hearts ($p<0.05$) (Figure 2B). When comparing WT and SQT1 hearts, there were significant differences in APD_{75} both at baseline (WT 141.6 ± 9.1 ms vs. SQT1 123.9 ± 10.0 ms, $p<0.01$) and with C16-Carnitine (WT 148.8 ± 7.1 ms vs. SQT1 126.1 ± 10.0 ms, $p<0.001$). Accordingly, APD_{75} in C16-Carnitine-treated SQT1 hearts remained shorter than WT APD_{75} at baseline ($p<0.05$).

L-Carnitine and C16-Carnitine effects on cellular action potential duration

Consistent with our observations in whole hearts, cellular APD was prolonged by L-Carnitine and by C16-Carnitine in isolated WT and SQT1 cardiomyocytes (WT: L-Carnitine, +10.4%, n=11/7, $p<0.05$; C16-Carnitine, +23.6%, n=17/7, $p<0.001$; SQT1: L-Carnitine, +9.5%, n=16/7, $p<0.01$; C16-Carnitine, +10.0%, n=19/5, $p<0.01$; Figure 3A-C).

Similar to the *ex vivo* whole heart APD data and hence in contrast to the observations *in vivo*, cellular APD_{90} in L-Carnitine and C16-Carnitine treated SQT1 cardiomyocytes remained shorter compared to WT baseline APD_{90}.

Of note, in a small subset of SQT1 cardiomyocytes, C16-Carnitine effects were investigated at 1Hz and at 0.5Hz (Suppl. Figure 7). In those cardiomyocytes, a more pronounced APD-prolonging effect was observed at slower stimulation rates, demonstrating a reverse rate dependent modulation of APD_{90} with C16-Carnitine, which one would expect from drugs/metabolites that exert their effects via a blockade of I_{Kr}.

L-Carnitine and C16-Carnitine effects on cardiac ion currents

To investigate the mechanisms underlying the observed QT/APD-prolongation, the effects of L-Carnitine and C16-Carnitine on cardiac ion currents I_{Kr}, I_{Ks}, I_{K1}, I_{to} and I_{Ca} were measured in isolated WT and SQT1 rabbit cardiomyocytes. In all these experiments only one concentration was used for L-Carnitine and C16-Carnitine (10 µM), which is within the physiological and previously tested concentration range.[12,20]

I_{Kr} currents

Carnitine / C16-Carnitine did not cause any changes in peak **I_{Kr} tail** current densities in WT or SQT1 cardiomyocytes (Figure 4A-D). By contrast, **I_{Kr} end-pulse/steady current**, which is significantly increased in SQT1 and contributes to the accelerated repolarization in SQT1, was significantly reduced (-23%) by L-Carnitine (from 0.79±0.07 to 0.61±0.05 pA/pF) in WT and by -16% (from 1.25±0.31 to 1.05±0.27 pA/pF) in SQT1. Similar results were obtained to a lesser extent with C16-Carnitine in both genotypes (-8.3% / -9.3%) (Figure 4E-F) thereby contributing to APD prolongation.

The **voltage dependent activation** (characterized by the potential of half activation ($V_{0.5}$) and the slope factor (*dx*) of I_{Kr} was not changed in either WT or in SQT1 following L-Carnitine administration (Figure 5A-B). 10 µM C16-Carnitine produced a slight (3.9 mV) rightward shift in the voltage-dependent activation curve of I_{Kr}-tail in WT but not in SQT1 (Figure 5A-B), indicating that I_{Kr} channels are slightly slower to activate in the presence of C16-Carnitine.

In addition, both, L-Carnitine and C16-Carnitine accelerated the **deactivation kinetics of I_{Kr}**–tail (Figure 5C-D; Suppl. Figure 8). The most pronounced effect on the deactivation time constant was observed in SQT1 rabbits (SQT1, 631.0±51.9ms vs. 427.6±57.3ms). Qualitatively similar results were obtained in presence of L-Carnitine and C16-Carnitine in both the WT and the SQT1 groups.

I_{Ks} currents

I_{Ks} end-pulse / steady current was significantly decreased by L-Carnitine in SQT1 and WT cardiomyocytes – in the voltage range from +20 to +40/50mV (Figure 6A, C), thereby also contributing to the observed carnitine-induced APD-prolongation. This effect was also seen with C16-Carnitine at +40-50mV in WT (Figure 6B, D), but did not reach statistical significance in SQT1.

By contrast, I_{Ks} tail currents were only decreased significantly in SQT1 cardiomyocytes in the presence of L-Carnitine or C16-Carnitine (Figure 6E).

I_{to} currents and $I_{Ca,L}$ currents

L-Carnitine and C16-Carnitine did not cause any changes in I_{to} in WT and SQT1 cardiomyocytes (Suppl. Figure 9). Similarly, $I_{Ca,L}$ was not altered by L-Carnitine or C16-Carnitine (Suppl. Figure 10).

I_{K1} currents

Both L-Carnitine and C16-Carnitine decreased the inward component of I_{K1} in WT and SQT1 rabbits (WT, -17%, and SQT1, -13.8%, Suppl. Figure 11) at very negative voltages of -120mV. Interestingly, C16-Carnitine also significantly decreased the outward component of I_{K1} in the voltage range between -60mV and 0mV in WT (Suppl. Figure 11) and may thereby contribute to the prolongation of APD in WT cardiomyocytes.

Anti-arrhythmic effects of L-Carnitine-induced ion current changes in SQT1 in 2D *in silico* models

The computational model was able to reproduce the effects of L-Carnitine on I_{Kr} in WT and SQT1 (Figure 7A), with a significant reduction in both, I_{Kr} steady and tail currents from -10 mV to +30 mV (Figure 7A). Consistent with cellular (Figure 3) and *ex vivo* monophasic APs (Figure 2), the model showed that L-Carnitine prolonged APD_{90} in SQT1 (Figure 7B). A sensitivity analysis of the effects of L-Carnitine, selectively excluding the effects on I_{Kr}, I_{Ks}, or I_{K1} in separate simulations, showed that the inhibition of I_{Kr} was primarily responsible for the APD prolongation in SQT1 (Suppl. Figure 12).

Moreover, the 2D tissue simulations revealed that sustained re-entry (i.e., re-entrant electrical activation lasting for > 9000 ms) can be induced in the SQT1 phenotype for an S_1S_2 interval of 240-290 ms; but cannot be induced in WT tissue (Figure 7C). Similar results were obtained in the absence of an apicobasal gradient (not shown). Strikingly, simulated L-Carnitine application prevented sustained re-entry formation in SQT1 (Figure 7C). Finally, the total arrhythmogenic risk was quantified by summing the reentry duration over all the S_1S_2 intervals for each phenotype, which was approximately 10 times larger for untreated SQT1 than for WT and SQT1 with L-Carnitine treatment, with virtually no difference between the latter two.

