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Exposure to Secondhand Smoke and Arrhythmogenic Cardiac Alternans in a Mouse Model

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Background: Epidemiological evidence suggests that a majority of deaths attributed to secondhand smoke (SHS) exposure are cardiovascular related. However, to our knowledge, the impact of SHS on cardiac electrophysiology, Ca^{2+} handling, and arrhythmia risk has not been studied.

Objectives: The purpose of this study was to investigate the impact of an environmentally relevant concentration of SHS on cardiac electrophysiology and indicators of arrhythmia.

Methods: Male C57BL/6 mice were exposed to SHS [total suspended particles (THS): $3.0 \pm 0.1\ mg/m^3$, nicotine: $0.4 \pm 0.2\ mg/m^3$, carbon monoxide: 12.4 ± 1.6 ppm, or filtered air (FA) for 4, 8, or 12 wk ($n = 4–5$/group]. Hearts were excised and Langendorff perfused for dual optical mapping with voltage- and Ca^{2+}-sensitive dyes.

Results: At slow pacing rates, SHS exposure did not alter baseline electrophysiological parameters. With increasing pacing frequency, action potential duration (APD), and intracellular Ca^{2+} alternans magnitude progressively increased in all groups. At 4 and 8 wk, there were no statistical differences in APD or Ca^{2+} alternans magnitude between SHS and FA groups. At 12 wk, both APD and Ca^{2+} alternans magnitude were significantly increased in the SHS compared to FA group ($p < 0.05$). SHS exposure did not impact the time constant of Ca^{2+} transient decay (τ) at any exposure time point. At 12 wk exposure, the recovery of Ca^{2+} transient amplitude with premature stimuli was slightly (but nonsignificantly) delayed in SHS compared to FA hearts, suggesting that Ca^{2+} release via ryanodine receptors may be impaired.

Conclusions: In male mice, chronic exposure to SHS at levels relevant to social situations in humans increased their susceptibility to cardiac alternans, a known precursor to ventricular arrhythmia. https://doi.org/10.1289/EHP3664

Introduction

Secondhand smoke (SHS) is a major source of indoor air pollution (Tárnoki et al. 2009; Weschler 2009). It is estimated that about 35% of nonsmokers were exposed to SHS worldwide in 2004 (Oberg et al. 2011). Although smoking rates in the United States are declining, 58 million people were exposed to SHS in 2011–2012 (Homa et al. 2015). SHS contains more than 4,000 chemicals, and exposure increases the risk of multiple diseases, including lung cancer, stroke, and coronary heart disease (Oberg et al. 2011). Approximately 63% of deaths associated with SHS exposure are attributed to cardiovascular disease, including sudden cardiac death (SCD), which is often due to ventricular arrhythmias (Oberg et al. 2011).

In addition to atherogenesis, impaired autonomic function and activation of the inflammatory cascade play important roles in adverse cardiovascular outcomes with SHS exposure (Chiu et al. 2011; Pope et al. 2001; Raupach et al. 2006; Zhang et al. 2013). Several studies have reported decreased heart rate variability (HRV, an indicator of autonomic control of the heart) after SHS exposure in both humans and animal models (Dinas et al. 2013). Our group previously showed that SHS exposure decreased HRV and increased the susceptibility to arrhythmias in mice, particularly the susceptibility to pacing-induced ventricular tachycardia/fibrillation (VT/VF) (Chen et al. 2008), a life-threatening arrhythmia associated with SCD (Monserrat et al. 2003; Vismara et al. 1975; Zipes and Wellens 1998). In addition to autonomic dysfunction, SHS exposure also increases markers of inflammation (Menzies et al. 2006; Panagiotakos et al. 2004) and oxidative stress (Van der Vaart et al. 2004; Zhang et al. 2002), both of which can lead to chronic myocardial remodeling (Kuster et al. 2010), altered electrophysiological function (Combes et al. 2002; De Jesus et al. 2017; Terentyev et al. 2008), and increased susceptibility to ventricular arrhythmias (De Jesus et al. 2015; Francis Stuart et al. 2016; Jeong et al. 2012). However, to our knowledge, the impact of SHS on myocardial electrophysiology has not been investigated, and how these changes may impact the susceptibility to ventricular arrhythmias remains unknown.

Many studies employing animal models of SHS exposure use relatively high doses for short periods of time (Otsuka et al. 2001; Pope et al. 2001). For example, our previous study exposed mice to high-dose SHS [particulate matter (PM): $30\ mg/m^3$ and nicotine: $5\ mg/m^3$ for 6 h/d] for 3 d. This dose may be achieved in a poorly ventilated, enclosed space (e.g., automobile), but is relatively high compared to general environmental SHS exposure in public places or workplaces (Hyland et al. 2008; Ong and Glantz 2000). For instance, the average fine PM concentration in smoking restaurants and bars has been reported to be $0.2–0.6\ mg/m^3$, with the maximal level reaching approximately $3\ mg/m^3$ (Liu et al. 2011; Pastore et al. 1999; Semple et al. 2007). In a study comparing tobacco smoke–derived particle levels in 1,822 bars, restaurants, retail outlets, airports, and other workplaces from 32 countries, Hyland and colleagues reported that $PM_{2.5}$ concentration in smoking establishments ranged from 0.001 to $3.764\ mg/m^3$ (Hyland et al. 2008). Since high doses of SHS are not typically encountered and a considerable portion of nonsmokers are exposed to relatively lower doses of SHS ($PM_{2.5} \leq 3\ mg/m^3$) for a lifetime (Jaakkola and Samet 1999; Jaakkola and Jaakkola 2006), investigation of the effects of long-term exposure to lower, environmentally relevant doses of SHS is particularly important.

Therefore, the goal of this study was to expose mice to an environmentally relevant concentration of SHS ($3\ mg/m^3$), and determine the time-dependent impact of exposure on cardiac electrophysiology, Ca^{2+} handling, and the susceptibility to arrhythmogenic activity. To isolate potential autonomic dys-

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function from direct cardiac effects, isolated Langendorff-perfused hearts were studied with high-resolution optical mapping of transmembrane potential (V_m) and intracellular Ca^{2+}. Action potential (AP) and Ca^{2+} transient properties were quantified at: *a*) relatively slow pacing rates [150-ms interval, equivalent to 400 beats per minute (BPM)] to assess baseline properties; *b*) in response to premature pacing stimuli to assess recovery of AP and Ca^{2+} properties as a function of diastolic interval; and *c*) at faster pacing rates (up to ~ 850 BPM) to induce AP duration (APD) and Ca^{2+} alternans (beat-to-beat alternation in duration or amplitude), which are sensitive indicators of increased arrhythmia risk and a predictor of SCD (Pastore et al. 1999; Rosenbaum et al. 1994).

