HHS Public Access

Author manuscript

Heart Rhythm. Author manuscript; available in PMC 2015 April 15.

Published in final edited form as:

Heart Rhythm. 2012 May ; 9(5): 823–832. doi:10.1016/j.hrthm.2012.01.009.

Estradiol Promotes Sudden Cardiac Death in Transgenic Long-QT Type 2 Rabbits while Progesterone is Protective

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Abstract

Background and Objective—Postpubertal women with inherited long-QT syndrome 2 (LQT2) are at increased risk for polymorphic ventricular tachycardia (pVT) and sudden cardiac death (SCD), particularly during the postpartum. We aimed at investigating whether sex hormones directly modulate the arrhythmogenic risk in LQTS.

Methods and Results—Prepubertal ovariectomized transgenic LQT2 rabbits were treated with estradiol (EST), progesterone (PROG), dihydrotestosterone (DHT), or placebo (OVX). During eight weeks of treatment, major cardiac events – spontaneous pVT or SCD – occurred in 5/7 EST rabbits, contrasting with 2/9 in OVX ($p<0.05$) and no events in 9 PROG and 6 DHT rabbits ($p<0.01$ vs. PROG, $p<0.05$ vs. DHT). Moreover, EST increased the incidence of pVT ($p<0.05$ vs. OVX), while PROG reduced PVCs, bigeminy, couplets, triplets, and pVT ($p<0.01$ vs. OVX, $p<0.001$ vs. EST). *In vivo* ECG monitoring, *in vivo* electrophysiological and *ex vivo* optical mapping studies revealed that EST promoted SCD by steepening the QT/RR slope ($p<0.05$), by prolonging cardiac refractoriness ($p<0.05$), and by altering the spatial pattern of APD dispersion. Isoproterenol-induced Ca^{2+} oscillations resulted in early afterdepolarisations (EAD) in EST-treated hearts (4/4), while PROG prevented SCD by eliminating this EAD formation in 4/7 hearts ($p=0.058$ vs. EST, $p<0.05$ vs. OVX). Analyses of ion currents demonstrated that EST increased the density of $I_{Ca,L}$ compared to OVX ($p<0.05$), while PROG decreased it ($p<0.05$).

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The authors declare no conflict of interests.

Conclusion—This study reveals the pro-arrhythmic effect of EST and the anti-arrhythmic effect of PROG in LQT2 *in vivo*, outlining a new potential anti-arrhythmic therapy for LQTS.

Keywords

long-QT syndrome; sex hormones; arrhythmogenesis; sudden cardiac death; transgenic rabbit model; ion channels; early afterdepolarisation; in vivo electrophysiological study

Introduction

The inherited long-QT syndrome (LQTS) is characterized by an impaired cardiac repolarization resulting in QT prolongation, polymorphic ventricular tachycardia (pVT), and sudden cardiac death (SCD)[1]. Importantly, patients with LQTS exhibit pronounced gender differences in cardiac repolarization and their arrhythmogenic risk. Data from the international LQTS registry show longer QT intervals, a steeper QT/RR ratio, and a higher risk for pVT and SCD in postpubertal women with LQTS type 2 (loss of I_{Kr}[2])[3]. In contrast, before puberty, the arrhythmia incidence is higher in boys[4]. Moreover, both the menstrual cycle and the postpartum period are associated with changes in the incidence of pVT. LQT2 patients have a reduced risk during pregnancy and a markedly increased risk during the postpartum period[5, 6]. In addition, in the acquired drug-induced LQTS variant the risk for pVT is higher during menses and the follicular phase with high serum estradiol levels than during the luteal phase with high progesterone levels[7]. These observations strongly suggest a potential pro-arrhythmic role for estradiol and an anti-arrhythmic effect of progesterone. However, these postulated pro- and anti-arrhythmic sex hormone effects in LQTS have never been demonstrated *in vivo* and their underlying mechanisms have yet to be characterized.

We recently generated transgenic LQT2 rabbits over-expressing a loss-of-function pore mutation of the human HERG channel (HERG-G628S) in the heart, mimicking the human LQT2 phenotype with QT prolongation, steeper QT/RR ratio in females, spontaneous pVT, and SCD – with a particularly high incidence in the postpartum[8, 9]. Mechanisms underlying these arrhythmias include a pronounced spatial dispersion of action potential duration (APD) and dynamic APD changes that lead to discordant alternans[8, 10], as observed in LQTS patients[11]. Here we demonstrate in prepubertal ovariectomized female LQT2 rabbits chronically treated with different sex hormones that estradiol and progesterone have direct and contrasting effects on arrhythmias and SCD by modulating the arrhythmogenic substrate and the generation of triggered activity.

Methods

(A detailed method description can be found in an accompanying online supplement.)

Ovariectomy, hormone treatment

Prepubertal LQT2 rabbits underwent ovariectomy or sham surgeries and 90-day release pellets (Innovative Research of America) containing 17β-estradiol, dihydrotestosterone, progesterone, or placebo were implanted subcutaneously resulting in similar estradiol levels

as during follicular phase, progesterone levels comparable to pregnant rabbits, and dihydrotestosterone levels as in males[12, 13] (Fig. 1 supplement).

Telemetric ECG monitoring: QT/RR ratio and arrhythmia screening

Using telemetric ECG devices (F70-EEE, DSI) QT/RR ratio and heart rate corrected QT indices were calculated[8, 9]. Arrhythmias and major cardiac events – pVT and SCD – within corresponding 2-hour intervals were analyzed and classified using Lown's classification[14].

In vivo electrophysiological studies (EPS)

Catheter-based *in vivo* EPS were performed to assess ventricular effective refractory periods (VERP) in RVapex and base at baseline and during isoproterenol infusion (ISO, 0.10 - 0.25 μg/min)[15].

Optical mapping

Dual voltage-calcium optical mapping (100x100 pixels, Ultima-L, Scimedia)[16] was performed using fluorescence probes PGH1 for membrane potential (generously provided by Dr. Guy Salama, University of Pittsburgh) and rhod-2 for Ca_i (Invitrogen). Images were acquired from the LV anterior surface and the field of view was set to 1.5 × 1.5 cm^2 with a spatial resolution of 150 × 150 μm^2 [8, 16]. To investigate hormone effects on early afterdepolarisation (EAD) formation, hearts were exposed to an intra-coronary isoproterenol bolus (140 nM) after AV-ablation.