Discussion

The observation of a connection between PCD – a metabolic disease leading to impaired mitochondrial β-oxidation – and acquired SQTS[12,13] with a subsequent normalization of the electrical phenotype (QT interval) after oral supplementation of carnitine, prompted us to investigate whether carnitine might also have direct – non-metabolic – cardiac electrophysiological effect(s) that could similarly normalize QT/APD in inherited SQTS, and, if so, which mechanisms might be involved.

As we have previously demonstrated that other metabolites (such as propionic acid) may – in addition to their well-documented effects on cellular metabolism and oxidative stress – acutely modulate repolarizing ion current densities and their kinetics, thereby directly affecting cardiac repolarization and QT duration,[27] we similarly investigated (direct, acute) electrophysiological effects of L-Carnitine and its metabolite C16-Carnitine on cardiac repolarization *in vivo, ex vivo* on the whole heart, and *in vitro* at the cellular/ion current levels.

Effects of L-Carnitine and C-16-Carnitine on QT/APD

We studied the effects of L-Carnitine/C16-Carnitine *in vivo* in their physiological plasma concentration range,[12,20] which is around 10-40 µM for L-Carnitine and around 1-10 µM for C16-Carnitine. WT and transgenic SQT1 rabbit models (HERG-N588K) mimic the human SQTS disease phenotype on all levels due to impaired I_{Kr} inactivation and subsequent shortening of cellular and whole-heart APD and *in vivo* QT-duration.[17] Here, we demonstrated a significant QT- and APD-prolonging effect of both L-Carnitine and C16-Carnitine in WT and SQT1. Notably, while baseline QT-interval on the surface ECG,

as well as APD in whole hearts and isolated cardiomyocytes were shorter in transgenic SQT1 rabbits compared to WT controls, there was no difference between the QTi of SQT1 rabbits treated with L-Carnitine/C16-Carnitine and baseline QTi of WT rabbits, indicating a L-Carnitine/C16-Carnitine-induced normalization of QT in SQT1. Importantly, regional QT dispersion and short-term variability of the QT were not enhanced by L-Carnitine, indicating a safe and homogenous prolongation of cardiac repolarization. In the *ex vivo* APD measurements – both in Langendorff-perfused hearts and in freshly isolated cardiomyocytes – a significant APD prolongation was similarly observed after perfusion with both L-Carnitine and C16-Carnitine in SQT1 and WT. This, however, did not lead to a complete normalization of APD in SQT1 animals at the applied L-Carnitine/C16-Carnitine concentrations in our experiments. These discrepancies between *in vivo* and *ex vivo* data might be partially due to the lack of autonomic control *ex vivo*, which removes sympathetic activation of I_{Ks} and hence the importance of I_{Ks} for cardiac repolarization. This might thereby reduce the contribution of L-Carnitine/C16-Carnitine induced I_{Ks}-alterations to APD-prolongation compared to *in vivo* conditions.

The QT prolongation in surface ECGs could already be observed around 5 minutes after L-Carnitine injection, and APD prolongation in patch-clamp recordings was already apparent after 90 seconds of perfusion, indicating an acute, direct effect of L-Carnitine and C16-Carnitine on cardiac ion channel properties. This acute and direct QT/APD-prolonging effect of L-Carnitine – and its mechanisms that will be detailed later – are novel results as the electrophysiological effects of this compound have not been studied before. Some data on the effects of C16-Carnitine and other long chain acylcarnitines on cardiac ion currents and Ca^{2+} homeostasis, in contrast, have previously been published.[20,28]

These, however, investigated mostly pathophysiologically high concentration ranges, because their myocardial accumulation in certain diseased conditions – such as in heart failure or myocardial ischemia – have been related/linked to increased arrhythmogenesis and impaired cardiac pump function.[29]

L-Carnitine and C16-Carnitine effects on cellular APD

We observed APD-prolonging effects on whole heart and cellular APD by both, physiological L-Carnitine and C16-Carnitine concentrations. While no other studies have investigated L-Carnitine effects on APD, the previously available data on C16-Carnitine effects on APD seem to be conflicting and dose-dependent. High doses of C16-Carnitine (30-75 µM) have been reported to shorten APD in guinea pig and rabbit papillary muscles.[28,30] By contrast, at lower, more physiological doses (10 µM), a biphasic effect on APD (initial prolongation followed by shortening of APD) was observed in guinea pig cardiomyocytes,[31] similar to our study in rabbit cardiomyocytes. This APD-prolonging effect of 10 µM C16-Carnitine was even more pronounced when applied after internal dialysis,[32] and was attributed to an inhibition of the Na^+/K^+ ATPase pump current.[33]

Ionic mechanism of APD prolongation

To determine the potential mechanisms underlying the acute APD/QT prolonging effects of L-Carnitine and C16-Carnitine, we investigated their (direct) electrophysiological effects on the main repolarizing potassium ion currents and the depolarizing $I_{Ca,L}$ responsible for shaping the AP in healthy and SQT1 cardiomyocytes.

I_{Kr} end-pulse/steady current is significantly increased in SQT1 due to impaired inactivation and contributes to the accelerated repolarization in SQT1.[17] I_{Kr}-steady was significantly reduced by L-Carnitine in WT and in SQT1 and to a lesser extent also by C16-Carnitine, thereby contributing to an APD prolongation. In addition, both L-Carnitine and C16-Carnitine led to faster deactivation of I_{Kr} in WT and SQT1 cardiomyocytes. Finally, C16-Carnitine even caused a slight rightward shift in the steady state activation curve of WT I_{Kr}. A similar change in activation / deactivation kinetics has been previously described for the LQTS-causing variant *KCNH2*-R56Q, in which accelerated deactivation kinetics resulted in a rightward shift in the voltage-dependent steady-state activation curve with slower I_{Kr} activation and subsequent prolongation of repolarization.[34] Thus, the observed changes in I_{Kr} induced by L-Carnitine/C16-Carnitine likely contribute to the observed APD prolongation in WT and SQT1 cardiomyocytes.