Methods

Male C57BL/6J mice (10 wk old) were purchased from Charles River Laboratories and were acclimated for 1–2 wk before study. Mice were housed in an exposure chamber at the Center for Health and Environment, University of California, Davis, with temperature control ($21 \pm 2°C$) and a 12-h light/dark cycle. Each mouse was housed in single polycarbon cage. Mice had *ad libitum* access to standard rodent chow and water. C57BL/6J mice were selected for this study because this is the strain or background strain used in nearly all of our previous studies (De Jesus et al. 2015; De Jesus et al. 2017; Francis Stuart et al. 2018), allowing for assessment of parameters with respect to previously collected results. All procedures involving animals were approved by the institutional animal care and use committee of the University of California, Davis (protocol #20207) and adhered to the *Guide for the Care and Use of Laboratory Animals* (NRC 2011).

Secondhand Smoke Exposure

Adult male C57BL/6 mice were randomly assigned at 12 wk of age to either filtered air (FA) or SHS exposure for 6 h/d (0900 hours to 1500 hours), 5 d/wk (Monday to Friday) for 4, 8, or 12 wk ($n = 4$–5/group). SHS-exposed mice were switched to FA during nonexposure hours. SHS was generated as described in previous studies (Chen et al. 2008; Joad et al. 2004; Sekizawa et al. 2008). We used 3R4F cigarettes from the University of Kentucky Tobacco and Health Research Institute (Lexington, Kentucky) for this study. Two cigarettes were combusted in a staggered fashion (1 puff/min, 35 mL/puff, 2 s duration) using a Teague E10 tobacco combustion system (Teague Enterprises). The smoke was diluted with FA in a mixing chamber to the appropriate concentration before delivering to the exposure chamber (stainless steel and glass Hinners-type, $0.44\ m^3$ in size). Total suspended particle (TSP) concentration was sampled gravimetrically twice each day with chamber particulate samples taken in the morning and again in the afternoon. Carbon monoxide concentration was measured every 30 min using a carbon monoxide analyzer (X-STREAM Gas Analyzer, Rosemount Analytical). Nicotine was sampled for 15 min every day during the exposure period. The SHS exposure condition was: total suspended particles: $3.0 \pm 0.1\ mg/m^3$, nicotine: $0.4 \pm 0.2\ mg/m^3$, and carbon monoxide: 12.4 ± 1.6 ppm.

Dual Optical Mapping of Ca^{2+} and V_m

The day following the fourth, eighth, or 12th week of exposure, mice were euthanized with an overdose of pentobarbital sodium (I. P. injection, 150 mg/kg) containing 120 IU of heparin. Hearts were rapidly excised and Langendorff perfused at $37 \pm 0.5°C$ with Tyrode's solution (NaCl 128.2 mmol/L, $CaCl_2$ 1.3 mmol/L, KCl 4.7 mmol/L, $MgCl_2$ 1.05 mmol/L, NaH_2PO_4 1.19 mmol/L, $NaHCO_3$ 20 mmol/L, and glucose 11.1 mmol/L) as previously described (De Jesus et al. 2017; Gardner et al. 2015). Perfusion flow rate (2.0–3.0 mL/min) was adjusted to maintain a perfusion pressure of 60–80 mmHg. Heart rate was monitored with a 2-lead electrocardiogram in the perfusion bath. Hearts were stained with voltage-sensitive dye [RH237, 10 μL of 5 mg/mL in dimethylsulfoxide (DMSO)] and an intracellular Ca^{2+} indicator (Rhod-2AM, Biotium, Inc., 50 μL of 1 mg/mL in DMSO and containing 10% pluronic acid), delivered over 5 min, in order to visualize epicardial transmembrane potential (V_m) and intracellular Ca^{2+}, respectively. Blebbistatin (10–20 μM), an excitation–contraction uncoupler, was added to the perfusate to reduce motion artifacts during optical recordings (Fedorov et al. 2007). Excitation light was produced with two continuous-wave LED sources centered at 531 nm and bandpass filtered from 511–551 nm (LEX-2; SciMedia). The emitted fluorescence from the anterior epicardium was collected through an objective (Leica Plan APO 1.0x; Leica), and the signals were split with a dichroic mirror at 630 nm. The longer wavelength moiety, containing the V_m signal, was longpass filtered at 700 nm, and the shorter wavelength signal moiety, containing the intracellular Ca^{2+} signal, was bandpass filtered with a 32-nm filter centered at 590 nm. Images were acquired at 1 kHz from a 10×10 mm field of view (100×100 pixels) with complementary metal oxide cameras (MiCam Ultima-L; SciMedia).

Baseline electrophysiological parameters were measured during left ventricular epicardial pacing at a cycle length (PCL) of 150 ms (heart rate = 400 BPM). To assess indicators of arrhythmia, two additional pacing protocols were performed as previously described (De Jesus et al. 2017): *a*) standard S1–S2 (12 S1 stimuli at 150 ms followed by a premature S2 stimulus decreasing in 5–10 ms until loss of capture), and *b*) continuous pacing at progressively faster frequencies (starting at PCL = 150 ms, decreasing in 10-ms increments until loss of capture or induction of arrhythmia) to induce APD and Ca^{2+} alternans.

Optical Mapping Data Analysis

Data analysis was performed using a commercially available analysis program (Optiq, Cairn Research Ltd). Optical signals were processed using a spatial Gaussian filter (3×3 pixels). Activation times were determined as the time at 50% between peak and baseline amplitude and rise times (TRise) as the time from 10–90% of the upstroke. APD at 80% repolarization (APD_{80}) was calculated as repolarization time − activation time. Ca^{2+} transient duration at 80% recovery ($CaTD_{80}$) was calculated similarly. Dispersion of APD (APDD, an indicator of relative APD distribution across the surface of the heart) was calculated from the inner 95th percentile (IP95) of all APDs in the mapping field of view (the shortest 2.5% and longest 2.5% of APDs were removed, and IP95 was the range of the remaining APDs) and was normalized to the area of the mapping field of view. The time constant of decay (τ) of the intracellular Ca^{2+} transient [an indicator of sarcoplasmic reticulum (SR) Ca^{2+} reuptake by SR ATPase (SERCA)] was quantified using the time constant of a single exponential fit to the recovery of the portion of the Ca^{2+} transient from 30–100% recovery. Recovery of relative Ca^{2+} release in response to premature stimuli was calculated as S2/S1 ratio of Ca^{2+} transient amplitude. Conduction velocity (CV; the speed with which the activation wave front spreads across the tissue) was measured by first fitting a polynomial surface to the space–time coordinates of local activation. Speed and direction of propagation (CV) were then computed from the gradient of the local polynomial surface (Bayly et al. 1998). Spectral methods were used to determine the presence and significance of APD and Ca^{2+} alternans (beat-to-beat alternation in duration or amplitude, respectively) as previously described (Wang et al. 2014). Briefly, for each pixel, each AP or Ca^{2+} transient (CaT) in the optical trace

was segmented and aligned at the activation time. For each time point in the repolarization phase (for APD alternans) or the peak phase (for CaT alternans), a fast Fourier transform was used to compute the power spectra across beats. The power at a frequency of 0.5 cycles/beat was then summed across each time point in the repolarization phase (for APD alternans) or peak phase (for CaT alternans) and divided by the length of the phase to produce a value for spectral alternans magnitude (unitless).