Patch clamp

Whole-cell recordings in cardiomyocytes isolated from LVapex were obtained with an Axopatch-200B amplifier (Axon Instruments) with standard patch-clamp techniques[8].

Western Blot

Western blot experiments on crude membrane preparations of LV apex were performed[8] using the following antibodies: anti-SERCA2a (Thermo Scientific, MA3-919), anti-PLN (Thermo Scientific, MA3-922), and anti-NCX (Thermo Scientific, MA3-926) as primary antibodies and HRP conjugated goat-anti-mouse (IgG polyclonal, Thermo Scientific) as secondary antibodies.

Statistical analysis

For normally distributed values, we used Student's *t*-test (paired and unpaired). Chi-square test was used for categorical variables. Analysis was performed with Prism 4.03 (Graphpad) and a p value ≤ 0.05 was considered significant.

Results

Sex hormone effects on arrhythmogenesis

To investigate hormone effects on arrhythmogenesis in LQTS, we treated prepubertal ovariectomized transgenic LQT2 rabbits with estradiol (EST), progesterone (PROG), dihydrotestosterone (DHT), or placebo (OVX) for 8 weeks. We first compared arrhythmia

incidences within corresponding 2-hour intervals one week before and within 96 hours following EPS using telemetric ECG monitoring (Fig. 1A). In the week before EPS, no arrhythmias besides isolated sinus pauses occurred in either group. In the 96 hours after EPS, however, arrhythmia incidences were higher in all groups but varied significantly among groups. PROG significantly reduced the incidence of PVCs and couplets compared to OVX and EST rabbits, and importantly, bigeminy and triplets did not occur in any PROG rabbit (Fig. 1A), strongly indicating an anti-arrhythmic effect in PROG rabbits. Moreover, no single episode of non-sustained or sustained pVT occurred in any PROG rabbit further underlining an anti-arrhythmic PROG effect (Fig. 1A). The incidences of PVC and bigeminy were similar in EST and OVX rabbits. However, couplets tended to occur more often in EST rabbits ($p=0.06$) and nsVT occurred significantly more frequently in EST than in OVX rabbits, suggesting a pro-arrhythmic EST effect. Of note, all lethal pVTs were initiated by short-long-short sequences and an early PVC coinciding with the T wave (R-on-T phenomenon), as described in LQTS patients[17], suggesting that EADs likely underlie arrhythmia initiation (Fig. 1B). Moreover, prior to the initiation of pVTs the heart rate slowed down, due to short-long-short sequences and AV block (in 5/8 rabbits) (Fig. 1B). The incidence of AV blocks, however, did not differ between groups.

We consecutively ranked the severity of arrhythmias using Lown's classification[14]: 9/9 PROG rabbits had either Lown 0 (no PVCs) or Lown 1 (less than 30 PVCs/hour) arrhythmias, while 5/9 OVX rabbits demonstrated at least Lown 3 arrhythmias (e.g., bigeminy) ($p<0.01$ vs. PROG), and 3/9 even had Lown 4 arrhythmias (e.g., couplets, triplets, or nsVT) ($p=0.05$ vs. PROG). In EST rabbits, the rate of Lown 4 arrhythmias (5/7) was even higher ($p<0.01$ vs. PROG).

Finally, we compared the incidence of major cardiac events – defined as spontaneous pVT and SCD – between hormone groups. In EST rabbits, the incidence of major cardiac events (5/7) was significantly higher than in OVX (2/9, $p<0.05$), PROG (0/9, $p<0.01$), and DHT (0/6, $p<0.05$) rabbits (Fig. 1C). Most lethal pVT occurred within 96-hours after EPS in rabbits that were fully awake similar to observations in previous studies[9, 15]. In a follow up study of rabbits that were not exposed to anesthesia, 3/13 EST rabbits died of pVT contrasting with no SCD in any other hormone group (0/9 DHT, 0/12 PROG, 0/12 OVX). All these observations strongly indicate a pro-arrhythmic effect of EST and an anti-arrhythmic effect of PROG in LQT2 rabbits.

Sex hormone-induced changes in cardiac repolarization

To investigate mechanisms that account for these pro- and anti-arrhythmic hormone effects, we first compared hormone effects on QT duration and QT/RR ratio in free-moving ECG monitored LQT2 rabbits. Heart-rate corrected QT indices were significantly longer in EST than in OVX, PROG or DHT rabbits, and tended to be longer in sham-operated females (SF) than in sham-operated males (SM) ($p=0.1$) (Fig. 2B). These differences in QT duration were particularly pronounced at slow heart rates. As demonstrated in representative ECG tracings in Figure 2A, at RR intervals of 300 ms, QT duration in EST rabbits was similar to SF, while in DHT rabbits the QT was shortened as observed in SM. In OVX and PROG rabbits QT duration was intermediate. Furthermore, EST steepened the QT/RR slope (Fig. 2C),

mimicking the adult LQT2 female phenotype (Fig. 2G). In contrast, DHT decreased the steepness of the QT/RR slope (Fig. 2D), thus mimicking the male phenotype (Fig. 2G). Similarly, OVX decreased QT/RR steepness (Fig. 2E), while PROG did not alter QT/RR ratio (Fig. 2F).

To further examine hormone effects on rate dependent changes of QT and VERP, we performed *in vivo* EPS. Figure 3 A-B depicts representative ECG recordings in EST and PROG rabbits during VERP determination in RVapex at a stimulation cycle length of 300 ms (S1 train). In the top panel, the coupled extra stimulus (S2) was captured, whereas in the lower panel the 10 ms shorter S2 stimulus failed to capture (at 240 ms in EST and 150 ms in PROG rabbit). In RVapex, VERPs were longer in EST than in OVX, PROG or DHT rabbits (Fig. 3C). In RVbase, however, hormone effects on the VERP were not as pronounced (Fig. 3C). Continuous infusion of ISO shortened the VERP in SF more than in SM. Similarly, the ISO-induced VERP-shortening was more pronounced in EST than in OVX, PROG, or DHT rabbits (Fig. 3D).