Interestingly, Ferro et al.[20] also reported accelerated deactivation kinetics induced by long-chain-acyl carnitines in recombinant HEK-293 cells. The general effect on I_{Kr}, however, contrasted with our findings as they reported that C16- and C18-Carnitine induced a dose-dependent increase in I_{Kr} – (both end-pulse and tail current) while L-Carnitine did not affect I_{Kr} in their mammalian expression system.[20] One possible explanation may be different properties and drug-susceptibilities of native HERG channels in cardiomyocytes versus cloned channels overexpressed in heterologous expression systems. This can be due to the presence of native subunits and other intracellular modulators in cardiomyocytes as described by Sanguinetti et al.[35]

I_{Ks} end-pulse current was also decreased (particularly at more positive potentials) by L-Carnitine and C16-Carnitine in WT and SQT1 cardiomyocytes, which is expected to

partially reduce its function as a repolarization reserve current and may also contribute to the overall APD prolongation we observed.

L-Carnitine and C16-Carnitine had no effect on I_{to} in WT and SQT1 cardiomyocytes. This is in agreement with previous reports on the effects of extracellular and intracellular L-Carnitine application.[32,36] C16-Carnitine, however, reduced I_{to} currents in that study, but only when it was dialyzed in rat ventricular myocytes.[32] This observation might play a role in long-term drug effects under pathological conditions, in which C16-Carnitine may accumulate in the sarcolemma.

In WT and SQT1 cardiomyocytes, I_{K1}, which plays an important role in stabilizing the diastolic membrane potential and shaping phase 3 of the cardiac AP was slightly but significantly decreased in the presence of L-Carnitine and C16-Carnitine both in WT and SQT1 at voltage ranges between -120 and -100 mV (inward component). This is in line with the findings of Sato et al.[37] showing that C16-Carnitine inhibits I_{K1} in guinea pig cardiomyocytes and thereby can slightly depolarize resting membrane potential – an effect that we did, however, not observe in our study.

An *in silico* sensitivity analysis of the effects of L-Carnitine, selectively excluding the effects on I_{Kr}, I_{Ks}, or I_{K1} in separate simulations, supports the notion that inhibition of I_{Kr} is primarily responsible for the L-Carnitine-induced APD prolongation in SQT1.

In sum, we identified an acute reduction of I_{Kr}-steady, which is pathologically increased in SQT1, and an accelerated I_{Kr} deactivation as main mechanisms accounting for the L-Carnitine-induced APD/QT normalization in SQT1.

In this study we focused on investigating carnitine's (acute) impact on repolarizing K^+ currents, as major drivers of the AP duration. Due to carnitine's effects on the membrane

lipid composition, which can also affect the expression and function of cardiac ion channels[38], a more comprehensive assessment of both acute and chronic effects, including the modulation of Na^+ currents, would be required to fully elucidate the impact of carnitine on cardiac electrophysiology.

Anti-arrhythmic effects of L-Carnitine in SQTS

These experimentally observed ionic changes were incorporated into WT and SQT1 *in silico* models to investigate potential anti-arrhythmic effects of L-Carnitine. Multi-scale *in silico* analyses of the acute effects of L-Carnitine on human ventricular electrophysiology confirmed 1) the increased pro-arrhythmic propensity in SQT1 2D tissues due to facilitated re-entry-formation based on the shortened APD and abbreviated refractory periods, which allow for the formation of full, sustained re-entry, and 2) the anti-arrhythmic effects of L-Carnitine in SQT1: While in SQT1 2D tissues, sustained re-entry could be induced readily at S_1S_2 intervals of 240-290 ms, the incorporation of L-Carnitine-induced changes in I_{Kr} (and I_{Ks} and I_{K1}) into the 2D model prevented the inducibility of sustained re-entry due to its APD-prolonging/normalizing effect, which prevented formation of re-entry due to longer tissue refractoriness, resulting in insufficient excitable tissue for re-entry formation. In addition, this wavelength prolongation relative to tissue size would be expected to reduce re-entry stability in line with the "critical mass theory" [39,40], further supporting an anti-arrhythmic effect of L-Carnitine in genetic SQTS. While antiarrhythmic mechanisms may be slightly different in 3D, data from class III antiarrhythmic drugs have shown that prolongation of repolarization duration (in the absence of EADs) has similar antiarrhythmic effects *in vivo* [41].

Clinical implications

To date, therapeutic strategies in the rare inherited channelopathy SQTS are limited. ICD implantation is recommended, particularly in symptomatic patients,[6] but only treats the arrhythmias once they occur and may be associated with complications such as inappropriate ICD shocks due to T-wave oversensing, electrode dysfunction, electrode dislocation or infection. Due to the young age of patients, non-surgical alternative treatment options are warranted. (Hydro)quinidine has been demonstrated to be effective in prolonging QT and reducing arrhythmia burden; but carries pronounced gastrointestinal side effects.[42]

Carnitine might be a good addition in the treatment of SQTS as it has been demonstrated that carnitine supplementation may normalize the pathologically shortened QT interval in patients with PCD (and concomitant acquired SQTS).[12,13] Here, we expand these data to genetic SQTS in the absence of intrinsic carnitine deficiency, demonstrating a prolongation of cardiac repolarization in SQTS without the induction of any (potentially pro-arrhythmic) regional or temporal heterogeneity in cardiac repolarization, further underlining its suitability for therapeutic QT/APD-prolongation in SQTS. Further studies with SQTS patients are required to investigate whether similar QT normalization effects can be observed in human SQTS patients. Importantly, in our study, we applied carnitine intravenously; but for long-term treatment of human SQTS patients, oral applications would be desirable – as already applied in PCD patients.[12,13] Thus, optimal oral carnitine dosages and long-term (beneficial and potentially harmful) effects need to be investigated in SQTS patients. This is particularly important as carnitine may also affect the membrane

lipid composition – particularly in the context of pathologically high carnitine and acylcarnitine concentrations[38] – which may modulate cardiac electrophysiology and thus needs to be considered when assessing the suitable therapeutic carnitine dosage in SQTS. Last but not least, these studies need to be complemented by long-term assessment of anti-arrhythmic effects in patients to confirm the beneficial reduction of reentry-based arrhythmias that we observed in our *in silico* modelling.

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Conflict of interest

None declared.

Author contributions

Ilona Bodi conducted and analyzed the shown patch clamp experiments, created figures and wrote the manuscript. Lea Mettke conducted and analyzed ECG and MAP experiments, made figures and wrote the manuscript. Konstantin Michaelides also

conducted and analyzed some patch clamp experiments, conducted and analyzed ECG and MAP experiments and wrote the manuscript. Tibor Hornyik conducted and analyzed patch clamp experiments and wrote the manuscript. Stefan Meier conducted all *in silico* modelling, made the corresponding figures and wrote the manuscript. Saranda Nimani analyzed ECG and MAP experiments and wrote the manuscript. Stefanie Perez-Feliz was responsible for the rabbit breeding, genotyping and helped with all animal procedures. Ibrahim el-Battrawy, Heiko Bugger, Manfred Zehender and Michael Brunner made critical revisions of the manuscript. Jordi Heijman and Katja E. Odening conceived and designed the experiments, secured funding and wrote the manuscript. All authors made a critical review of the manuscript, approved the final version of the manuscript, and agreed to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Figure legends

Figure 1: Carnitine effects on QT interval *in vivo*.