Statistics

For recovery of Ca^{2+} release, data were first fit with a one-phase exponential (nonlinear regression) and then subject to the extra sum of squares (ESS) F-test to compare whether the two curve fits were statistically different. For all other data, two-way analysis of variance (ANOVA) followed by, when appropriate, Tukey's post-hoc testing was used for analyzing the difference between FA- and SHS-exposed mice, with exposure (FA or SHS) as one factor and PCL, S2 interval, or exposure duration (4, 8, or 12 wk) as the other factor. There are four to five hearts per group at each exposure duration and PCL/S2 interval with two exceptions where $n = 3$ at the shortest S2 interval only (Figures 4C and 4E) because the interval decreased below the refractory period of the tissue, thus making it physiologically impossible to collect that data point. Exact n numbers are indicated in the figure legends. Data are presented as mean ± standard error of the mean except Figure 4C–4E where data points in each group were fitted with a regression curve. $P < 0.05$ was considered statistically significant. Sample size was based on a power analysis ($\alpha = 0.05$; 80% power).

Results

To assess the impact of SHS on cardiac electrophysiology and intracellular Ca^{2+} handling, dual optical mapping of V_m and intracellular Ca^{2+} was performed on isolated hearts. Baseline AP properties at a PCL of 150 ms, including APD, APDD, and TRise, were not altered by SHS exposure at any time point (Table 1). Baseline CV and CaTD were also similar between SHS and FA groups at all time points (Table 1). To evaluate the susceptibility to premature stimuli-induced arrhythmias, hearts were paced at a PCL of 150 ms followed by a single premature stimulus (S1–S2 protocol), with decrementing S2 intervals until loss of capture (refractory period of the tissue). No sustained VT/VF was induced with this protocol in either the FA or SHS group (Table 1). Extra beats were induced with the S1–S2 protocol in some of the hearts; however, there were no statistical differences in the number of extra beats induced between the two groups at any time point (Table 1). At 4 and 8 wk of exposure, SHS and FA groups had similar mean APDs at all S2 intervals (Figure 1A and B). After 12 wk of exposure, the SHS group tended to have slightly longer mean APDs compared to FA as the S2 interval shortened (Figure 1C); however, these differences did not reach statistical significance, potentially due to the relatively small sample size and/or variation among hearts within each group. In addition, at 12 wk, some hearts in the SHS group tended to have slower CV than the FA group (Figure 1D), especially at shorter S2 coupling intervals; however, this trend was not statistically significant.

To assess the effect of SHS on the susceptibility to APD and Ca^{2+} transient alternans [beat-to-beat alternation of APD (long-short-long-short) and Ca^{2+} transient amplitude (large-small-large-small)], hearts were continuously paced at progressively shorter PCLs. The severity of both APD and Ca^{2+} alternans (i.e., how different the APD and Ca^{2+} amplitude are from beat to beat) was assessed in the frequency domain and is presented as a spectral magnitude in arbitrary units, where the larger the spectral magnitude, the bigger the beat-to-beat difference (Myles et al. 2011). This approach is the same approach used to assess alternans in the clinical setting (Verrier et al. 2011). Decreasing PCL increased the average APD alternans magnitude at all exposure time points (Figure 2A–C). At 4 and 8 wk, there were no significant differences in APD alternans magnitude between the FA and SHS groups (Figure 2A–2B). At 12 wk, the SHS group had significantly increased APD alternans magnitude compared to the FA group (Figure 2C). Of note, at 12 wk, arrhythmia was induced in one SHS heart at PCL = 70 ms; therefore, analysis of alternans was not possible. This arrhythmia was not sustained following cessation of pacing, and no such arrhythmias were observed in any other hearts at any other exposure durations or PCLs. As shown in the example, in alternans magnitude maps and optical traces (12 wk exposure, Figure 2D–2E), at a PCL of 100 ms, there was minimal APD alternans in both groups (APD did not vary much from beat to beat), whereas at a PCL of 80 ms, the APD alternans magnitude in the SHS group was increased. These data suggest that SHS exposure increases the susceptibility to APD alternans and that the effect is dependent on exposure duration.

Similar to APD alternans, Ca^{2+} transient alternans magnitude was significantly increased with shorter PCL (Figure 3A–3C). At 4 and 8 wk of exposure, the SHS group tended to have larger Ca^{2+} alternans magnitude than the FA group, but this difference did not reach statistical significance (SHS vs. FA: $p = 0.081$ and 0.095 for 4 wk and 8 wk, respectively; Figure 3A and 3B). With longer exposure duration (12 wk), this trend became more robust, reaching statistical significance (SHS vs. FA: $p = 0.021$; Figure 3C). As shown in the example maps of Ca^{2+} alternans magnitude and corresponding optical traces (12 wk of exposure, Figure 3D–3E), the SHS group had increased beat-to-beat alternation in Ca^{2+} transient amplitude compared to FA at each PCL. These data indicate that, consistent with APD alternans, SHS may increase the susceptibility to Ca^{2+} alternans after 12 wk of exposure.

Table 1. Cardiac electrophysiological parameters of FA and SHS hearts (PCL = 150 ms).