Sex hormones and APD dispersion

We next investigated whether sex hormones modulate spatial APD dispersion – a known mechanism underlying arrhythmogenesis in LQT2 syndrome[11] – using optical mapping. Figure 4A shows representative APD maps of the LV anterior surface of hormone- treated LQT2 rabbit hearts with basal regions represented in the upper part of the image and apical regions in the lower part. Isolines of APD are drawn every 5 ms and dark regions represent long APD while bright regions represent short APD. In all OVX, PROG, and DHT rabbits (*n=4-7* each) the region with the longest APD was found in the LV mid-septal region and the shortest APD in the apex. Yet, EST altered the pattern of APD dispersion, and the longest APD region was shifted towards the LVapex. However, we found no significant differences in mean APD and APD dispersion among groups (Fig. 4B). Programmed ventricular stimulation induced VF in all five EST rabbits, contrasting with 3/6 OVX ($p<0.05$), 2/7 PROG ($p<0.05$), and 0/4 DHT rabbits ($p<0.01$). In all EST rabbits, during VF, activation waves propagated around the apical region of prolonged APD. As illustrated in Figure 4C, activation waves encountered refractoriness in the apical region with prolonged APD, which caused unidirectional block and wave propagation around this region. Conduction velocity measured on the anterior surface of the left ventricle was similar in all groups (OVX, 0.55 ± 0.12 m/s; EST, 0.59 ± 0.2 m/s; PROG, 0.56 ± 0.1 m/s), indicating a lack of structural changes with hormone treatment.

Sex hormones and EAD formation

Since it is well known that pVT is initiated by EADs triggered by sympathetic surge[18], we examined the response to a bolus of ISO using dual voltage-calcium optical mapping. In all EST (4/4), OVX (5/5), SF (4/4), and SM (4/4) rabbits, both membrane potentials and Ca^{2+} transients began to oscillate within 5-10 s after ISO bolus (Fig. 5A). Ca^{2+} started to rise prior to changes in membrane potential during the early phase of EAD formation, yet in later EADs with larger amplitude the membrane potential changes led to Ca^{2+} rise (Fig. 5A), suggesting that Ca^{2+} oscillations may be an important trigger for EAD formation in this LQT2 model. In contrast, in 4/7 PROG and in 2/4 DHT rabbits, Ca^{2+} transient oscillations

failed to initiate EADs, resulting in a significantly reduced EAD formation rate in PROG rabbits (*p=0.058* vs. EST, *p<0.05* vs. OVX) (Fig. 5B).

Sex hormone effects on ion currents

To elucidate underlying mechanisms on the cellular level, we performed patch clamp experiments with cells harvested from the LVapex, where we observed the most pronounced differences in APD maps and Ca^{2+} oscillations. I_{Ks} current density was significantly increased in all hormone groups, SF, and SM as compared to OVX rabbits. However, this increase was most pronounced in EST rabbits resulting in a significantly higher I_{Ks} current in EST than in PROG or DHT-treated cardiomyocytes (Fig. 5C). Importantly, EST increased the density of $I_{Ca,L}$ currents while PROG decreased $I_{Ca,L}$ as compared to OVX resulting in a significantly higher $I_{Ca,L}$ in EST than in PROG rabbits (Fig. 5D). No differences were observed in I_{to} and I_{K1}. Due to the dominant negative effect of the HERG pore mutant in LQT2 rabbits, I_{Kr} was absent in all groups[8].

Sex hormone effects on Ca^{2+} cycling proteins

To test whether changes in Ca^{2+} cycling proteins may account for the differences in the propensity to develop EADs, we compared their expression in the LVapex. We observed a significantly increased expression of the sarcoplasmic reticulum calcium ATPase2a (SERCA2a) polypeptides in PROG as compared to EST and OVX rabbits (*p<0.05*; Fig. 5E) that may contribute to an increased Ca^{2+} reuptake into the SR thereby shortening Ca^{2+} transient duration. No hormone-induced differences were apparent in sodium-calcium exchanger (NCX) (Figure 5F), and phospholamban (PLN) (Figure 5G).

Discussion

Pro- and anti-arrhythmic effects of sex hormones *in vivo*

Sex differences in long-QT-related arrhythmias with a higher risk for polymorphic VT and SCD in women than in men and a particularly increased risk during the postpartum period have been well documented in the clinical setting[3, 6]. This study takes advantage of a transgenic LQT2 rabbit model that develops spontaneous pVTs and SCD[8] to investigate the role of sex hormones in arrhythmogenesis *in vivo.*

Here we show for the first time a direct link between sex hormones and the incidence of arrhythmias and SCD. Our telemetry recordings of hormone-treated LQT2 rabbits demonstrate that PROG significantly reduces potential triggers for polymorphic VTs – such as bigeminy and couplets – and completely abolishes the occurrence of pVT. Moreover, we show that PROG is protective and prevents SCD, suggesting that high PROG levels during pregnancy likely account for the reduced risk in pregnant LQT2 patients[5]. Additionally, the marked reduction of PROG during the postpartum period likely promotes postpartal arrhythmias and SCD in LQT2 patients[6]. EST, in contrast, increases both, triggers and the sustainability of pVT and thereby promotes SCD, indicating that EST likely underlies the increased arrhythmogenic risk in postpubertal women with LQT2[3].

As in LQT2 patients[3], SCD is a rare event in LQT2 rabbits. However, similar to observations in previous studies[9, 15], lethal pVT occurred more frequently within 96-hours after isoflurane anesthesia. It may be possible that anesthesia-induced slowing of the heart rate[15] or the I_{Ks}-blocking properties of isoflurane[9] may contribute to the overall increased risk. However, all rabbits had recovered from anesthesia and were fully awake at the time of SCD. Since similar anesthetic dosages were used, it is likely that anesthesia increased the likelihood of events similarly in all groups and therefore enabled us to uncover the protective effect of PROG and the pro-arrhythmic effect of EST. Moreover, in a group of rabbits not exposed to anesthesia, we observed 3 SCD in 13 EST rabbits while no SCD was observed in any other group, further underlining the pro-arrhythmic effect of EST.