Representative ECG recordings at similar heart rates before and after L-Carnitine (**A.**) and C16-Carnitine) (**B.**) in WT and in SQT1 rabbits. Right lane: Dot plot diagrams of heart rate corrected QT-index (QTi) in individual rabbits at baseline and 35 minutes after application of L-Carnitine (**A.**) and C16-Carnitine (**B.**) demonstrate significant prolongation in WT and SQT1 rabbits. Numbers of rabbits are indicated as N. Paired t-tests, *** *p<0.001*.

Figure 2: Effects of Carnitine on action potential duration in whole hearts *ex vivo*.

Representative monophasic action potentials acquired in whole heart recordings at baseline and during L-Carnitine (**A.**) and C16-Carnitine (**B.**) perfusion in WT and SQT1 rabbit. Right lane: dot plots indicating changes in $APD75_{mean}$ between and L-Carnitine

(**A.**) or C16-Carnitine (**B.**) in individual WT and SQT1 rabbit hearts. Numbers of rabbits are indicated as N. Two-way ANOVA for Carnitine, paired t-tests for C16-Carnitine, *** *p<0.001*, ** *p<0.01*, * *p<0.05*.

Figure 3: Carnitine effects on cellular action potential duration (APD). Representative action potential tracings recorded at 1 Hz pacing frequency demonstrate effects of L-Carnitine (L-Carn, **A.**) and Palmitoylcarnitine (C16-Carn, **B.**) on APD_{90} in ventricular cardiomyocytes isolated from wild-type (WT, upper lane, black) and short QT syndrome 1 (SQT1, lower lane, blue) rabbit hearts. **C.** Dot plots show significant prolongation of APD_{90} in WT and SQT1 cardiomyocytes after 10 µM L-Carn or C16-Carn administration. Indicated are numbers of cardiomyocytes (n) and numbers of rabbits, from which the cardiomyocytes are isolated (N). Paired t-tests, *** *p<0.001*, ** *p<0.01*, * *p<0.05*.

Figure 4: Carnitine and C16-Carnitine effects on I_{Kr} tail and end-pulse. **A. and B.** Representative recordings of I_{Kr} from WT (left panel) and SQT1 (right panel) at baseline (upper line) and after application of 10 µM L-Carnitine in the continued presence of Nisoldipine (Nis, to eliminate $I_{Ca,L}$) and Chromanol (Chro, to inhibit I_{Ks}) (lower line). Voltage protocol indicated in inset. **C. and D.** Current density-voltage (*I-V*) relationships for WT and SQT1 at baseline and after L-Carnitine (**C.**) as well as after C16-Carnitine (**D.**) were obtained by plotting the tail current peak amplitude measured at -40 mV as a function of the respective test pulse potential preceding repolarization. Current amplitude corrected for cell capacitance observed in the absence and presence of drugs was plotted

against the test potentials. **E. and F.** Dot plot graphs for L-Carnitine (**E.**) and C16-Carnitine (**F.**) effects on I_{Kr} end-pulse current at 30 mV and at 40 mV. Indicated are numbers of cardiomyocytes (n) and numbers of rabbits, from which the cardiomyocytes are isolated (N). Paired t-tests for different voltages, p-values are indicated.

Figure 5: Carnitine and C16-Carnitine effects on I_{Kr} activation and deactivation.

A. and B. Voltage-dependent activation curves in WT and SQT1 before and after application of L-Carnitine (**A.**) and C16-Carnitine (**B.**). To obtain the activation curves for I_{Kr} tail currents, the amplitudes of the tail currents for various depolarizing step potentials (V_m) were normalized to the maximum tail current and plotted against V_m. The relationship between normalized I_{Kr}-tail current and V_m were fitted to a Boltzmann equation: $g/g_{max} = 1/(1+\exp[(V_{0.5} - V_m)/k])$, where $V_{0.5}$ is the half-maximum activation voltage and k is the slope factor of the steady-state activation curve. **C. and D.** Deactivation of the I_{Kr}-tail currents in WT and SQT1 rabbits were analyzed in the absence and presence of 10 µM L-Carnitine and C16-Carnitine, respectively. The current decay of I_{Kr}-tail was fitted to a single exponential to obtain deactivation time constants tau, which are indicated as dot plots. Both compounds accelerated deactivation kinetics. Indicated are numbers of cardiomyocytes (n) and numbers of rabbits, from which the cardiomyocytes are isolated (N). Paired t-tests for baseline vs. Carnitine or C16-Carnitine, p-values are indicated.

Figure 6: Carnitine and C16-Carnitine effects on I_{Ks}.

A. and B. Representative current recordings demonstrate the effect of L-Carnitine and C16-Carnitine on I_{Ks} in WT and SQT1 ventricular cardiomyocytes, pretreated with E4031

and Nisoldipine to block I_{Kr} and $I_{Ca,L}$, respectively. **C. and D.** Voltage-dependent I_{Ks} end-pulse current density in WT (**C.**) and SQT1 (**D.**) rabbits. **E. and F.** I-*V* curves for I_{Ks} tail-current density in WT (**E.**) and SQT1 (**F.**) ventricular cardiomyocytes. Indicated are numbers of cardiomyocytes (n) and numbers of rabbits, from which the cardiomyocytes are isolated (N). Paired t-tests for baseline vs. Carnitine or C16-Carnitine, p-values are indicated.

Figure 7: *In silico* analysis of the anti-arrhythmic effects of L-Carnitine in SQT1. **A**. I_{Kr} steady and tail currents in WT and SQT1 model versions together with the fitted effects of the L-Carnitine treatment shown as average reduction in I_{Kr} steady and I_{Kr} tail in experiments (grey bars with dots representing individual cardiomyocytes) and model (light grey bars). **B**. SQT1 and L-Carnitine effects on action potential repolarization in a simulated human endocardial ventricular cardiomyocyte during 1 Hz pacing (left), together with the changes in I_{Kr} (right). **C**. Reentry sensitivity analysis was performed in a 2D homogenous 9 x 9 cm endocardial tissue through a S_1S_2 protocol. Sustained reentries (> 9000 ms) could be induced for the untreated SQT1 phenotype (S_1S_2 interval of 240-290 ms), but not for the WT and SQT1 with L-Carnitine treatment groups (see exemplary simulations for WT, SQT1, and SQT1+L-Carnitine in upper panel of C). The sum (Σ) of reentry durations for all S_1S_2 intervals shows an approximately 10-fold increase in total arrhythmogenic risk for the SQT1 phenotype compared to the WT and the SQT1 with L-Carnitine phenotypes (bottom right panel). No statistical comparisons were performed for the modelling data given the deterministic nature of the model, resulting in zero variation if simulations are repeated under the same conditions.