Duration	4 wk		8 wk		12 wk	
Exposure	FA ($n = 4$)	SHS ($n = 4$)	FA ($n = 4$)	SHS ($n = 4$–5)	FA ($n = 4$)	SHS ($n = 5$)
APD (ms)	68.5 ± 5.3	66.3 ± 10.1	61.1 ± 7.6	60.7 ± 4.7[a]	61.1 ± 10.1	64.1 ± 7.8
APDD (ms/mm^2)	0.57 ± 0.12	0.48 ± 0.09	0.41 ± 0.09	0.28 ± 0.06[a]	0.43 ± 0.09	0.43 ± 0.07
Trise (ms)	3.5 ± 0.4	3.3 ± 0.2	3.9 ± 0.2	3.7 ± 0.1[a]	3.3 ± 0.2	3.6 ± 0.3
CV (cm/sec)	45.4 ± 2.6	48.0 ± 1.9	48.3 ± 1.1	47.3 ± 1.5[b]	49.5 ± 0.4	44.9 ± 1.8
CaTD (ms)	69.2 ± 2.2	65.8 ± 2.9	66.3 ± 1.0	63.9 ± 1.8[b]	67.2 ± 2.0	68.3 ± 2.2
VT/VF incidence	0/4	0/4	0/4	0/5	0/4	0/5
Extra beats	0.8 ± 0.5	1.5 ± 0.6	2.3 ± 1.1	2.6 ± 1.2[b]	2.5 ± 1.2	0.8 ± 0.5

Note: Data were analyzed with two-way analysis of variance (ANOVA) with Tukey's posthoc test. No statistical differences were observed between groups, among exposure duration, or with group/duration interaction. APD, action potential duration; APDD, action potential duration dispersion; CaTD, Ca^{2+} transient duration; CV, conduction velocity; FA, filtered air; PCL, pacing cycle length; SHS, secondhand smoke; Trise, action potential rise time; VF, ventricular fibrillation; VT, ventricular tachycardia. VT/VF incidence is shown as the ratio of hearts per group with VT/VF. Extra beats, the maximum number of extra beats observed in each heart during a complete S1–S2 pacing protocol.
[a]$n = 4$/group.
[b]$n = 5$/group.

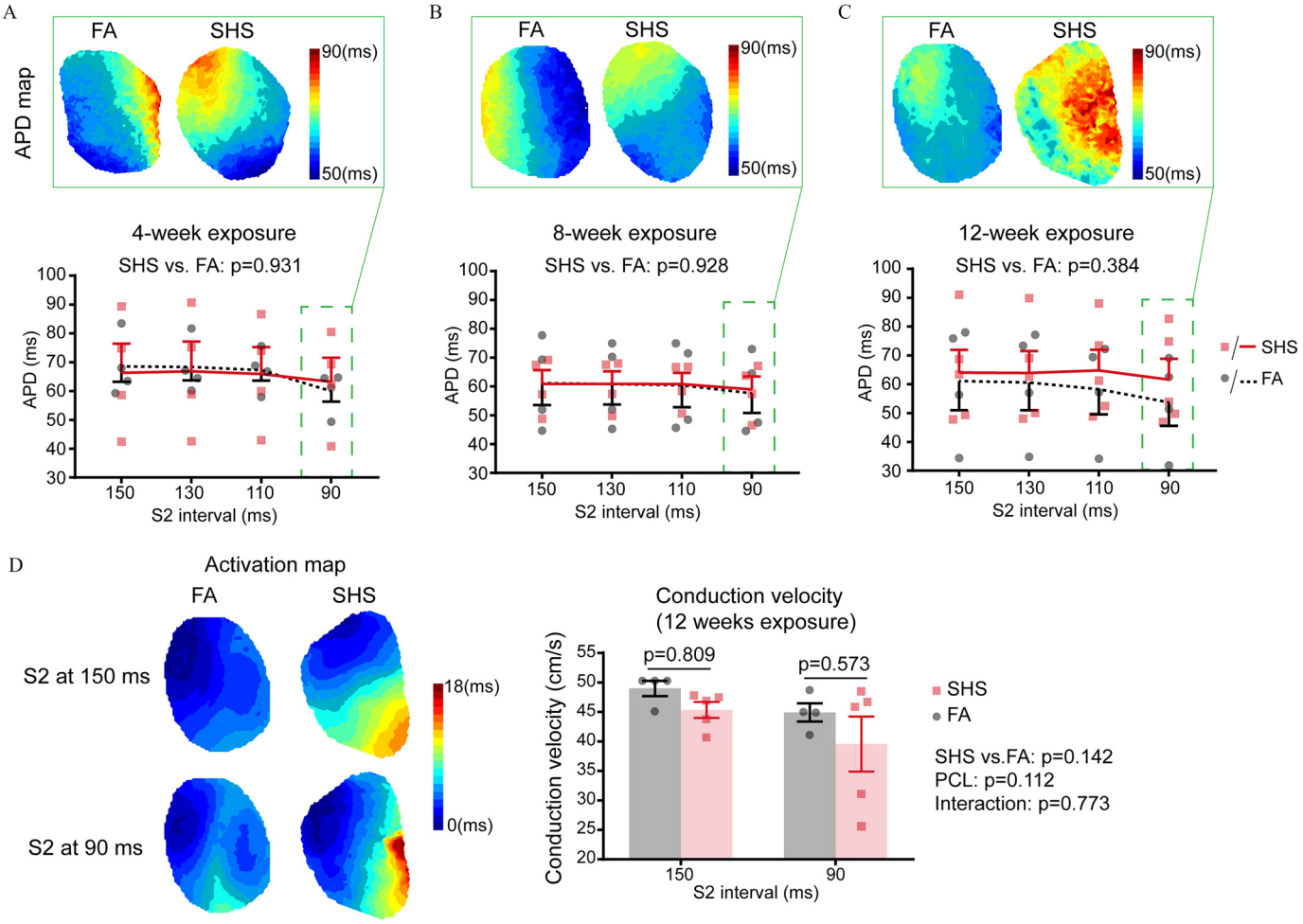

Figure 1. Action potential duration (APD) and conduction velocity (CV) following SHS or FA exposure. (*A–C*) APD as a function of premature stimulus interval (S2 interval) after (*A*) 4, (*B*) 8, or (*C*) 12 wk exposure. Data were analyzed with two-way analysis of variance (ANOVA) with Tukey's posthoc test. In (*A*), $p=0.830$ for PCL; $p=0.981$ for interaction between PCL and exposure; and $n=4$/group. In (*B*), $p=0.964$ for PCL; $p=0.999$ for interaction; and $n=4$/group. In (*C*), $p=0.927$ for PCL, $p=0.988$ for interaction; FA $n=4$; and SHS $n=5$. Top panel shows representative optical APD maps from SHS and FA groups at S2 = 90 ms (in the rectangle). (*D*) Left: representative activation maps (used to calculate CV) at a long (150-ms) and short (90-ms) coupling interval following 12 wk of exposure. Right: the corresponding CV graph. Data were analyzed with two-way ANOVA with Tukey's posthoc test. FA $n=4$; SHS $n=5$. Data is shown as mean ± standard error of the mean (SEM). Note: FA, filtered air; PCL, pacing cycle length; SHS, secondhand smoke.

