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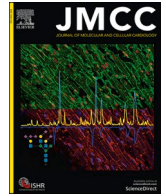
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Optogenetic termination of atrial tachyarrhythmias by brief pulsed light stimulation

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ABSTRACT

Aims: The most efficient way to acutely restore sinus rhythm from atrial fibrillation (AF) is electrical cardioversion, which is painful without adequate sedation. Recent studies in various experimental models have indicated that optogenetic termination of AF using light-gated ion channels may provide a myocardium-specific and potentially painless alternative future therapy. However, its underlying mechanism(s) remain(s) incompletely understood. As brief pulsed light stimulation, even without global illumination, can achieve optogenetic AF termination, besides direct conduction block also modulation of action potential (AP) properties may be involved in the termination mechanism. We studied the relationship between optogenetic AP duration (APD) and effective refractory period (ERP) prolongation by brief pulsed light stimulation and termination of atrial tachyarrhythmia (AT).

Methods and results: Hearts from transgenic mice expressing the H134R variant of channelrhodopsin-2 in atrial myocytes were explanted and perfused retrogradely. AT induced by electrical stimulation was terminated by brief pulsed blue light stimulation (470 nm, 10 ms, 16 mW/mm²) with 68% efficacy. The termination rate was dependent on pulse duration and light intensity. Optogenetically imposed APD and ERP changes were systematically examined and optically monitored. Brief pulsed light stimulation (10 ms, 6 mW/mm²) consistently prolonged APD and ERP when light was applied at different phases of the cardiac action potential. Optical tracing showed light-induced APD prolongation during the termination of AT.

Conclusion: Our results directly demonstrate that cationic channelrhodopsin activation by brief pulsed light stimulation prolongs the atrial refractory period suggesting that this is one of the key mechanisms of optogenetic termination of AT.

1. Introduction

Atrial fibrillation (AF) is the most common tachyarrhythmia and has a large socioeconomic impact due to its associated morbidity, mortality, reduction in quality of life and health care costs [1]. The prevalence of AF is rapidly growing globally due to aging of the human population and adherence to unhealthy lifestyles. Although clinical trials have confirmed the efficacy of treating AF by pulmonary vein isolation [2],

many patients show refractory symptomatic AF despite multiple ablation procedures and drug therapies [3–5]. A considerable number of these patients require electrical cardioversion to terminate AF [6]. However, electrical cardioversion is distressing and requires adequate sedation [7]. To improve quality of life and simplify the care of patients with recurrent AF, we require a novel therapeutic approach that enables painless cardioversion [8].

Optogenetics is an innovative technology that includes the delivery

Abbreviations: AF, Atrial fibrillation; AP, Action potential; APD, Action potential duration; AT, Atrial tachyarrhythmia; CCS, Cardiac conduction system; ChR2, Channelrhodopsin-2; ERP, Effective refractory period; LED, Light-emitting diode.

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of microbial light-sensitive proteins called opsins to excitable cells, which enables either light-based depolarization or hyperpolarization [9,10]. Channelrhodopsin-2 (ChR2) is a light-gated cation channel activated by blue light. Early studies have shown that expressing ChR2 in cardiomyocytes and applying blue pulsed light to the myocardium resulted in depolarization of the sarcolemma, which enabled optogenetic pacing [11–15]. More recent studies have shown that long light illumination can terminate reentrant tachyarrhythmias [16–20] and could provide a method for painless cardioversion.

The mechanism(s) of optogenetic termination by depolarization with optogenetic tools is/are not well understood. Possible mechanisms include (i) establishment of a transient conduction block [17,21,22] and (ii) filling of the excitable gap [8]. Filling of the excitable gap involves only brief light exposure (of a few milliseconds [ms]) but requires global illumination of the excitable myocardium for optimal termination efficacy [23], which is difficult to achieve especially in anatomically complicated structures such as the atria [8]. In *in situ* experiments in transverse ventricular slices, the photostimulation of the depolarizing light-activated ion channel Ca^{2+} -translocating channelrhodopsin caused a complete conduction block and terminated reentrant arrhythmias [22]. It was proposed that the conduction block was achieved by the inactivation of voltage-dependent Na^+ channels via resting membrane potential elevation during light stimulation [8,13]. Based on this hypothesis, illumination should be conducted for the duration of the tachycardia cycle to terminate reentrant arrhythmias. However, local brief (10–30 ms) pulsed light stimulation without global illumination can also terminate reentrant arrhythmias with termination rates of ~80% [23,24]. Therefore, we presumed that an additional mechanism is involved in the optogenetic termination of arrhythmias.

ChR2 shows an inwardly rectifying current-voltage relationship with a reversal potential of approximately 0 mV [10]. As a consequence, light stimulation of ChR2 leads to influx of cations, even when the cell membrane is (partially) depolarized. It is hypothesized that brief pulsed light stimulation, even within the absolute and relative refractory period, may prolong the action potential (AP) duration (APD) and, therefore, the effective refractory period (ERP), effectively increasing the arrhythmia termination rate beyond that derived from the instant conduction block imposed by the elevated resting membrane potential. This phenomenon has been observed in *in silico* and *in vitro* experiments and has been hypothesized to modulate AP shape [25–27]. However, the effect of brief pulsed light stimulation on APD and ERP duration and the influence of refractory period on optogenetic termination rate have not yet been studied in whole hearts.

The aims of this study were to systematically investigate optogenetic APD and ERP prolongation by brief pulsed light stimulation and to describe the relationship between refractory period extension and arrhythmia termination rate in an *ex vivo* whole heart model of atrial tachyarrhythmia (AT).

2. Methods

2.1. Animals

Cx40-CreERT2 mice (Institut de Biologie du Développement de Marseille, Aix-Marseille Université) [28] were bred to B6.Cg-Gt(ROSA)26Sor^{tm2.1(CAG-COP4⁺H134R/tetTomato)Hze/J} mice (The Jackson Laboratory, Bar Harbor, ME) to generate transgenic mice harboring a tamoxifen-inducible Cre recombinase (CreERT2) gene under the control of the Cx40 promoter and a ChR2(H134R) ~ tdTomato fusion protein gene driven by the strong ubiquitously active CAG promoter [15]. The Cx40 promoter mediates CreERT2 expression in atrial myocytes and in cells of the cardiac conduction system (CCS). Exposure of double transgenic (DTg) adult animals to tamoxifen resulted in the nuclear translocation of CreERT2 followed by the removal of the floxed STOP sequence separating the CAG promoter from the ChR2(H134R) ~ tdTomato fusion protein gene and the onset of cardiac channelrhodopsin

expression in atrial myocytes and CCS cells (Fig. 1A). To induce Cre recombination and ChR2(H134R) ~ tdTomato expression, 3-month-old mice were given 100 mg/kg tamoxifen (Combi-Blocks, San Diego, CA) for 5 consecutive days by intraperitoneal injection. At two weeks after the tamoxifen treatment, the mice were used as the ChR2-positive group for the experiments described below. Age-matched wild type C57BL/6 mice were used as the ChR2-negative group. All animal husbandry protocols and experimental procedures were in accordance with the guidelines for the Care and Use of Laboratory Animals at the Hokkaido University Graduate School of Medicine, approved by our Institutional Animal Research Committee, and complied with relevant national and international guidelines, including the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.2. *Ex vivo* Langendorff-perfused hearts and electrophysiological examination