Figure 1: Carnitine effects on QT interval *in vivo*

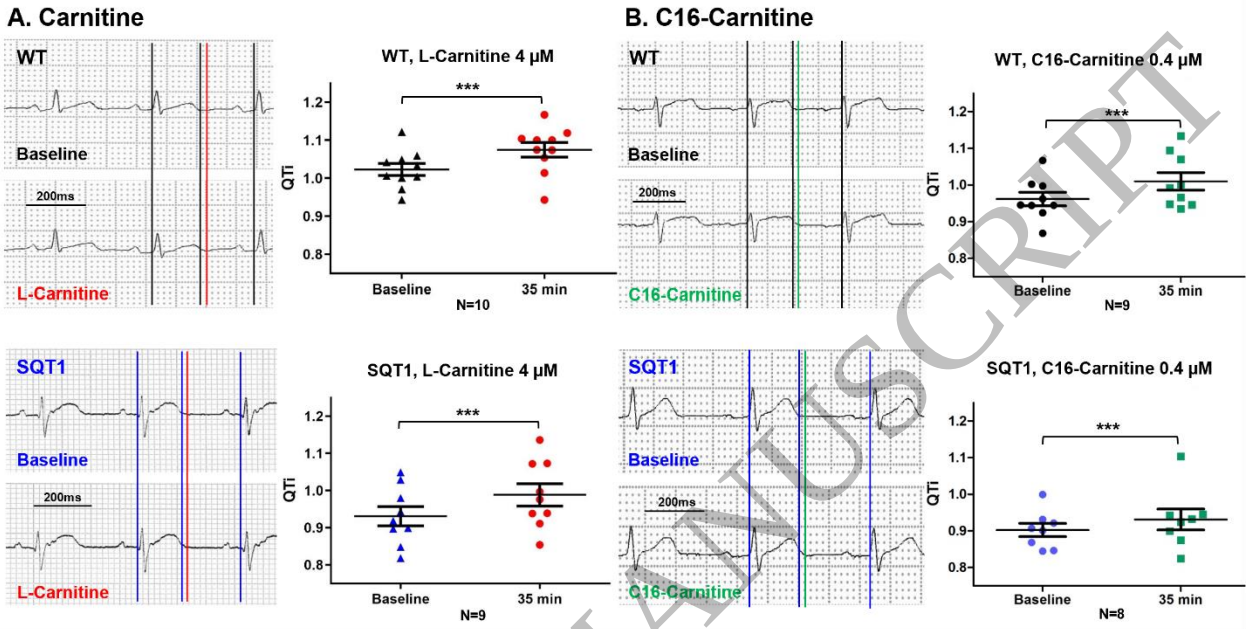


Figure 1
165x90 mm (x DPI)

Figure 2: Carnitine effects on APD in whole hearts *ex vivo*

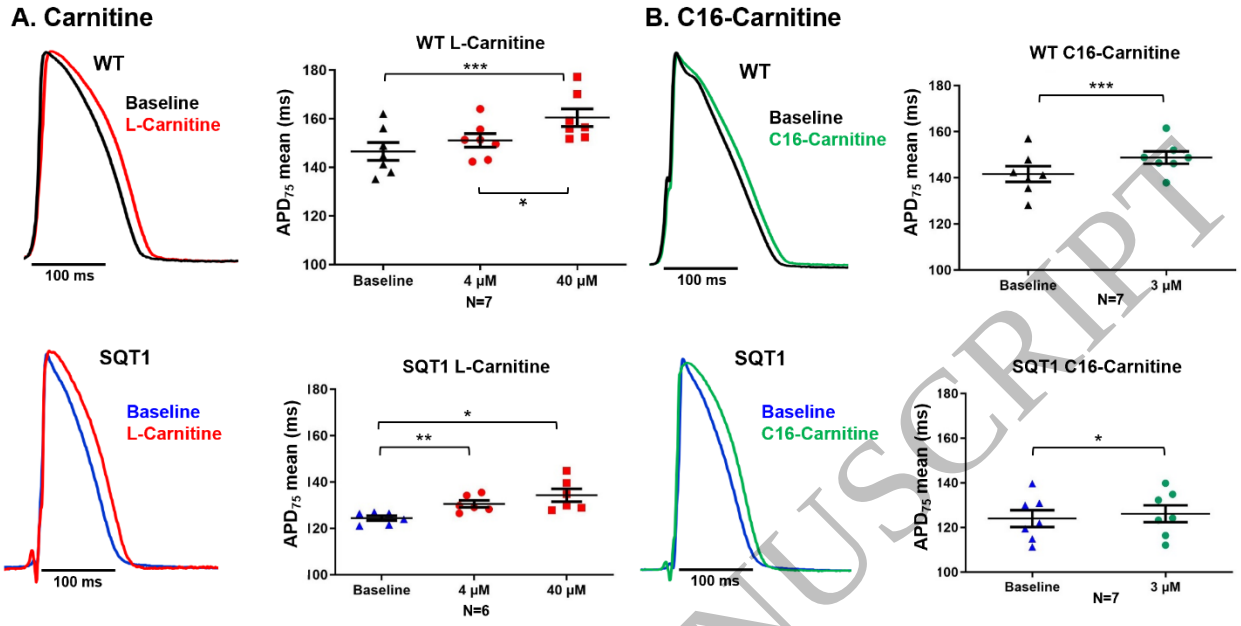


Figure 2
165x88 mm (x DPI)