To evaluate SR Ca^{2+} reuptake and the recovery of Ca^{2+} release, the time constant of the Ca^{2+} transient decay (τ; Figure 4A) and recovery of the Ca^{2+} transient amplitude with premature stimuli (Figure 4F) were analyzed, respectively. There was no difference in the rate of Ca^{2+} transient decay (τ) between SHS and FA groups at any exposure duration (Figure 4B), suggesting similar rates of SR Ca^{2+} reuptake and comparable SERCA function in all groups. There was also no significant difference in the recovery of Ca^{2+} release between SHS and FA groups at any exposure duration (Figure 4C–4E). However, the recovery of Ca^{2+} release in the SHS group was slightly attenuated compared to the FA group at 12 wk (i.e., the curve fits are marginally different between groups; Figure 4E–4F). This difference was not statistically significant, however, potentially due to the modest effect and small sample size.

Discussion

In this study, we showed that SHS exposure at levels relevant to social situations in humans increased the susceptibility to cardiac alternans, a known precursor for severe ventricular arrhythmias, including VT/VF (Gehi et al. 2005; Pastore et al. 1999; Verrier et al. 2011). These data may provide a critical mechanistic link between SHS exposure and arrhythmia susceptibility. The arrhythmogenic effect of SHS depended on the duration of exposure. Shorter-duration (4 and 8 wk) exposure did not alter electrophysiological parameters nor the susceptibility to cardiac alternans, whereas longer-term (12 wk) exposure significantly increased the susceptibility to both APD and Ca^{2+} transient alternans, suggesting that adverse electrophysiological remodeling begins to have a measurable impact on the isolated heart sometime between 8 and 12 wk of exposure. Since intracellular Ca^{2+} handling is a major driver of cardiac alternans (Chudin et al. 1999; Diaz et al. 2004; Shkryl et al. 2012), our findings suggest that SHS may adversely impact intracellular Ca^{2+} handling, yet we did not observe any significant differences in SR Ca^{2+} release or reuptake in this study (potentially due to modest effect size and low *n* number).

Chronic Secondhand Smoke Exposure

SHS exposure has been shown to have an acute impact on automatic function (Dinas et al. 2013), oxygen-carrying capacity of blood (Aronow et al. 1979; DeBias et al. 1976), and blood pressure (Celermajer et al. 1996; Mahmud and Feely 2004), which could all

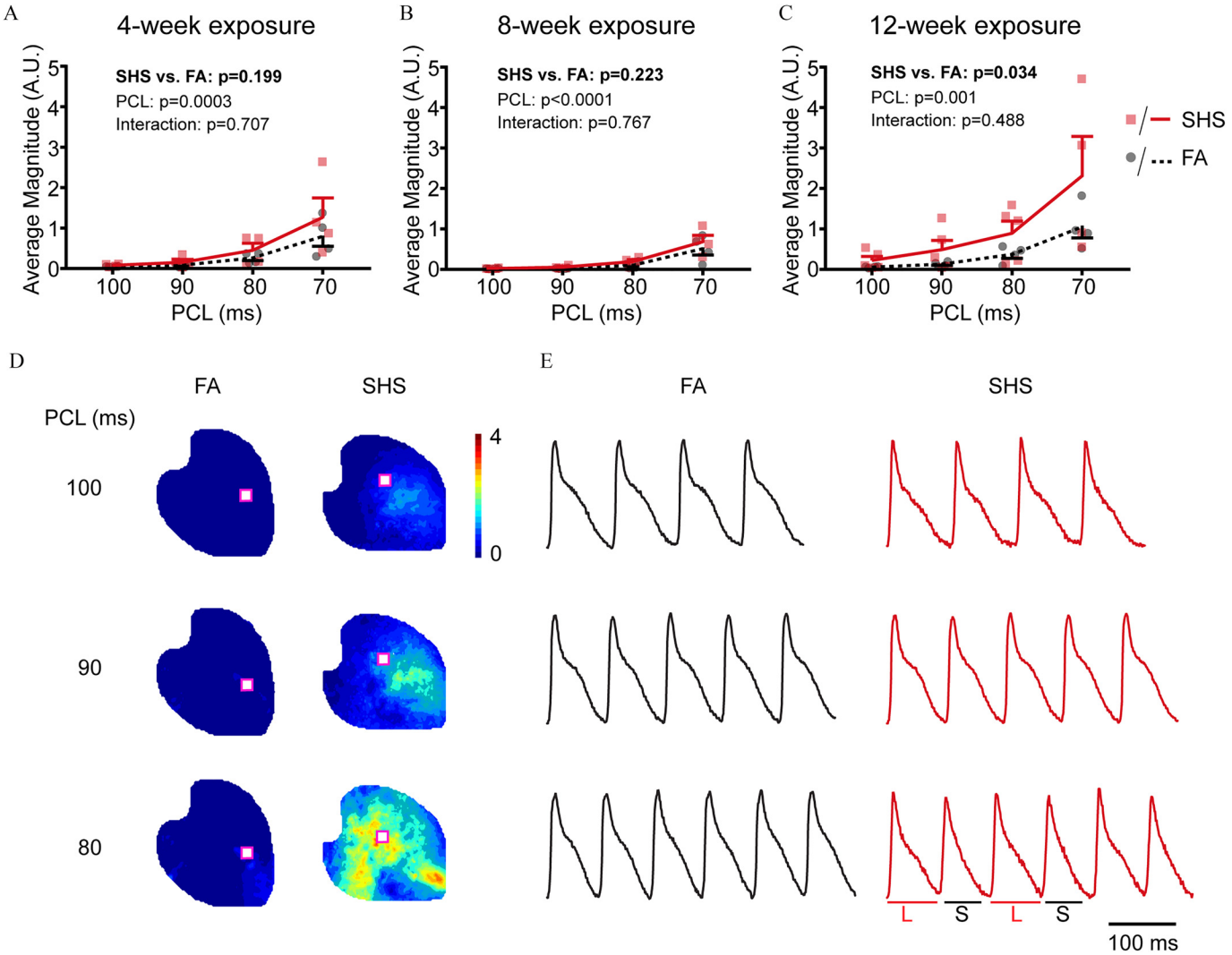

Figure 2. Effect of SHS on APD alternans. (*A–C*) Average APD spectral alternans magnitude is plotted against the pacing cycle length (PCL) following (*A*) 4- ($n=4$/group), (*B*) 8- ($n=4$/group), and (*C*) 12- (FA $n=4$; SHS $n=4$–5) wk of exposure to FA (circles) or SHS (squares). Data was analyzed with two-way analysis of variance (ANOVA) with Tukey's posthoc test. Data is shown as mean ± standard error of the mean (SEM). (*D*) Representative APD alternans magnitude maps following 12 wk of exposure at decreasing PCLs. (*E*) Representative optical V_m traces from the location indicated in square cutout red box in (*D*). Note: APD, action potential duration; FA, filtered air; L, longer APD; PCL, pacing cycle length; S, shorter APD; SHS, secondhand smoke.