Hormone effects on the arrhythmogenic substrate

Enhanced APD dispersion due to a spatially heterogeneous prolongation of cardiac repolarization is considered a major contributor to LQT-related arrhythmias[11]. Moreover, we have previously identified an increased spatial APD dispersion across the anterior surface of the LV as a major mechanism underlying arrhythmias in transgenic LQT2 rabbits[8, 10]. We thus further explored whether sex hormones alter dispersion of repolarization in RV and LV. In EST rabbits, the longest APD region was shifted towards the LVapex, in line with the more pronounced VERP prolongation in the RVapex, suggesting that EST may exert its proarrhythmic effects, at least partly, by differentially changing APD in different regions of LV and RV thus modifying the arrhythmogenic substrate. Indeed, programmed ventricular stimulation induced VF in all five EST rabbits but only rarely and significantly less frequently in OVX, PROG, and DHT rabbits, thus indicating the pro-arrhythmic significance of these EST-induced changes. Moreover, during VF the activation waves propagated around the apical island of prolonged APD.

We further investigated how sex hormones differentially affect rate dependent repolarization (restitution). Our *in vivo* ECG monitoring studies are the first to demonstrate that EST steepens the QT/RR slope in LQT2 by prolonging QT duration at slow heart rates and shortening QT at fast heart rates, whereas DHT decreases the steepness of QT/RR slope. EST and DHT treatment, thus mimic sex differences in cardiac repolarization in LQT2 rabbits[9], similar to observations in LQT2 patients[3]. $I_{Ca,L}$ and I_{Ks} play an important role in conferring these hormone effects on QT/RR: EST rabbits have higher density of $I_{Ca,L}$ than any other groups, resulting in prolongation of QT at slow heart rates, while the higher density of I_{Ks} in EST rabbits shortens QT at fast heart rates thus contributing to EST-induced steepening of QT/RR ratio.

Hormone effects on the susceptibility to pro-arrhythmic triggers

The initiation of pVTs in LQTS is known to be linked to EAD formation[18]. Moreover, previous animal studies demonstrated that hormones alter EAD formation in isolated cardiomyocytes[13, 19]. Here we demonstrate that sex hormones exert different effects on EAD formation in response to sympathetic stimuli at the organ level. In EST and OVX rabbits, ISO triggered both Ca^{2+} oscillations and EAD formation, while in PROG and DHT rabbits ISO triggered Ca^{2+} oscillations without EAD formation. Thus, PROG altered the response of the membrane potential to Ca^{2+} oscillations.

Experimental and simulation studies show that the reactivation of $I_{Ca,L}$ plays an important role in EAD formation[20, 21] and that reducing $I_{Ca,L}$ by Ca^{2+} -blocking drugs such as verapamil is most effective in preventing pVT formation in animal models of LQTS[22]. Previous experimental and simulation data strongly suggest that $I_{Ca,L}$ is a key player in the formation and propagation of EADs[24, 25]. Here we demonstrate that EST increased $I_{Ca,L}$ while PROG and DHT decreased $I_{Ca,L}$ as previously described[23, 24]. Thus, EST-induced increase in $I_{Ca,L}$ likely contributes to the higher propensity to develop EADs, contributing to the proarrhythmic effects of EST. By contrast, the PROG-induced decrease in $I_{Ca,L}$ reduces the likelihood of triggered activity. Our studies also demonstrate an increase of I_{Ks} in EST rabbits. Recent computer modeling studies show that both inward Ca^{2+} and outward K^+ currents are essential for generating oscillatory behavior of the membrane potential during the plateau phase[25] suggesting that the EST-induced increase of $I_{Ca,L}$ and I_{Ks} facilitates triggered activity.

To further delineate molecular mechanisms underlying these pro- and anti-arrhythmic effects on the susceptibility to triggers, we investigated hormone effects on Ca^{2+} cycling proteins, which may contribute to arrhythmogenesis by altering cytoplasmic and SR Ca^{2+} concentrations, and the initiation of EADs. While previous studies in rats reported a DHT- and EST-induced increase in NCX[26, 27], we observed no hormone effects on NCX or PLN expression in LQT2 rabbits. Yet, PROG increased SERCA2a expression in LQT2 rabbits, which may contribute to an increased Ca^{2+} reuptake into the SR thereby shortening Ca^{2+} transient duration. An increased expression of SERCA2a has previously been reported to reduce VT/VF in ischemia-reperfusion models[28] and to reduce the susceptibility to alternans-mediated ventricular arrhythmias[29]. Consequently, this increase in SERCA2a and the decrease in $I_{Ca,L}$ currents contribute to the anti-arrhythmic effect of PROG.

Limitations

Transgenic LQT2 rabbits over express the dominant-negative loss-of-function mutation HERG-G628S that is localized in the pore-region and leads to a complete loss of I_{Kr}[30]. Many missense mutations in HERG channels that are found in LQT2 patients, however, lead to a substantial decrease rather than a complete loss of I_{Kr}. The findings on hormone effects on arrhythmogenesis in this transgenic LQT2 rabbit model likely recapitulate the findings in human patients with pore mutations or other HERG mutations with a loss of functional I_{Kr} currents in the heart.

Conclusion

In this study, we demonstrate that EST promotes polymorphic VTs and SCD while PROG prevents arrhythmias and SCD in LQT2 *in vivo*. EST exerts this pro-arrhythmic effect by changing the arrhythmogenic substrate by steepening QT/RR ratio, prolonging cardiac refractoriness, and altering the spatial pattern of APD dispersion. The underlying mechanisms are an increased I_{Ks} current that contributes to the steepening of QT/RR by shortening QT at fast heart rates and notably a substantially increased $I_{Ca,L}$ that contributes to both a longer refractoriness and a higher propensity to depolarize the membrane in response to Ca^{2+} oscillations. PROG, in contrast, exerts an anti-arrhythmic effect by

preventing EAD formation in response to Ca^{2+} oscillations, likely due to an increase in SERCA2a and a decrease in the oscillatory $I_{Ca,L}$.

Clinical implications

To date, standard treatment of LQT2 patients consists of beta-blockade and implantation of ICDs[1]. Understanding the mechanisms that underlie sex hormones' deleterious or protective effects could help to develop specific, hormone-based therapies. The experimental observation of an anti-arrhythmic PROG effect in transgenic LQT2 rabbits suggests a potential use of oral progestins as a new class of anti-arrhythmic treatment in LQTS. Further prospective studies are needed to provide evidence-based data to support this treatment option in LQTS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Louise Organ-Darling, Alfred E. Buxton, and Gary F. Mitchell for their valuable comments in reviewing the manuscript and Megan Shearer for her help with the ovariectomy surgeries.