Mice were euthanized by cervical dislocation, 10 min after intraperitoneal administration of 200 U heparin sodium (AY Pharmaceuticals, Tokyo, Japan). The hearts were quickly excised and immediately placed in Tyrode's solution of 4 °C containing (in mM) 143 NaCl, 5.4 KCl, 0.33 NaH_2PO_4 , 5 HEPES, 5.5 glucose, 0.5 MgCl_2 , and 1.8 CaCl_2 (pH 7.4, adjusted using NaOH) to perform a careful cannulation [29,30]. The surrounding tissue was dissected, and the hearts were retrogradely perfused via the aorta with oxygenated (100% O_2) Tyrode's solution of 37 °C until the heart rate stabilized. The perfusion pressure was maintained at 80 mmHg using a peristaltic pump (MINIPULS 3 MP-2/HF; Gilson, Middleton, WI) together with an IN175 STH pump controller (AD Instruments, Sydney, Australia), DX-100 pressure transducers (Nihon Kohden, Tokyo, Japan) and a FE221 Bridge Amp preamplifier (AD Instruments). A bipolar cardiac electrocardiogram (ECG) was recorded from electrodes placed in the right and left atrial appendages or apex of the heart using an FE132 Bio Amp amplifier and ML846 data acquisition system (both from AD Instruments). Data were processed with LabChart 8 software (AD Instruments). The timing and intensity of electrical and light stimulations were precisely programmed by an arbitrary waveform generator (AWG-10 K; Elmos, Aichi, Japan). Electrical stimulation of the right atrial appendage was performed using an arbitrary electrical stimulator and a handmade stimulation electrode. Photostimulation was delivered using a 470-nm light-emitting diode (LED), aspheric condenser lens and t-Cube LED driver (M470L4, ACL12708U-A, LEDD1B; Thorlabs, Newton, NJ). Light was applied to the epicardium of the atria over an area of 25 mm² (Fig. 1B). Light intensity was measured using an illuminometer (PD300-3 W; Ophir, Jerusalem, Israel) and StarLab 2.1 software (Ophir). The ERPs when light stimulation for ChR2 was not performed measured from electrocardiographic recordings. ERP was defined as the longest S1–S2 interval to result in the absence of new propagations.

2.3. AT induction protocol

To increase susceptibility to sustained episodes of AT, the perfusate was switched from normal Tyrode's solution to a modified Tyrode's solution with a low potassium concentration (2.7 mM) and containing 2 mM of the muscarinic receptor agonist and $\text{I}_{\text{K,ACh}}$ activator carbachol (PHR1511; Sigma-Aldrich, St. Louis, MO). Under these conditions, high-frequency electrical stimulation (cycle length: 10–50 ms, duration: 200–1000 ms, current: 0.1–0.3 mA) of the right atria with a handmade monopolar electrode consistently induced AT. The counter electrode was placed on the perfusion needle. Attempts to optogenetically terminate ATs were started after 3000 ms of sustained arrhythmic activity.

Optogenetic termination by long light exposure involved illumination of the Langendorff-perfused hearts for three times 1000 ms with 1000-ms intermissions (a modification of the protocol of Brueggemann et al.) [23] using 470-nm light with an intensity of 16 mW/mm². The light was delivered to 25 mm² of the right atrial epicardial surface. We