contribute to the occurrence of cardiovascular events. In our study, SHS did not significantly alter the susceptibility to cardiac alternans in isolated hearts at 4 or 8 wk; the phenotype more strongly emerged following 12 wk of exposure. This delayed onset suggests a more chronic, time-dependent remodeling of the myocardium, but we cannot make definitive assessments of acute effects since 4 wk was the minimum exposure period studied. Further, our studies were performed on isolated perfused hearts, which are devoid of acute autonomic inputs, and blood pressure and oxygen delivery are controlled. Thus, our data suggest a novel mechanism of chronic SHS-induced arrhythmia susceptibility that likely involves myocardial remodeling, and that 12 wk was sufficient for SHS exposure to cause this remodeling. We have not yet studied longer SHS exposures; however, we hypothesize that myocardial remodeling would continue and that alternans and arrhythmia susceptibility may increase with longer exposure durations.

Mechanisms of Action Potential Duration Alternans and Arrhythmogenesis

VT/VF is the most deadly ventricular arrhythmia and may be precipitated by cardiac alternans (Gehi et al. 2005; Pastore et al. 1999; Rosenbaum et al. 1994; Verrier et al. 2011). Beat-to-beat alternation of the APD is reflective of repolarization alternans, which can be observed as T-wave alternans (TWA, a periodic beat-to-beat variation in the amplitude or shape of the T wave on the electrocardiogram) (Pastore et al. 1999). Repolarization alternans may cause severe dispersion of repolarization and conduction block, wherein a wave front meets an area of refractory tissue and fails to propagate in a particular direction (Cutler and Rosenbaum 2009; Narayan 2006; Pastore et al. 1999). This situation sets the stage for reentry, a tornado-like pattern of activation that underlies VT/VF (Cutler and Rosenbaum 2009; Narayan 2006; Pastore et al. 1999). Thus, the presence of TWA is a highly sensitive marker for SCD (Cutler and Rosenbaum 2009; Narayan 2006). TWA is rarely detected at resting heart rates or in healthy individuals but may be induced with elevation of heart rate (via exercise or pacing), especially in patients with high risk of SCD (Kaufman et al. 2000). In our study, the magnitude of APD alternans was negligible at slow pacing rates (PCL > 110 ms) and increased with rapid pacing in both groups at all exposure time points. In the 12-wk SHS group, APD alternans severity was significantly increased compared to FA, suggesting that susceptibility to VT/VF may be increased.

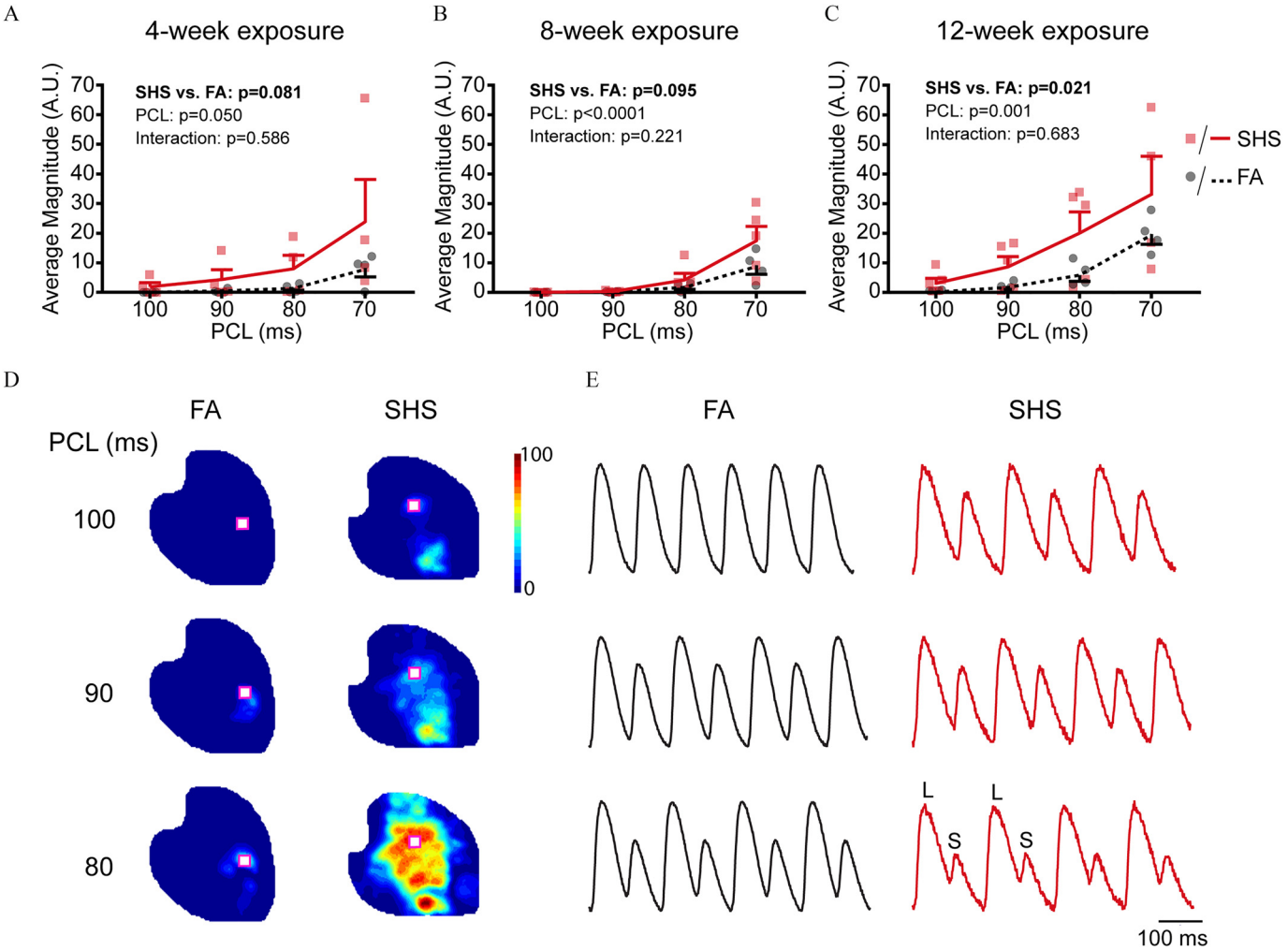

Figure 3. Effect of SHS on Ca^{2+} alternans. (*A–C*) Average Ca^{2+} alternans magnitude is plotted against the pacing cycle length (PCL) following (*A*) 4- ($n=4$/group), (*B*) 8- (FA $n=4$; SHS $n=5$), and (*C*) 12- (FA $n=4$; SHS $n=4$–5) wk exposure. Data was analyzed with two-way analysis of variance (ANOVA) with Tukey's posthoc test. Data is shown as mean ± standard error of the mean (SEM). (*D–E*) Representative Ca^{2+} alternans maps following 12 wk of exposure (*D*) and corresponding optical Ca^{2+} traces from locations indicated in square cutout (*E*). Note: FA, filtered air; L, larger Ca^{2+} transient; PCL, pacing cycle length; S, smaller Ca^{2+} transient, SHS, secondhand smoke.