Figure 3: Carnitine effects on cellular APD

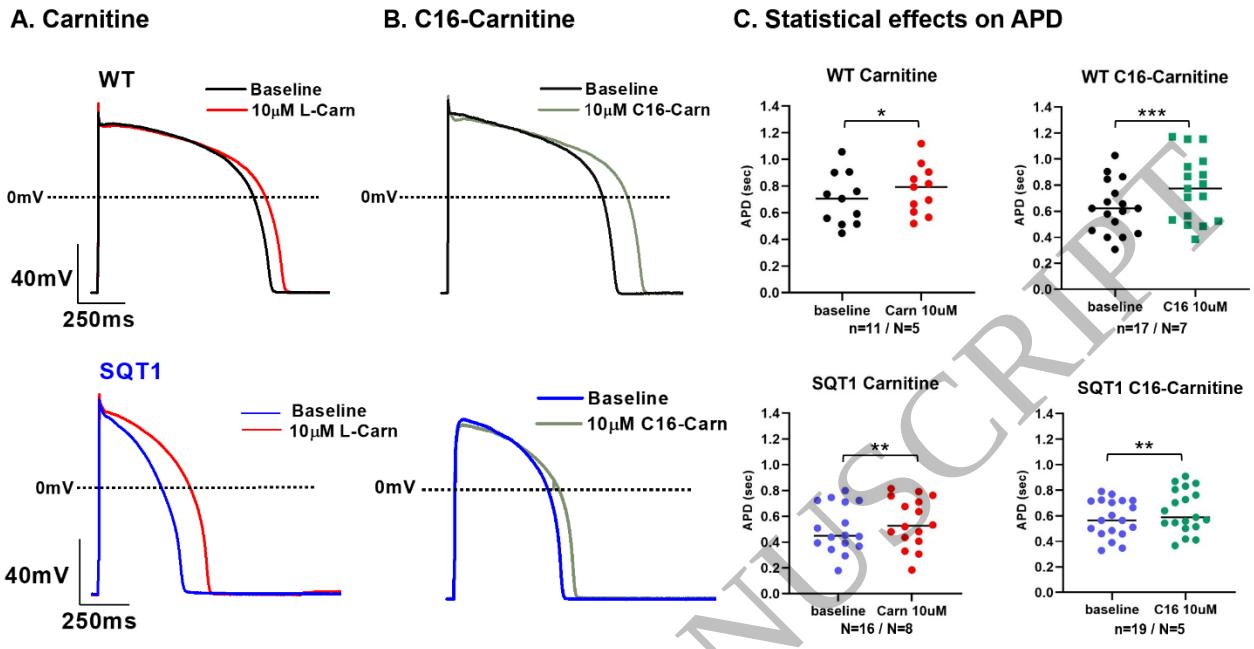


Figure 3
165x91 mm (x DPI)

Fig. 4: Carnitine and C16-Carnitine effects on I_{Kr} tail and steady

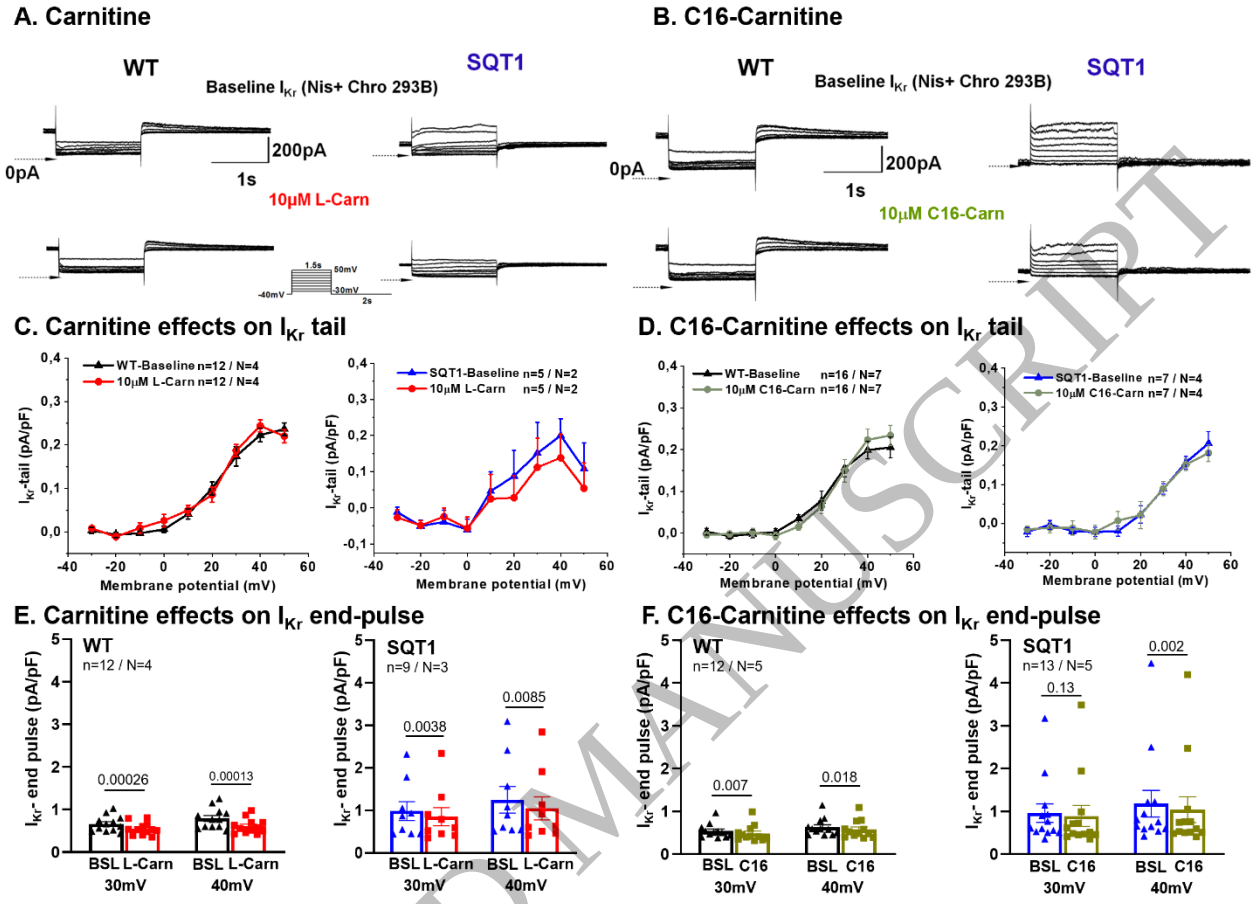


Figure 4
165x123 mm (x DPI)

Fig. 5: Carnitine and C16-Carnitine effects on I_{Kr} activation and deactivation