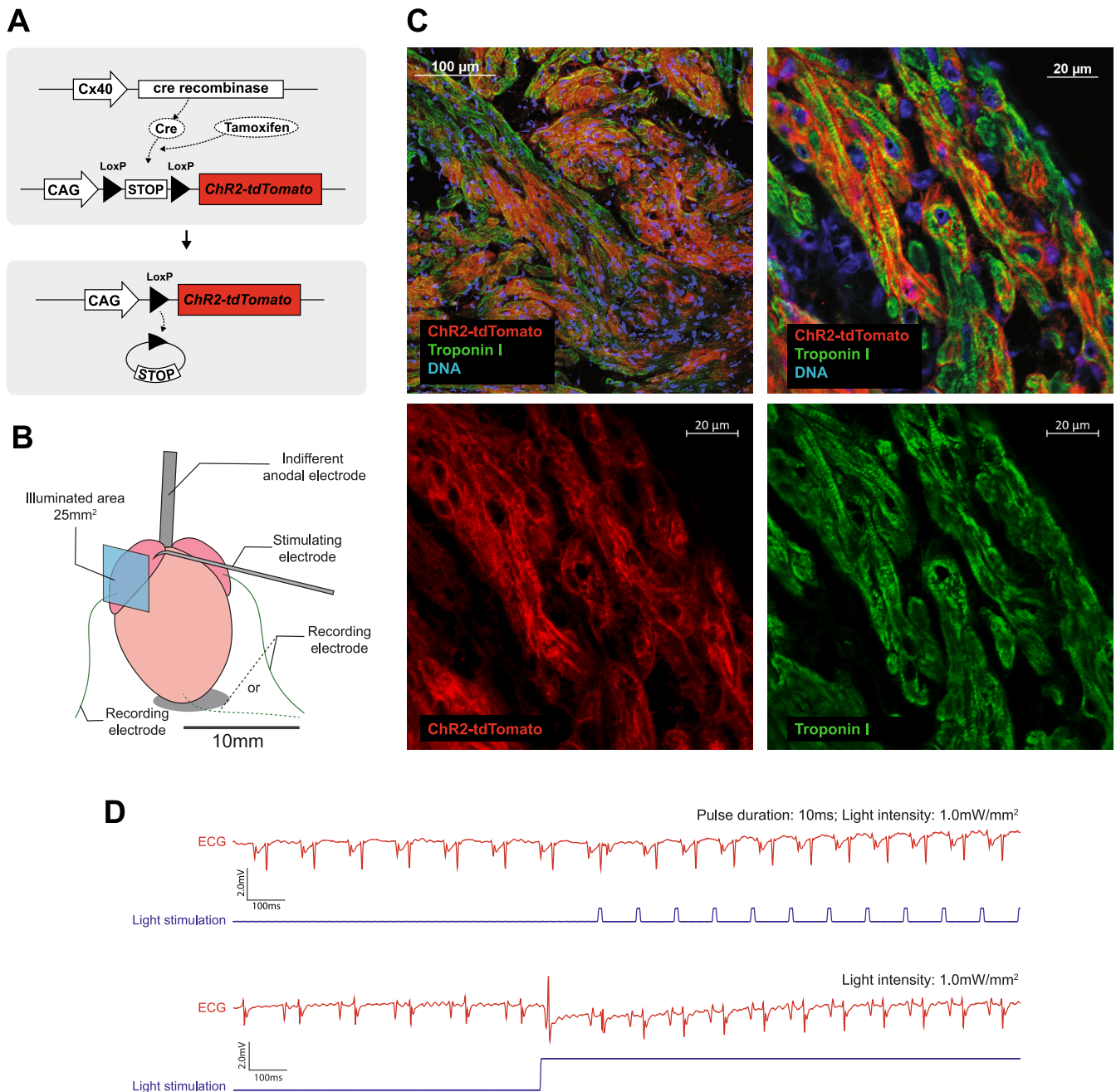


Fig. 1. Expression and function of ChR2(H134R) in atrial myocardium. (A) Transgenic mouse model conferring atrium (and cardiac conduction system)-specific expression of *tdTomato*-tagged ChR2(H134R). (B) Hearts were explanted and retrogradely perfused in a Langendorff configuration. An ECG was recorded using electrodes (green) placed at the right and left appendages or apex. Electrical pacing was performed using a handmade electrode as unipolar cathode on the right atrium. The stainless microtube used for perfusion was also used as an indifferent anodal electrode. Rectangular illumination was performed with a blue LED (470 nm) focused on a 25 mm² area of the epicardial atrial surface. (C) Histology showing *tdTomato*-ChR2 (red) expression in the atria. Blue indicates the nuclei of cardiomyocytes and green indicates cardiac troponin I. (D) Representative ECG traces during brief pulsed light stimulation and during long light exposure of the atrial surface. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

considered optogenetic termination to be successful if AT ceased within 1000 ms of irradiation.

Attempts to optogenetically terminate ATs by brief light stimulation were performed using pulse widths of 5–100 ms and light intensities of 1–16 mW/mm² as indicated. The irradiated area was the same as before. We considered optogenetic termination to be successful if AT ceased within 100 ms after light stimulation. To allow multiple cycles of arrhythmia induction and termination in the same heart, 2000 ms after each attempt to terminate AT by brief light exposure, the right atrial epicardial surface was exposed for 1000 ms to 16 mW/mm² of 470-nm

LED light (Fig. 3A). Each brief light illumination period and intensity was tested at least three times per heart.

2.4. Optical voltage mapping, APD and ERP measurement

To visualize changes in membrane potential, we used voltage-sensitive dye imaging technology according to previously described methods [31,32]. After the beating rate of the isolated hearts stabilized, the perfusate was switched from normal Tyrode's solution to normal Tyrode's solution containing 10 μM blebbistatin (Toronto Research,

Toronto, Canada). This selective myosin II inhibitor, efficiently blocks cardiac contractions and therefore eliminates motion artifact during data acquisition [33]. However, because blebbistatin affects cardiac electrophysiology in a dose- and time-dependent manner, measurements were completed within 1 h of starting blebbistatin perfusion [34]. In case of AT induction, hearts were perfused with the aforementioned modified Tyrode's solution containing carbachol and a low potassium concentration, which was supplemented with 10 μ M blebbistatin. A total of 75 nmol of the voltage-sensitive dye RH237 (Invitrogen, Waltham, MA) was diluted in 1 ml normal Tyrode's solution and injected into the heart over a period of 10 min. Next, the heart was illuminated with quasi-monochromatic light (530 ± 20 nm) from a 150 W halogen light source (MHF-G150LR; MORITEX, Saitama, Japan). The emitted fluorescence was captured using an image-intensified complementary metal oxide semiconductor camera (Mi-CAM02-CMOS; BrainVision, Tokyo, Japan) through a long-pass filter (FF01-795/188-25; Semrock, Rochester, NY), which let through 93% of the light between 701 and 889 nm. The optical signals were obtained at 0.60-ms sampling intervals, acquired from 96×80 pixels. The region of interest for APD and ERP measurement was set at 5×5 pixel (approximately 1 mm^2) near the stimulating electrode (Supplemental Fig. S1A). To avoid the influence of excitations caused by light for ChR2 and minimize post-acquisition artifacts, optical signals were detrended using polynomial fit 3×3 spatial and cubic filters (Supplemental Fig. S1B–D). Optical APDs were measured at 80% or 90% repolarization (APD₈₀ or APD₉₀). ERPs were measured using optical voltage mapping data instead of electrocardiographic recordings when light stimulation for ChR2 was performed to avoid misdiagnosis due to electrical noise. ERP was defined as the longest S1–S2 interval to result in the absence of new propagations.

2.5. Histological analysis

tdTomato, associated with ChR2, was observed by a laser-scanning confocal microscope (LSM-710, Carl Zeiss, Oberkochen, Germany). Explanted hearts with intact atria were perfused with phosphate-buffered saline and cryopreserved. The specimens were cut into 10- μ m-thick sections using a CM1950 cryostat (Leica Microsystems, Tokyo, Japan) and attached to glass slides. After 30 min of air drying, the sections were incubated overnight at 4 °C with polyclonal goat antibodies specific for cardiac troponin I (sc-8118; 1:200; Santa Cruz Biotechnology, Dallas, TX) diluted in antibody diluent (DAKO, Glostrup, Denmark). After primary antibody incubation, the sections were washed twice with PBS-0.05% Tween 20 and incubated for 30 min with fluorescein-conjugated AffiniPure F(ab')₂ Fragment donkey anti-goat IgG (H + L) (1:100; Jackson Immuno Research, Penn, PA). Finally, samples were counterstained with 4',6-diamidino-2-phenylindole dihydrochloride (Thermo Fisher Scientific, Waltham, MA) to visualize cell nuclei.

2.6. Statistical analysis

All data are presented as the mean \pm standard error of the mean. Two-sided paired Student's *t*-tests were performed to compare the electrophysiological characteristics of hearts exposed to different perfusion solutions and to compare APD₉₀ before and during light stimulation. Fisher's exact test and log-rank test was performed to compare the termination rates in the long light exposure experiment. Owing to non-normal data distribution, unequal variances, and repeated measures of the same experimental unit, comparison of termination rates at different illumination parameters (i.e., light intensity and light pulse duration) were made using Friedman test and Dunn's multiple comparison test. Comparisons of APD₈₀ and ERP between the three groups or under different illumination conditions were performed using a one-way or one-way repeated measures analysis of variance. Tukey's multiple comparison test was carried out post hoc to describe the significant variables. Differences were considered statistically significant at

$p < 0.05$. Statistical analyses were performed using GraphPad Prism version 8 (GraphPad Software, San Diego, CA).