No sustained ventricular arrhythmias were induced in this study; however, this is not unexpected, given that ventricular arrhythmias are relatively rare in isolated, Langendorff-perfused mouse hearts (De Jesus et al. 2015). Constant perfusion, uncoupling of mechanical contraction, and lack of autonomic inputs are all potential antiarrhythmic mechanisms in this experimental preparation. *In vivo*, however, these experimental protective mechanisms are absent. Autonomic inputs to the heart are also altered with SHS exposure and may have proarrhythmic effects *in vivo* (Chen et al. 2008; Pope et al. 2001). Compounds found in SHS, including particulate matter and semivolatile aldehydes, can increase oxidative stress and inflammation (Andrè et al. 2008; Rhoden et al. 2008), as well as activate sensory afferent neurons in the lung (Taylor-Clark and Undem 2011). Together, these responses all contribute to a systemic reflex resulting in increased sympathetic outflow to the heart (Middlekauff et al. 2014). In addition, a previous study by our group reported impaired parasympathetic regulation of the heart (measured by HRV) in mice following short-term (3 d), higher-dose (30 mg/m^3) SHS exposure (Chen et al. 2008). Acutely, these changes in autonomic control of the heart may precipitate arrhythmias *in vivo*. Indeed, our previous study demonstrated an increase in pacing induced VT/VF in mice *in vivo*. Chronic alterations in autonomic activity can also lead to changes in expression and function of myocardial ion channels, Ca^{2+} handling proteins, and adrenergic receptors, which may further exacerbate arrhythmias following SHS exposure (Gardner et al. 2016). Thus, even though we did not observe any statistically significant electrophysiological changes following 4 or 8 wk of SHS exposure in isolated hearts, it is possible that proarrhythmic remodeling has already begun and may manifest *in vivo* at these earlier time points. Indeed, Ca^{2+} alternans magnitude was already trending higher in the SHS group at 4 and 8 wk.

Mechanisms of Intracellular Ca^{2+} Alternans

Beat-to-beat alternation of the intracellular Ca^{2+} transient amplitude can be a primary driver of APD alternans (Clusin 2008). Our data are consistent with this mechanism, as Ca^{2+} alternans emerged at slower pacing frequencies, followed by the appearance of APD alternans as pacing frequency increased. Impaired Ca^{2+} cycling can contribute to the presence and severity of Ca^{2+} alternans (Chudin et al. 1999; Diaz et al. 2004; Shkryl et al. 2012). Under normal steady-state conditions, the amount of Ca^{2+} released from the SR via the ryanodine receptors (RyRs) is