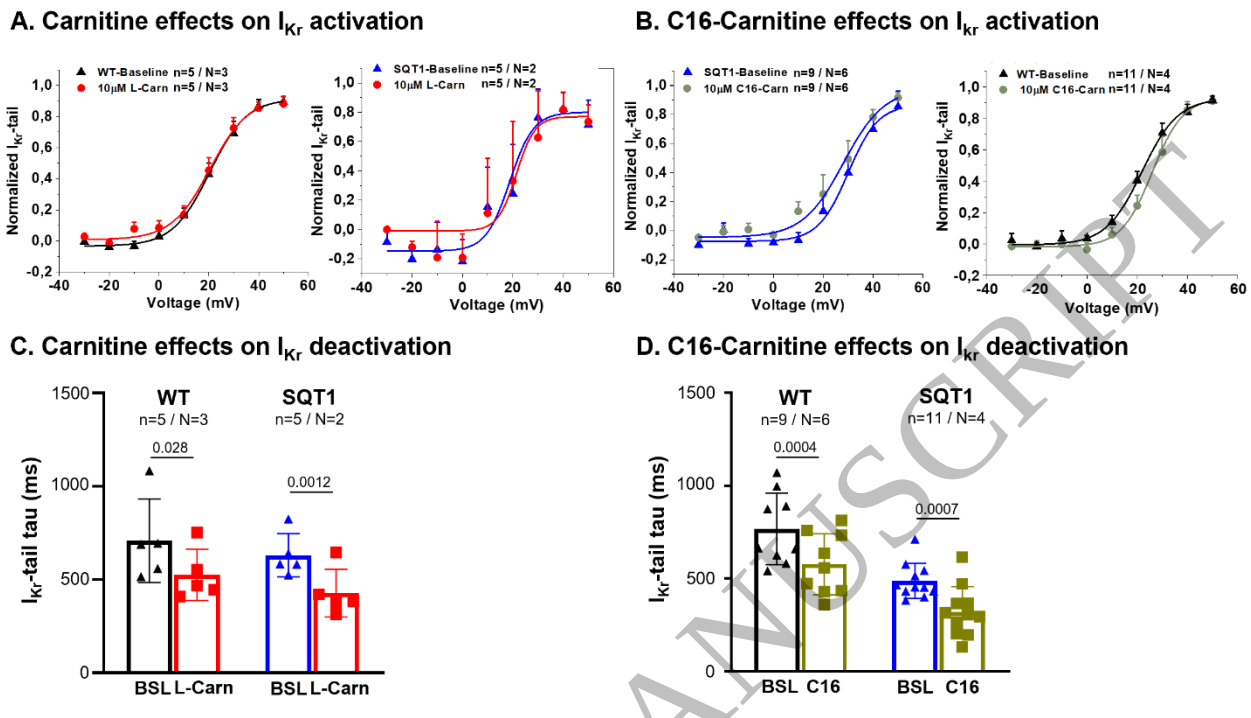


Figure 5
165x99 mm (x DPI)

Fig. 6: Carnitine and C16-Carnitine effects on I_{Ks}

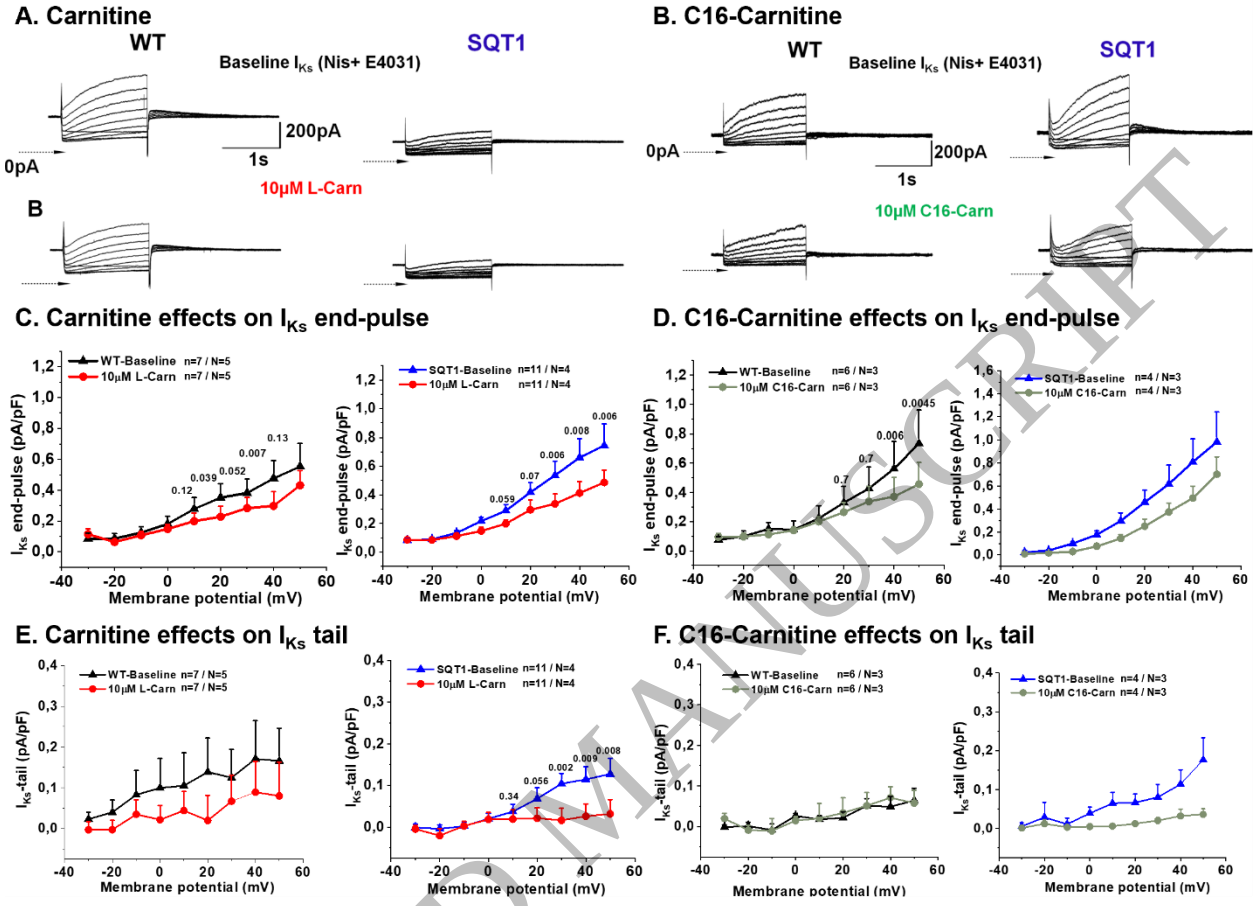


Figure 6
165x123 mm (x DPI)