3. Results

3.1. Histological and electrophysiological characterization of ChR2-expressing atrial tissue

ChR2 expression was histologically assessed by tdTomato fluorescence, which confirmed that ChR2 was expressed in the atria of mice after tamoxifen treatment and was mainly located at the plasma membrane (Fig. 1C). To confirm the function of ChR2, the epicardium of the right atria was irradiated with blue light (470 nm, 10 mW/mm²) during sinus rhythm to examine photostimulation (Fig. 1B). As expected, pulsed light stimulation onto the right atrial surface resulted in consistent atrial pacing. Prolonged light exposure did not block atrial excitation but induced an accelerated atrial rhythm (Fig. 1D). To investigate possible cardiotoxic effects of Cre on atrial myocytes and whether C57BL/6 wild type mice are appropriate as a control, we performed electrophysiological studies in DTg mice treated with and without tamoxifen as well as in wild type mice. There were no significant differences in cycle length, atrioventricular conduction, and ERP between the three groups of animals (Supplemental Fig. S2A–C), justifying the use of age-matched wild type mice as ChR2-negative control group in our study. In all hearts, pulsed light of ≥ 10 ms and ≥ 1.0 mW/mm² allowed for 10 Hz atrial optogenetic pacing and 1:1 ventricular conduction (Supplemental Fig. S3A–B). Photostimulation caused synchronous depolarization within the irradiated area that propagated toward the adjacent areas, whereas electrical impulses from the sinus node or from an external electrode resulted in centrifugal propagation (Supplemental Fig. S3C). To look for possible the side effects of strong light exposure, ERPs were measured in ChR2-positive mice before and after three sessions of light stimulation with a light intensity of 16 mW/mm² and a pulse width of 1000-ms. The results showed no difference in ERP before and after light irradiation (Supplemental Fig. S3D).

3.2. Characterization of ATs

To increase the inducibility of AT, a modified Tyrode's solution with low potassium concentration (2.7 mM) and with the I_{KACH}-increasing muscarinic receptor agonist carbachol (2 mM) was used, as repeated AT induction could not be achieved without APD shortening. The modified perfusate caused a significant shortening of the atrial wave duration, with no change in atrioventricular conduction duration (Figs. 2A–C). Replacement of the normal Tyrode's solution by the modified Tyrode's solution also caused a large increase in cycle length (Fig. 2D). Burst pacing of the right atrial appendage at 20–100 Hz for 200–1000 ms was used to induce atrial arrhythmias (Fig. 2E). The mode of propagation of the induced atrial arrhythmias was investigated by optical voltage mapping. The propagation pattern was not uniform, indicating a reentrant arrhythmia with irregular rotation (Fig. 2F).

3.3. Efficacy of optogenetic AT termination by brief pulsed light stimulation

To assess the suitability of our experimental model for examining the efficacy of optogenetic arrhythmia termination, we first evaluated optogenetic termination using three consecutive 1000-ms light pulses (470 nm, 16 mW/mm²) with 1000-ms intermissions onto 25 mm² of the right atrial epicardial surface (Supplemental Fig. S4A). AT was terminated in seven out of eight ChR2-positive mice and in only one out of eight ChR2-negative mice ($p < 0.01$, Supplemental Fig. S4B–D). In addition, when ChR2-positive mice were not exposed to light, AT was terminated in only two out of eight mice. From these results, we concluded that ChR2 expression itself did not affect arrhythmia termination rate.