Funding sources

G. Koren is the recipient of NIH grant RO1 HL046005-18 and HL093205; K.E. Odening was supported by grants from the German Cardiac Society (St. Jude Medical Stipendium), the German Research Foundation (DFG Forschungsstipendium OD 86/1-1), and by an AHA postdoctoral fellowship award (AHA 0826071D).

List of abbreviations

LQT2	long-QT syndrome type 2
pVT	polymorphic VT
SCD	sudden cardiac death
EST	estradiol
OVX	ovariectomy
PROG	progesterone
DHT	dihydrotestosterone
SF	sham-operated female
SM	sham-operated male
PVC	premature ventricular contraction
RV	right ventricle
LV	left ventricle
EAD	early afterdepolarisation
APD	action potential duration

AV	atrioventricular
EPS	electrophysiological study
CL	cycle length
AVWCL	AV Wenckebach cycle length
AVNRP	AV nodal refractory period
VERP	ventricular effective refractory periods
ISO	isoproterenol
VF	ventricular fibrillation

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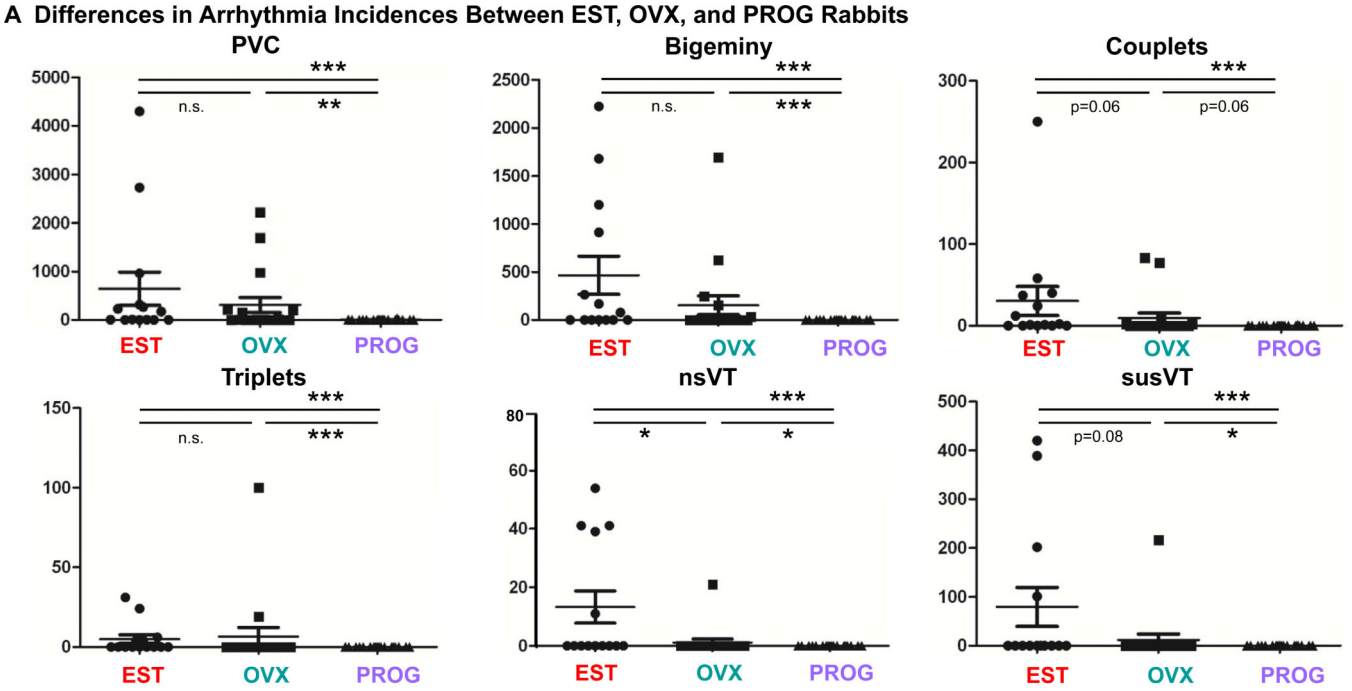

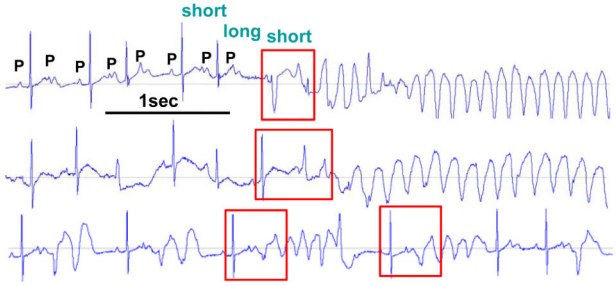

C Sex Hormones and Incidence of Cardiac Events

	SF	SM	EST	DHT	OVX	PROG
n	6	6	7	6	9	9
Cardiac Events (SCD)	1 (1)	1 (1)	5 (4)	0 (0)	2 (2)	0 (0)

Figure 1. Effect of Sex Hormones on Incidence of Arrhythmias

A. Dot blots of differences in arrhythmia incidences (PVC, bigeminy, couplets, triplets, non-sustained VT (nsVT), all presented as beats/two hours; sustained VT (susVT), duration in seconds). Each dot represents a 2-hour interval of an individual rabbit (*n=14* in EST, *n=18* in OVX and PROG rabbits). * *p<0.05*, ** *p<0.01*, *** *p<0.001*. **B.** Telemetric ECG recordings of the initiation of lethal pVT in two EST rabbits, top two rows. Indicated are R-on-T (red square), short-long-short sequences, and P waves (P) during episodes of AV 2:1 block. Bottom row shows several episodes of nsVTs following couplets in an EST rabbit. **C.** Incidence of major cardiac events during 8 weeks of hormone-treatment. Incidences of SCD are indicated in brackets. * *p<0.05*, ** *p<0.01*.