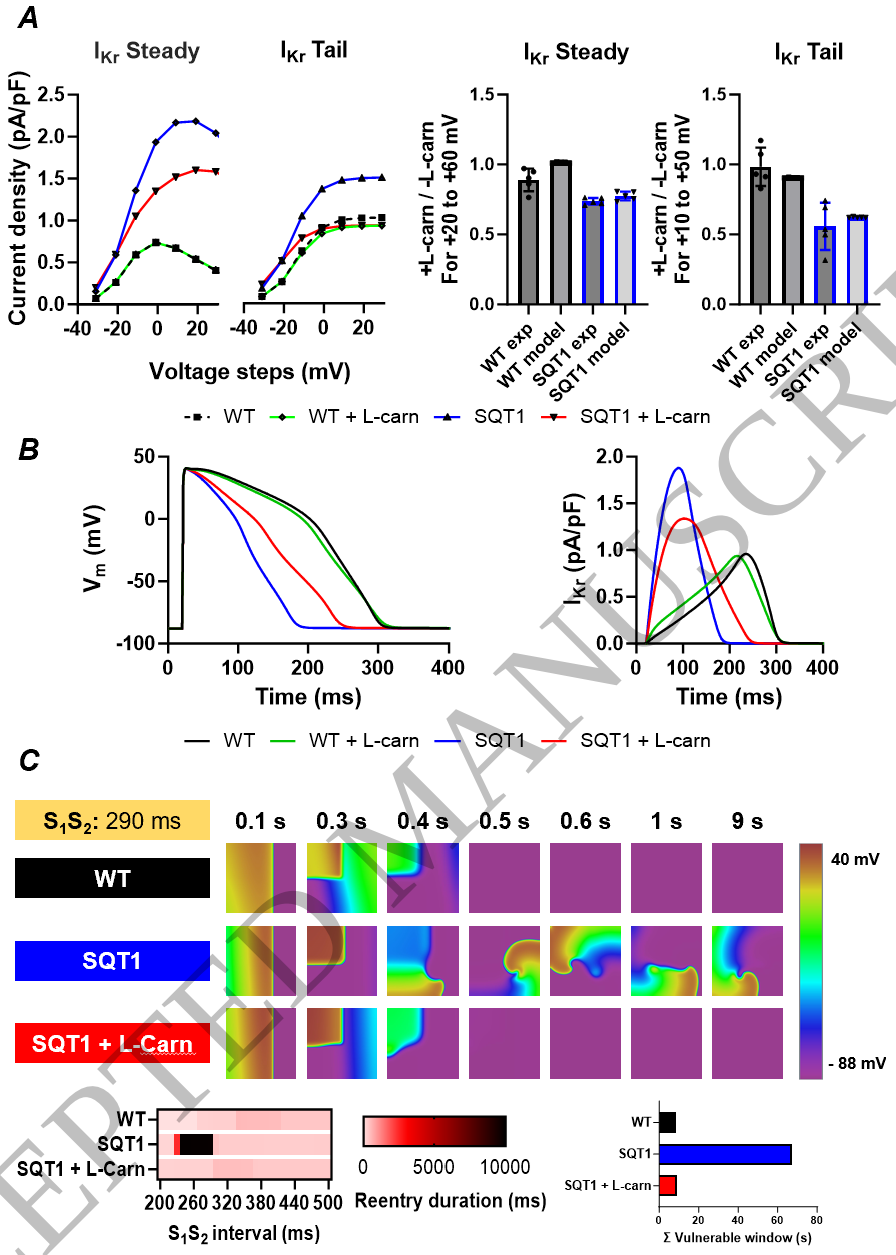
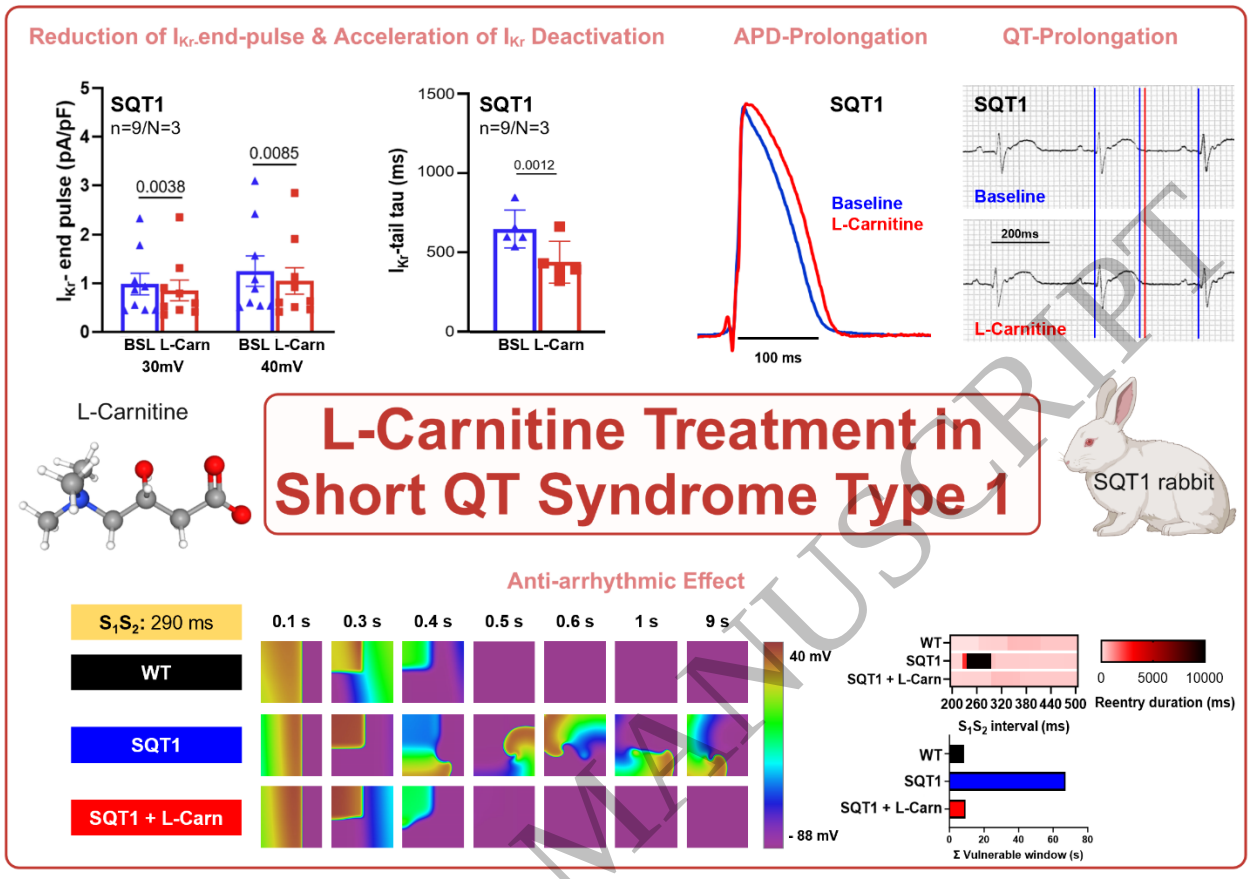


Figure 7
117x166 mm (x DPI)



Graphical Abstract