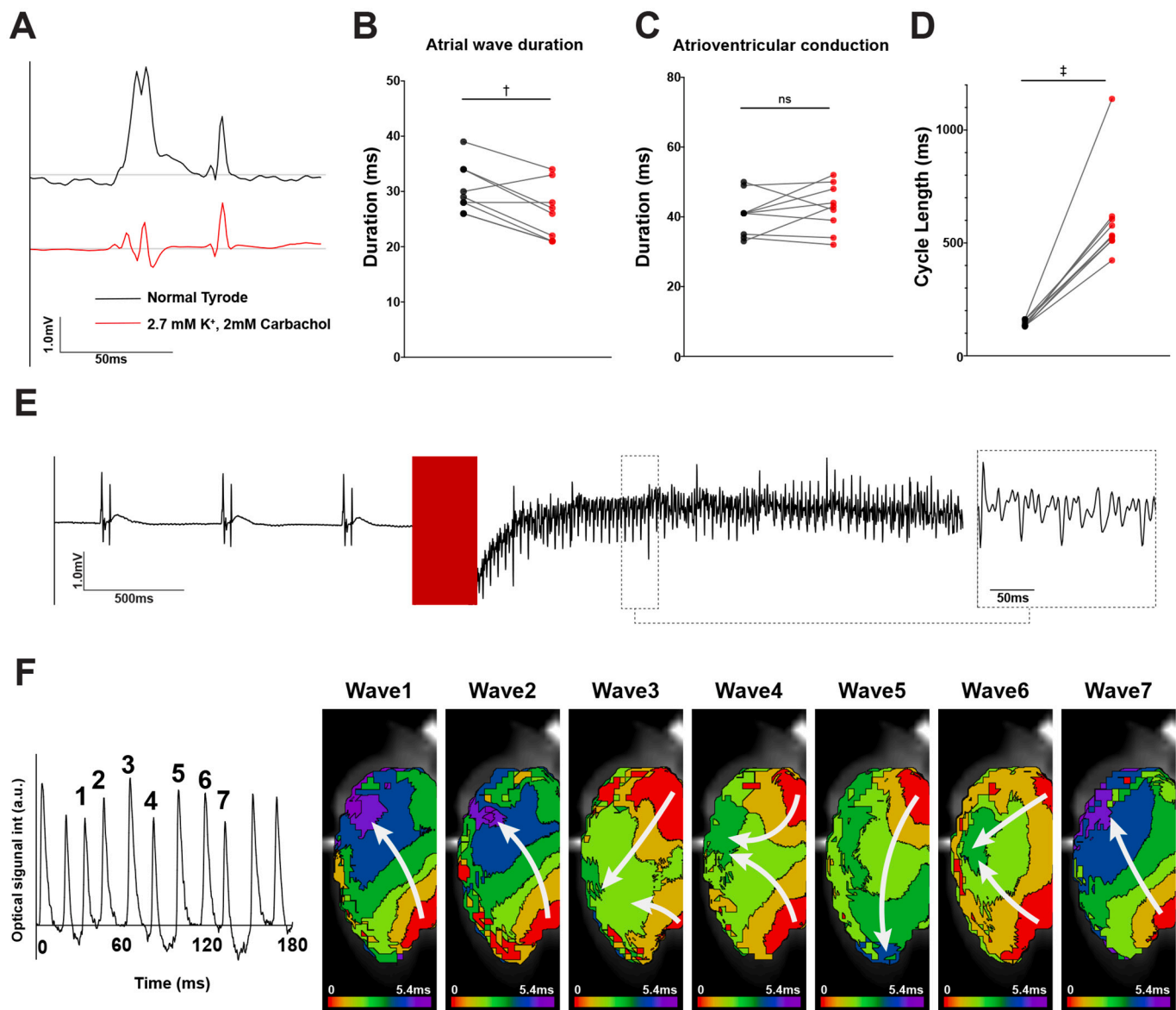


Fig. 2. Induction of AT in ex vivo mouse hearts. (A) Representative example of ECG from a Langendorff-perfused mouse heart using normal Tyrode's solution (black) and modified Tyrode's solution with a lower K^+ concentration (2.7 mM) and containing 2 mM carbachol. (B–D) Comparison of atrial wave duration ($n = 9$, $p = 0.0059$), atrioventricular conduction duration ($n = 9$, $p = 0.3408$), and cycle length ($n = 9$, $p = 0.0001$) between normal (black) and modified Tyrode's solution with 2.7 mM K^+ and 2 mM carbachol (red). (E) An ECG recording of an AT induced by high-frequency atrial electrical stimulation (300 ms, 50 Hz; red box). A magnified ECG recording is shown on the right. (F) A series of activation maps during burst pacing-induced AT. $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

We next examined the AT termination rate following different protocols of light stimulation. To allow multiple measurements in the same animal, light exposure for 1000 ms with a maximum light intensity (16 mW/mm²) was automatically provided 2000 ms after each episode of induced AT and subsequent arrhythmia termination attempt by brief pulsed light stimulation (Fig. 3A). After 2000 ms of light irradiation, we waited for at least 1 min before the next AT induction for the atrial tissue to recover from the effects of long light exposure. First, we fixed the pulse width and illuminated area to 10 ms and 25 mm², respectively, and raised the light intensity from 1 to 16 mW/mm². The arrhythmia termination rate increased with increasing light intensity (Fig. 3C). Next, the light intensity and illumination area were fixed at 10 mW/mm² and 25 mm², respectively, and the pulse duration was stepwise increased from 5 to 100 ms. As shown in Fig. 3D, the AT termination rate increased as the pulse duration increased up to 20 ms. Importantly, pulse durations > 20 ms (i.e., pulse durations larger than the murine atrial APD

in our model) did not further increase the AT termination rate. Moreover, in all successful cases, AT was terminated immediately after the onset of brief pulsed light stimulation (Fig. 3B).

3.4. Effect of brief pulsed light stimulation on APD in different AP phases

Next, we examined how brief pulsed light stimulation modulated the AP waveform by illuminating the atrial tissue for 10 ms during a train of electrical pacing with 150 ms intervals, which did not interfere with the visualization of APs by optical voltage mapping (Fig. 4A). The same test was repeated three times per animal, from which average APD₈₀ values were calculated.

In ChR2-positive mice, brief pulsed light stimulation (10 ms, 6 mW/mm²) with different time delays to electrical stimulation always modulated AP shape and prolonged APD₈₀ (Fig. 4B), whereas APs remained unaltered in ChR2-negative mice (Fig. 4C) and in ChR2-