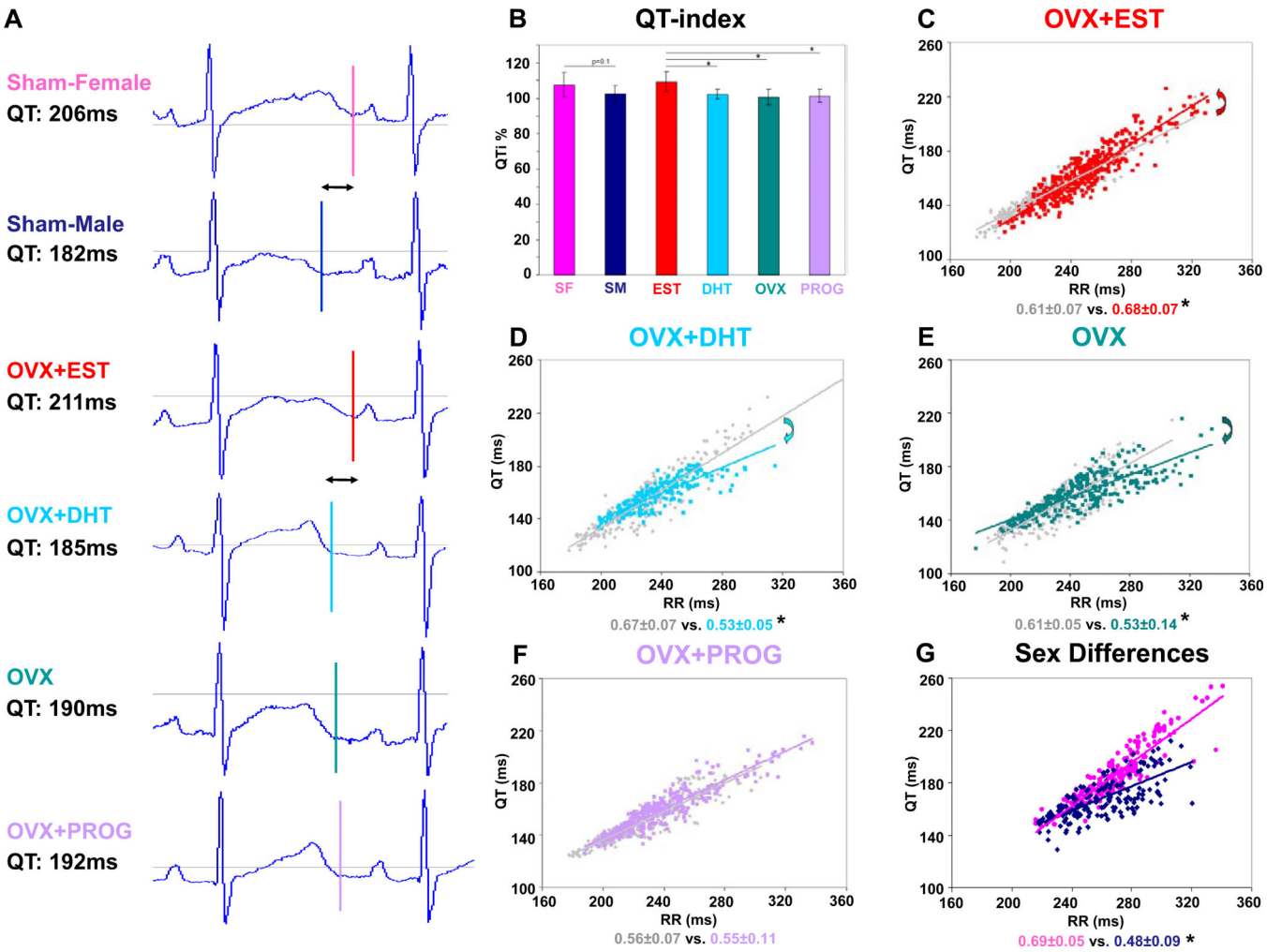

Figure 2. Effect of Sex Hormones on QT Duration

A. Exemplary, representative ECG traces of individual rabbits at 300 ms RR intervals. QT durations are indicated. **B.** QT indices in *n*=6 rabbits after 4 weeks of hormone-treatment calculated based on QT and RR intervals acquired over 24-hours of ECG monitoring. * *p<0.05.* **C-F.** QT/RR ratio in *n*=6 rabbits at baseline (grey) and after 4 weeks of treatment (color). Arrows indicate the direction of changes in QT/RR ratio. * *p<0.05.* **G.** QT/RR ratio in *n*=6 adult SF and SM. * *p<0.05.*