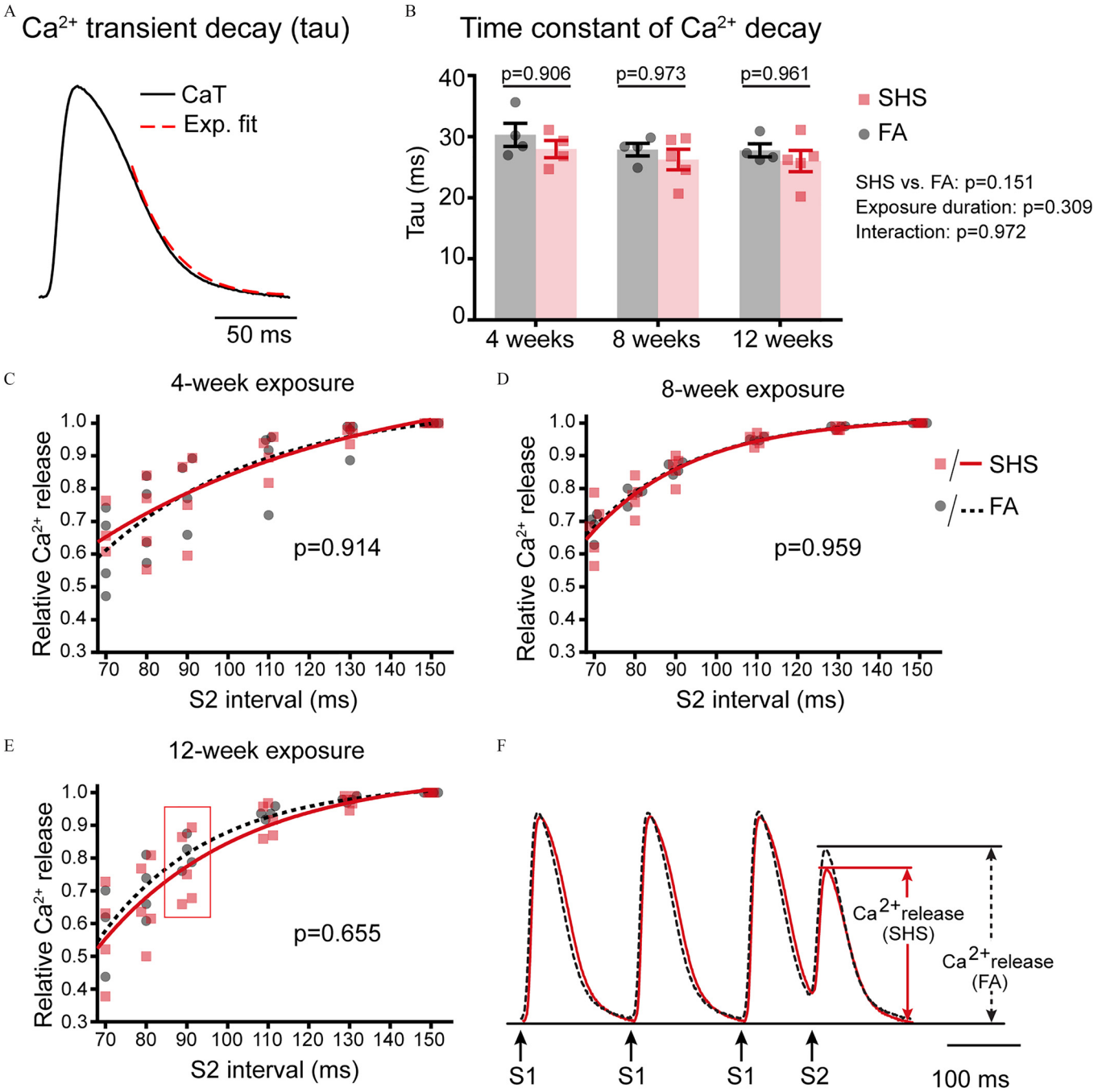

Figure 4. Time constant of Ca^{2+} decay (τ) and recovery of Ca^{2+} release. (*A*) Representative Ca^{2+} transient trace and corresponding exponential fit (PCL = 150 ms). (*B*) Time constant (τ) of Ca^{2+} decay after SHS or FA exposure for 4, 8, or 12 wk. Data was analyzed with two-way analysis of variance (ANOVA) with Tukey's posthoc test. Data is shown as mean ± standard error of the mean (SEM). For 4 wk, $n = 4$/group; for 8 and 12 wk, FA $n = 4$; SHS $n = 5$. (*C–E*) S2/S1 ratio of Ca^{2+} transient amplitude is plotted against S2 coupling interval after (*C*) 4- (FA $n = 4$; SHS $n = 4$ except PCL 70 ms $n = 3$), (*D*) 8- (FA $n = 4$; SHS $n = 5$), or (*E*) 12- (FA $n = 4$ except PCL 70 ms $n = 3$; SHS $n = 5$ except PCL 70 ms $n = 4$) wk of exposure to SHS or FA. Data were first fit with a one-phase exponential curve and then subject to the extra sum of squares (ESS) F-test. No significant differences were observed between the SHS and FA groups. Solid and dashed lines represent exponential fits. (*F*) Example Ca^{2+} transient traces at S2 = 90 ms (rectangle in E). Note, FA, filtered air; PCL, pacing cycle length; S1, normal pacing stimuli; S2, premature stimulus; SHS, secondhand smoke.

exactly equal to the amount of Ca^{2+} pumped back up into the SR via the SERCA pump. As pacing frequency increases, time for SR refilling (governed by SERCA activity) and recovery of SR Ca^{2+} release (governed in part by RyR refractoriness) are both reduced. Encroachment on either of these parameters can lead to a mismatch between SR Ca^{2+} release and reuptake, and beat-to-beat alternation of the intracellular Ca^{2+} transient. Thus, both RyR refractoriness and SERCA activity have been implicated in the development of Ca^{2+} alternans (Diaz et al. 2004; Shkryl et al. 2012; Wang et al. 2014).

In the present study, the time constant of Ca^{2+} decay (τ, reflecting SERCA activity) was not different between groups or exposure times. To assess recovery of SR Ca^{2+} release, restitution curves of the amplitude of Ca^{2+} release during a premature stimulus (S2) normalized to the amplitude of Ca^{2+} release at steady state (S1) were assessed. A slightly slower recovery of SR

Ca^{2+} release was observed in the SHS group following 12 wk of exposure, potentially indicating slower recovery of RyRs from refractoriness. Although this difference was not statistically significant, this trend only appeared following 12 wk of exposure and is consistent with the increasing severity of Ca^{2+} alternans at 12 wk in the SHS group. A previous study from our lab specifically investigated the role of RyR refractoriness on Ca^{2+} alternans. We showed that a slight alteration in recovery of SR Ca^{2+} release (approximate 10% change) resulted in a twofold difference in the magnitude of Ca^{2+} alternans (Wang et al. 2014). Therefore, it is possible that very slight changes in recovery of Ca^{2+} release (that are perhaps not yet detectable at the whole-heart level) are occurring with SHS exposure and contributing to alternans; however, this conclusion is speculative and emphasizes the need for future study in this area.

Impact of Secondhand Smoke on Ca^{2+} Handling

A number of potential candidates could contribute to increased Ca^{2+} alternans after 12 wk of SHS exposure. There are several reports showing that the individual components of SHS can directly modify Ca^{2+}-handling proteins. For example, long-term administration of nicotine has been shown to change expression levels of neuronal RyR2 in mice (Ziviani et al. 2011). Chronic carbon monoxide pollution has been shown to decrease myocardial SERCA2a levels, leading to prolongation of the Ca^{2+} transient decay in rats (Andre et al. 2010). In addition to these direct modifications, long-term exposure to SHS can also induce oxidative stress (Van der Vaart et al. 2004; Zhang et al. 2002) and inflammation (Flouris et al. 2009; Jefferis et al. 2010), both of which can alter myocardial Ca^{2+} handling (Combes et al. 2002; Jeong et al. 2012; Kuster et al. 2010; Terentyev et al. 2008). Therefore, a systematic evaluation of Ca^{2+}-handling proteins (e.g., SERCA2a, RyR2), and proteins regulating their function (e.g., phospholamban, Ca^{2+}/calmodulin-dependent protein kinase II) after 12 or more wk of SHS exposure will be an important area for future study.

Limitations

Although the group size for this study ($n = 4$–5) was based on a power analysis, this is a relatively small sample size and conclusions about significance—or lack thereof—must be interpreted accordingly. We did not expose any mice to SHS for less than 4 or more than 12 wk; thus, we cannot make definitive assessments of either acute or more chronic effects. It is likely that stronger phenotypes and/or baseline differences may emerge with longer SHS exposure durations, and this remains an important area for future study. Cardiac alternans are a sensitive indicator of VT/VF susceptibility (Gehi et al. 2005; Pastore et al. 1999; Rosenbaum et al. 1994; Verrier et al. 2011); however, no sustained arrhythmias were induced in the present study. This may be an artifact of the isolated mouse heart preparation, and several additional factors, including autonomic input (Chen et al. 2008; Pope et al. 2001), oxidative stress (Menzies et al. 2006; Panagiotakos et al. 2004), and inflammation (Van der Vaart et al. 2004; Zhang et al. 2002), may also contribute to arrhythmias *in vivo*. The optical mapping technique employed here does not allow for assessment of individual depolarizing or repolarizing ionic currents underlying the AP; therefore, patch clamp experiments in isolated myocytes remains an important area for future study.

Conclusions

The present study revealed that long-term exposure to an environmentally relevant concentration of SHS can increase the susceptibility to cardiac alternans in mice. This arrhythmogenic effect was not statistically significant at 4 or 8 wk of exposure but emerged more strongly after 12 wk, indicating that sometime between 8 and 12 wk of exposure may represent a pathophysiological threshold for myocardial remodeling at this concentration of SHS. The mechanisms by which low-concentration SHS exposure increases the susceptibility to cardiac alternans may involve modification of intracellular Ca^{2+} handling; however, future studies are warranted to further discern these underlying mechanisms.

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