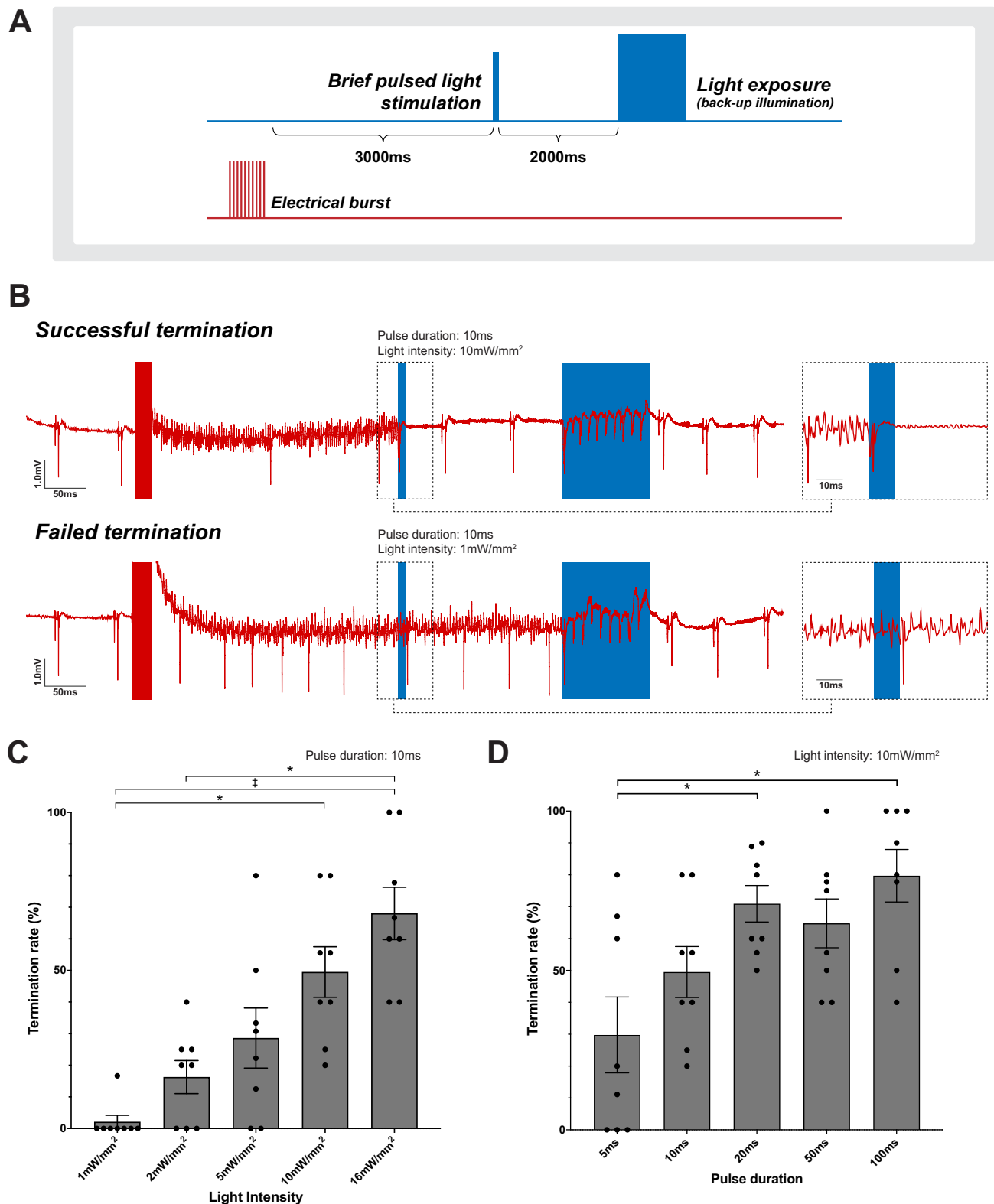


Fig. 3. Optogenetic termination of AT by brief pulsed light stimulation in ChR2(H134R)-expressing mouse hearts. (A) Protocol to investigate the AT termination rate by brief pulsed light stimulation. AT was induced by electrical burst pacing and, after confirming that it lasted 3000 ms, a brief pulsed blue light stimulation was performed (pulse width: 10–100 ms, light intensity: 1–16 mW/mm²; first blue square). Termination was considered successful if the arrhythmia was terminated within 100 ms after illumination. Back-up long light exposure (pulse width: 1000 ms, light intensity: 16 mW/mm²; second blue square) was automatically applied to ensure arrhythmia termination in each trial. The same trials were repeated at least three times in the same individual. (B) Representative ECGs of successful and failed optogenetic AT termination. (C–D) Rates of optogenetic arrhythmia termination at different light intensities ($n = 8$, pulse duration: 10 ms) and pulse durations ($n = 8$, light intensity: 10 mW/mm²). * $p < 0.05$, $\dagger p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

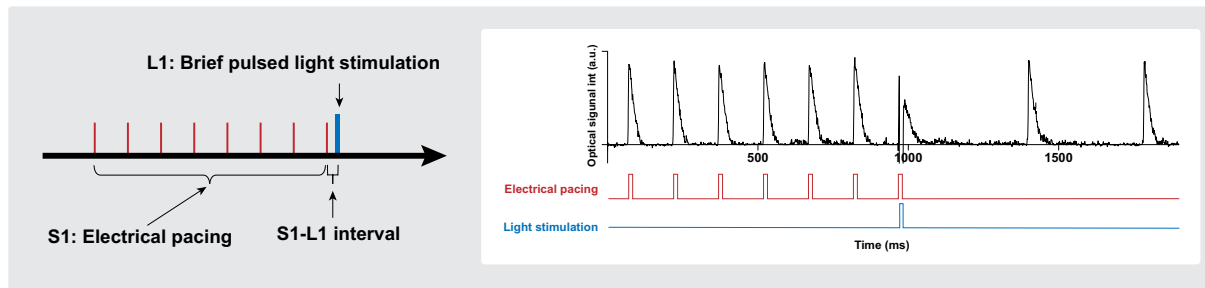
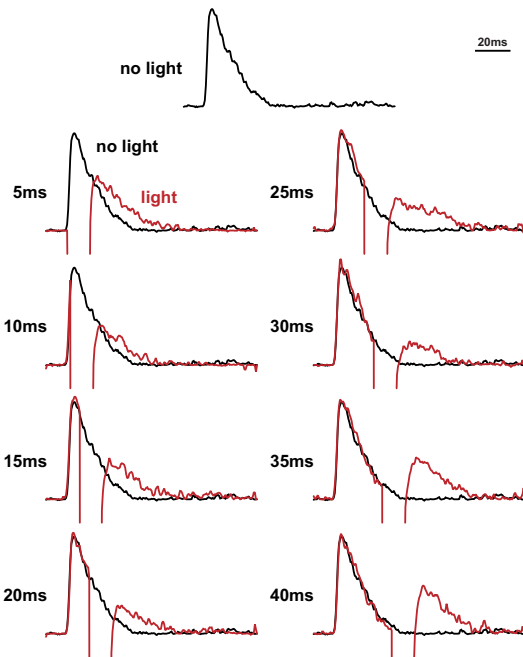
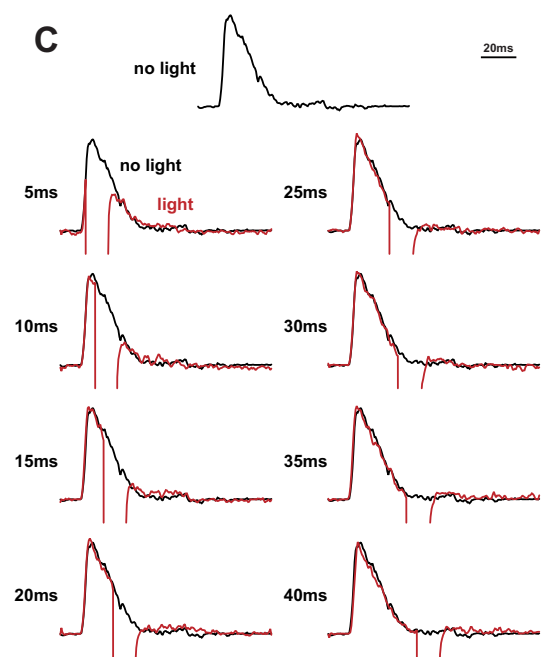
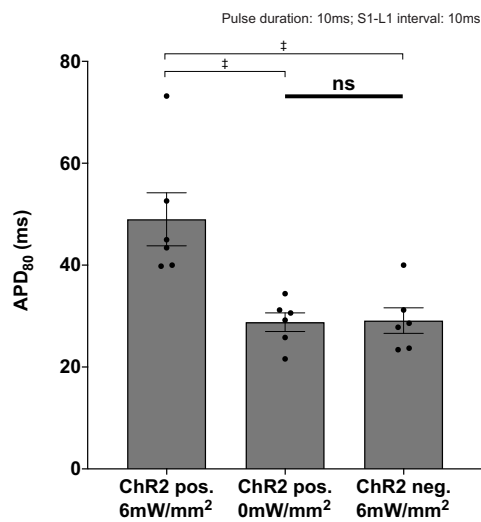
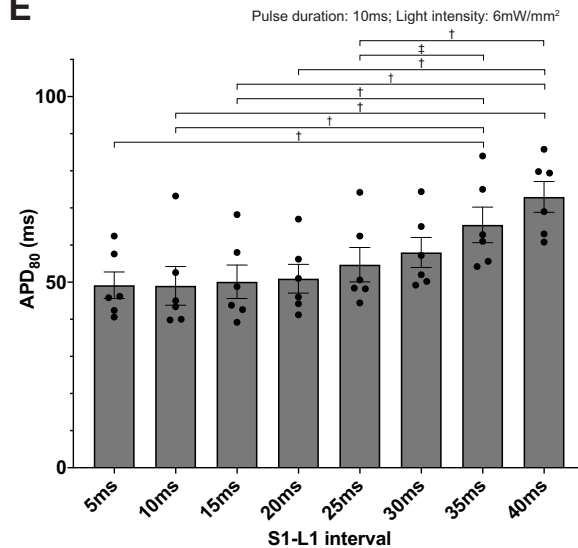
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Fig. 4. APD prolongation by brief pulsed light stimulation in ChR2(H134R)-expressing mouse hearts. (A) Protocol to measure the effect of timed light stimulation on APD (left) and an example of an APD measurement (right). Light stimulation (L1; pulse duration: 10 ms, light intensity: 6 mW/mm²) was applied with different time delays from the last electrical stimulation (S1) of 8 trains with a cycle length of 150 ms. The time delay (S1–L1 interval) varied between 5 and 40 ms. (B, C) Examples of APs modified by brief light stimulation. (B) In ChR2-positive mouse hearts, APD was prolonged when light was applied (red) compared to that without light (black) for all time delays. In contrast, APD was not affected by light in ChR2-negative mouse hearts (C). (D) Statistical analysis of APD₈₀ when the S1–L1 interval and pulse duration were both fixed at 10 ms, respectively ($n = 6$). (E) Quantification of APD₈₀ for different S1–L1 intervals. $N = 6$ in each group and each condition. $^{\dagger}p < 0.01$, $^{\ddagger}p < 0.001$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