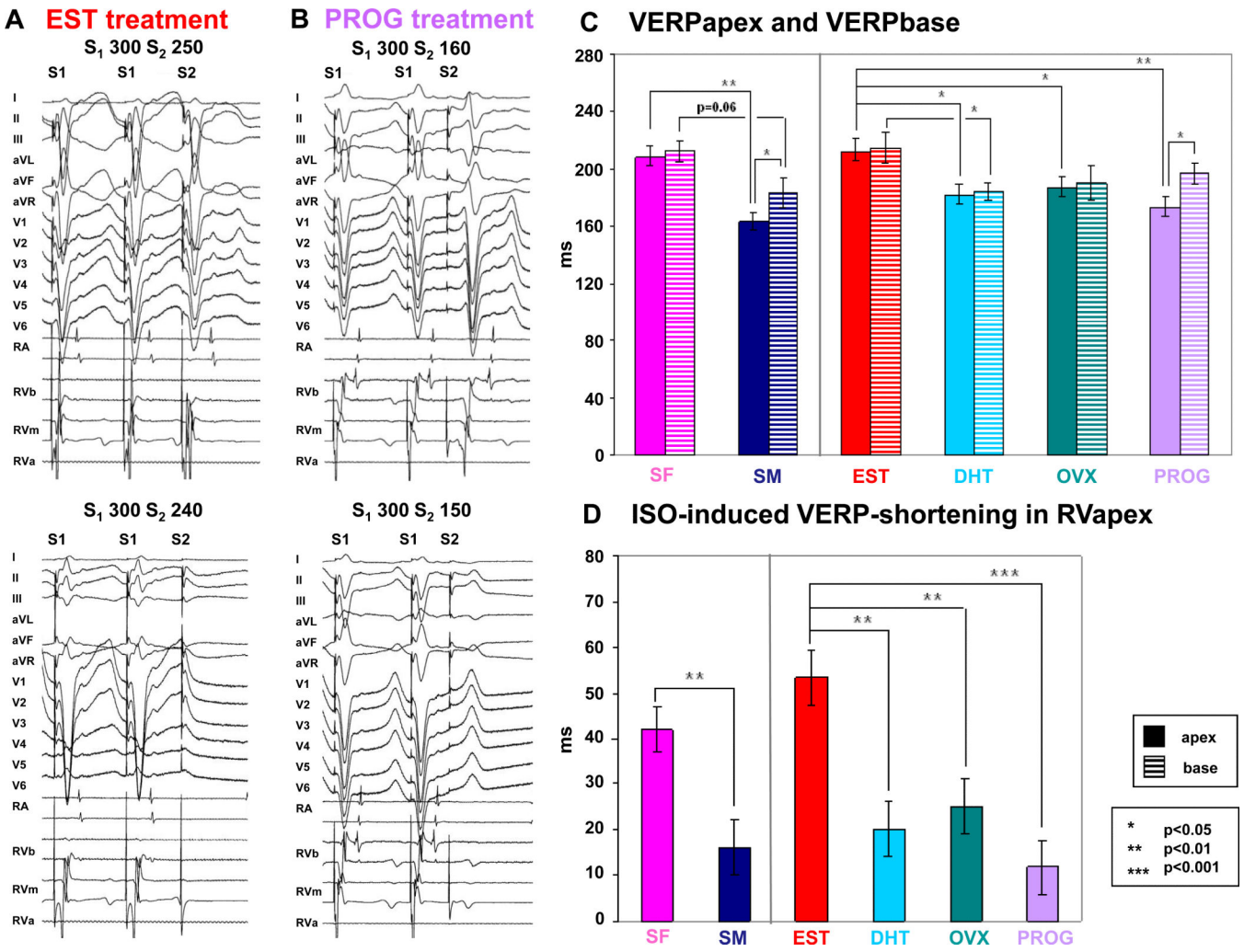

Figure 3. Effect of Sex Hormones on Cardiac Repolarization

A. and **B.** Surface and intra-cardiac ECG in individual EST and PROG rabbit during VERP determination: (top to bottom) 12 surface ECG leads, right atrium (RA) (2 recordings), RVbase (2 recordings), RVmid (2 recordings), and RVapex. Top panel shows stimulation with 300 ms CL (S1) and coupled extrastimuli (S2) that are captured. Lower panel shows shorter S2 extrastimuli that fail to capture. **C.** VERP in RVapex (filled bars) and base (hatched bars) in *n=6* rabbits per group. * *p<0.05*, ** *p<0.01*. All values are shown as mean ± SD. **D.** Delta-VERP baseline-isoproterenol in RVapex. ** *p<0.01*, *** *p<0.001*.

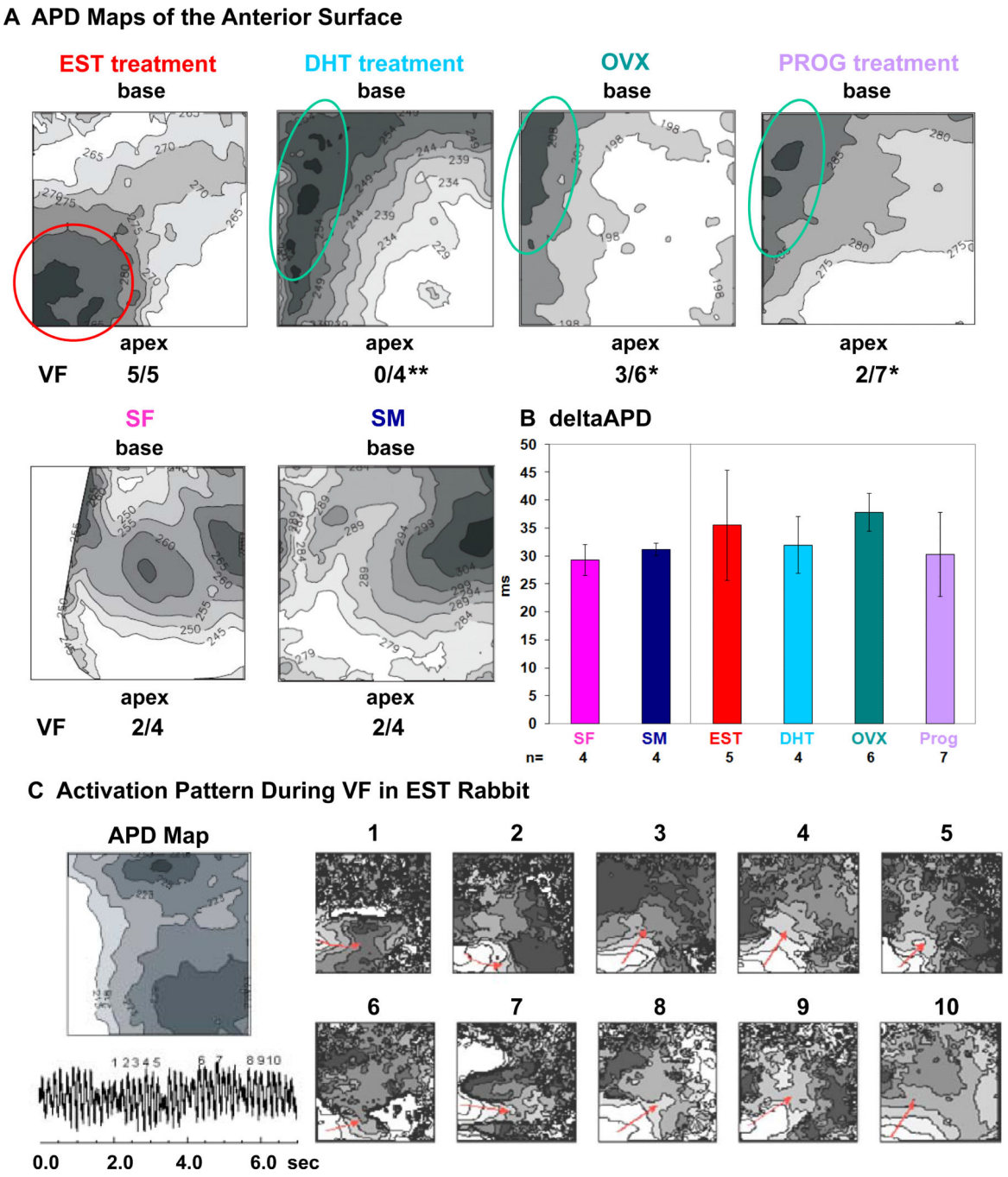

Figure 4. Effect of Sex Hormones on APD Dispersion

A. Representative APD maps of the anterior surface of the LV (field of view 1.5 x 1.5 cm^2) of individual rabbits. Isolines of APD are drawn every 5 ms, darker regions represent longer APD. Indicated are regions of long APD in LV mid-base region (green circle) and LVapex (red circle). Rates of VF inducibility are listed. * $p<0.05$, ** $p<0.01$. **B.** ΔAPD defined as longest – shortest APD. All values are shown as mean ± SD. **C.** Activation pattern during VF in EST rabbit. Displayed are APD map, ECG trace of VF (bottom left), and consecutive