positive mice without light stimulation (Fig. 4D). Notably, even light stimulation provided during the electrical refractory period prolonged APD. In addition, APD₈₀ prolongation was more pronounced when the light pulse was delivered 30–40 ms after electrical stimulation (Fig. 4E). Brief pulsed light stimulation consistently prolonged APD₈₀ in all experimental protocols using different time delays, that is, in all phases of the AP.

3.5. Effect of brief pulsed light stimulation on ERP

To investigate whether light stimulation was similarly effective in prolonging the ERP, a train of eight electrical stimuli (S1) was followed by brief pulsed light stimulation (L1) and an additional electrical stimulus (S2) (Fig. 5A).

First, we examined changes in ERP by brief pulsed light stimulation with a fixed S1–L1 interval (20 ms), duration (10 ms), and intensity (6 mW/mm²). As expected, ERP was significantly prolonged by light stimulation in Chr2-positive mice compared to that in unirradiated Chr2-positive animals and in blue light-exposed Chr2-negative mice (Fig. 5B).

Next, we evaluated ERP when the S1–L1 interval was varied between 10 and 40 ms while the light intensity and pulse width of illumination were fixed at 10 mW/mm² and 10 ms, respectively. ERP became longer with increasing S1–L1 interval (Fig. 5C). These light-induced ERP prolongations in Chr2-positive mice showed a similar trend as the light-induced prolongations of APD. We also evaluated ERP at different light intensities (0–6 mW/mm²) when the S1–L1 interval and pulse width of illumination were fixed at 20 ms and 10 ms, respectively. ERP was significantly longer during illumination with 1 mW/mm² than without illumination, i.e., 0 mW/mm². Interestingly, a further increase in light intensity did not cause additional ERP prolongation (Fig. 5D). Finally, we investigated the effect of different pulse widths of light stimulation on ERP at a constant S1–L1 interval of 20 ms and a constant light intensity of 6 mW/mm². ERP increased significantly with pulse width duration (Fig. 5E). Importantly, light-induced ERP prolongations were not observed in Chr2-negative mice under any of the tested conditions (Supplemental Fig. S5A–C).

3.6. Attenuation of light intensity as it passes through atrial tissue

Optical voltage mapping only allows ERP measurements at/near the atrial surface because of poor light penetration into the myocardium. Likewise, intense light might be required to affect the ERP of the deeper myocardial layers. We therefore measured the rate of light attenuation through atrial tissue. To this end, light with intensities of 1–20 mW/mm² was applied onto an atrial tissue piece and the intensity of the transmitted light was measured with an illuminometer (Supplemental Fig. S6A). Light intensity was attenuated by approximately 60% of that of the light source irrespective of the light intensity at the source (Supplemental Fig. S6B).

3.7. AP propagation during optogenetic AT termination

Finally, we optically analyzed the alteration of the AP shapes during optogenetic termination of ATs by brief pulsed light stimulation. In the optical voltage recording of optogenetic AT termination by a brief pulsed light stimulation in a Chr2-positive mouse (Fig. 6), a rotating wavefront was observed to disappear during light stimulation, and “depolarized optical fluorescence” was visible even after illumination ended (about +18 to +30 ms). In Chr2-negative mice, brief pulsed light stimulation did not terminate the reentrant arrhythmia or prolong the APD (Fig. 7).