maps (1-10) of the activation pattern during VF. Red arrows indicate the direction of activation waves rotating around the apical region of prolonged APD.

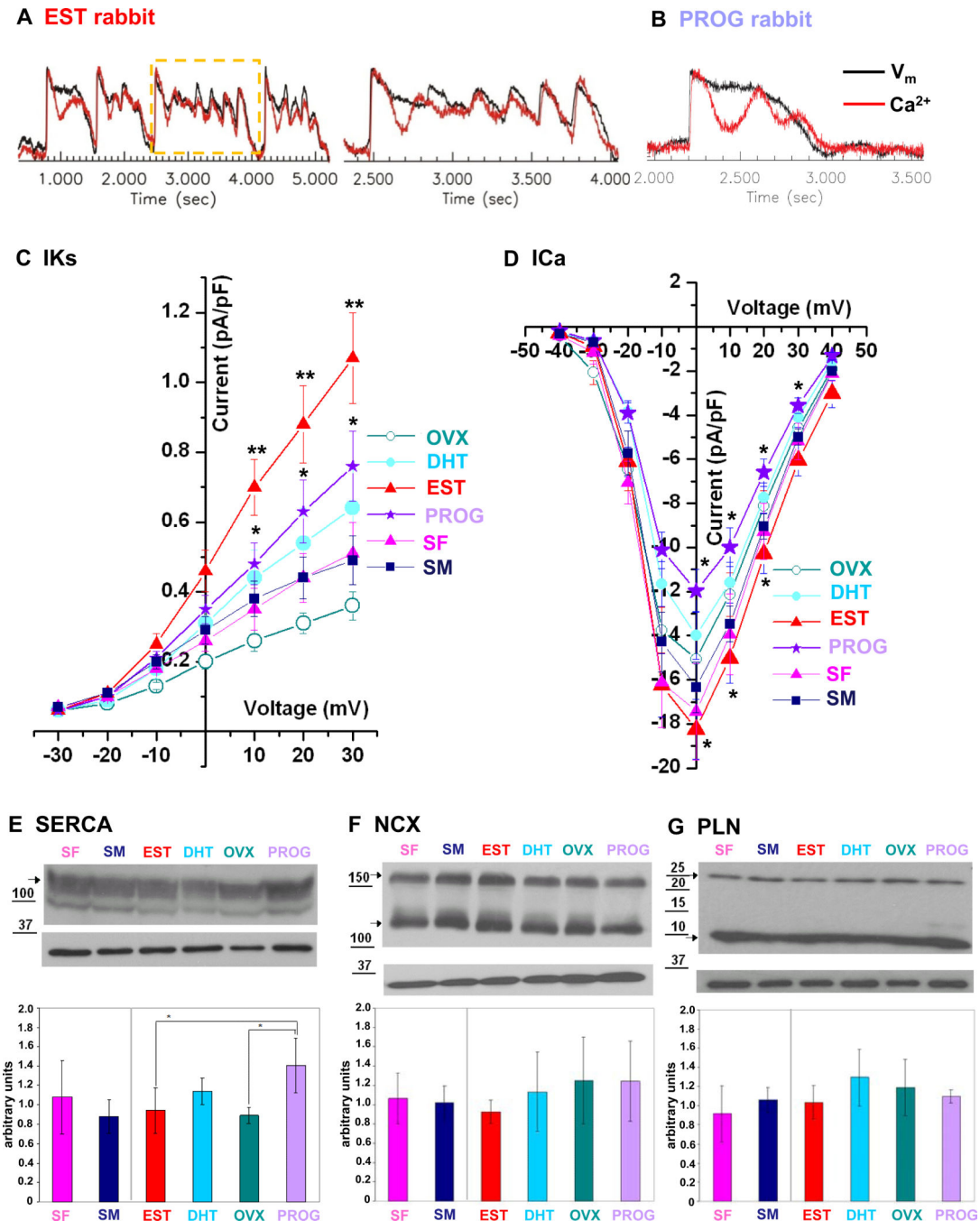

Figure 5. Effect of Sex Hormones on Ca^{2+} Oscillations, EADs, Ion Currents, and Ca^{2+} Cycling Proteins

A. Representative trace of Ca^{2+} oscillations and EADs in EST rabbit after ISO bolus. Black line indicates changes in voltage fluorescence signal (Vm); red line indicates changes in Ca^{2+} signal. The region shown in higher magnification in the right column is indicated by a yellow rectangle. **B.** Representative trace of Ca^{2+} oscillations and lack of EAD formation in PROG rabbit after ISO bolus. **C.-D**. Hormones effects on I_{Ks} and $I_{Ca,L}$ current densities measured in cardiomyocytes harvested from LVapex of EST (n=15 cardiomyocytes), DHT

(*n*=14), OVX (*n*=15), PROG (*n*=18), SF (*n*=6), and SM (*n*=6) rabbits. All values are shown as mean ± SEM. I_{Ks}: EST vs. OVX: *p<0.01*; EST vs. PROG, PROG and DHT vs. OVX *p<0.05*. $I_{Ca,L}$: EST vs. OVX, PROG vs. OVX: *p<0.05*; EST vs. PROG: *p<0.01*. **E.-G.** Representative western blots of SERCA2a, NCX, and PLN. Bar graphs indicate the expression levels of 3 independent experiments in 3 different rabbits per group in arbitrary units. All values are shown as mean ± SD. * *p<0.05*.