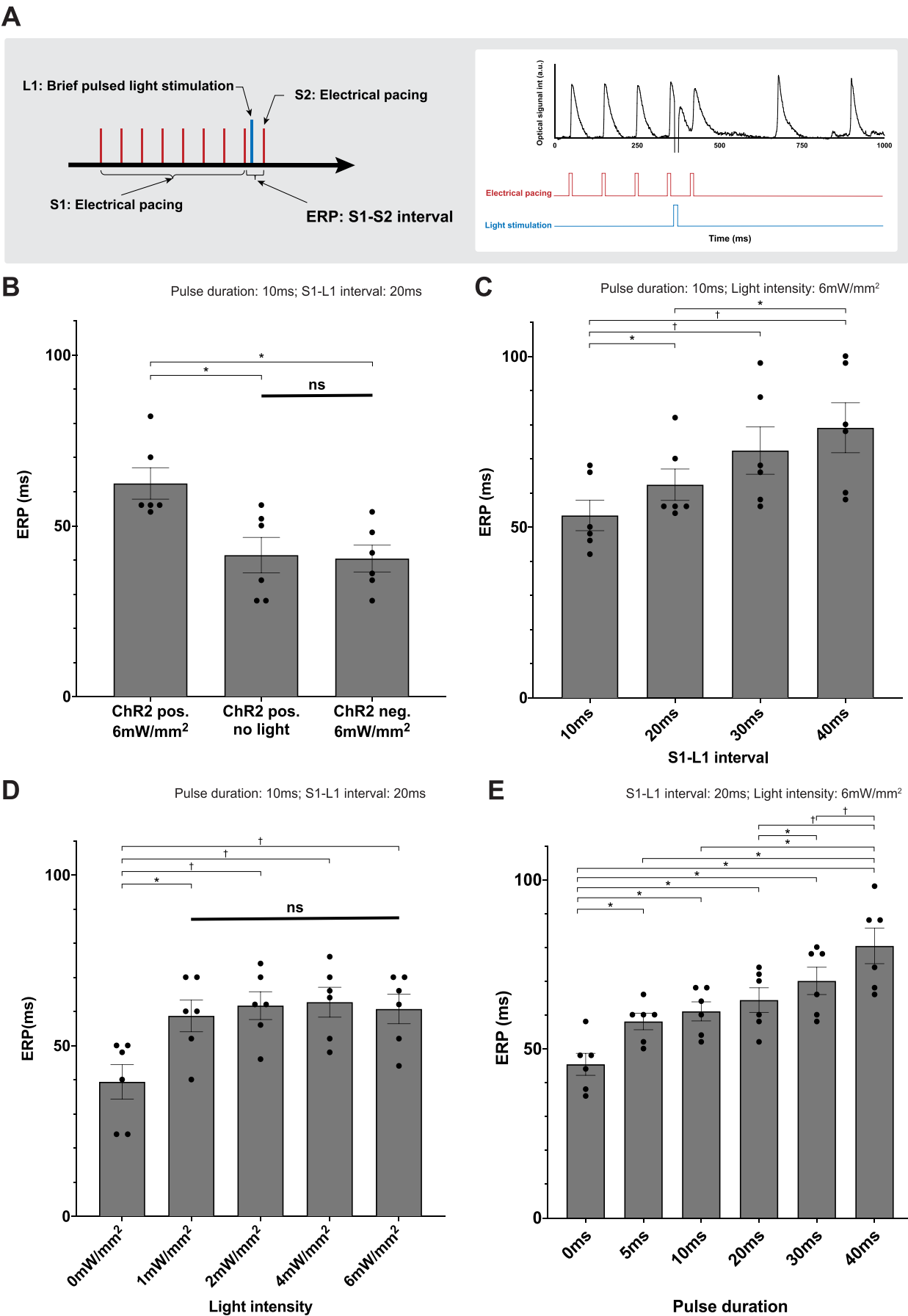
4. Discussion

In the present study, we systematically investigated the influence of

brief pulsed light stimulation on atrial APs in intact mouse hearts expressing the H134R variant of Chr2. We clearly demonstrated that brief pulsed light stimulation applied during an AP could prolong the APD and ERP. We also observed that optogenetic termination of ATs was achieved with the prolongation of APD. Based on the results of our study, we propose that ERP prolongation by light stimulation is one of the key mechanisms of optogenetic termination of ATs (Graphical Abstract).

AF causes palpitations and hemodynamic compromise, which often require patients to visit the emergency department. Although electrical cardioversion is the most effective way to acutely terminate AF, it could be painful if sedation is inadequately performed. In addition, myocardial damage by electrical cardioversion may lead to a poor prognosis and heart failure in patients with AF [35]. Therefore, a painless and less harmful approach for restoring sinus rhythm from AF is needed. Optogenetics is a new technology that can control electrophysiological phenomena in a cell-specific manner achieved by cell-specific promoters, depending on irradiation site, time and intensity [15]. The use of this technique was recently incorporated in the field of cardiology and has been proposed by Knollmann et al. as an alternative method of defibrillation [14]. Numerous studies using several animal models have since shown the efficacy of optogenetic termination in ventricular arrhythmias [16,17,19,20,22] and in atrial arrhythmias [23,24]. In corroboration with these previous studies, our results demonstrated that optogenetic termination of AT is achievable and that brief light exposures, requiring less energy than longer illumination periods, can terminate AT in an intact murine heart. In a recent simulation study, optogenetic arrhythmia termination was achieved in human MRI-based atrial and ventricular models [36]. Moreover, Nyns et al. recently reported on an effective automated hybrid bioelectronic device employing optogenetics for autogenous restoration of sinus rhythm in AF [24]. With the continued development of optogenetic technologies, drug- and ablation-resistant AF could in the near future be treated in a painless manner with light.

The efficacy of optogenetic termination has been demonstrated in both experimental and simulation studies, although its mechanism has not been fully elucidated. Conduction block is considered to be a major mechanism of optogenetic arrhythmia termination that is achieved by the inactivation of voltage-dependent Na⁺ channels via elevation of the resting membrane potential [8,13]. This is consistent with a recent study reporting that light stimulation at subthreshold intensity partially inactivates Na⁺ channels and reduces conduction velocity in intact mouse hearts [37]. However, in these studies, changes in action potential characteristics or conduction after light irradiation were not investigated. “Filling of the excitable gap” and “Modulation of electrophysiological properties” are considered as other possible mechanisms of optogenetic arrhythmia termination [19,20,38]. The former is a concept to activate the excitable gap creating a new wave front that collides with the arrhythmic wave front. To achieve an effective arrhythmia termination by a pulsed light, localized stimulation requires to know the extent of the excitable gap in time and space. Alternatively, global illumination of all myocardia is needed. Therefore, incorporation of conduction block with a prolonged light stimulation can provide robust arrhythmia termination [23]. The latter mechanism is assumed to terminate reentry termination through modulation of atrial conduction and excitability or refractoriness via constant optogenetic depolarization, similar to pharmacological approaches of arrhythmia termination [39]. In the current study, we demonstrated that pulsed light stimulation during the absolute refractory period prolonged ERP as well as APD and we also observed APD prolongation during light-induced AT termination. In other words, our study suggested that optogenetic arrhythmia termination can be achieved even after light stimulation has ended by prolongation of the refractoriness combined with filling of the excitable gap. The modulation of AP shape by optogenetics has until now been demonstrated only in isolated rodent cardiomyocytes and in human induced pluripotent stem cell-derived cardiomyocytes [25–27].



(caption on next page)

Fig. 5. ERP prolongation by brief pulsed light stimulation in ChR2(H134R)-positive mouse hearts. (A) Protocol to measure the effect of timed light stimulation on ERP (left) and an example of an optically recorded AP (right). A photostimulus (L1) was applied at different times after the last of 8 electrical stimuli with a cycle length of 100 ms each (S1). An additional electrical stimulus (S2) was applied and the ERP, measured by optical voltage mapping, was defined as the longest S1–S2 interval to result in the failure of propagation. The experiment was conducted with one of three parameters fixed: time delay (S1–L1 interval), light intensity, or pulse duration. (B) Statistical analysis of ERP when the S1–L1 interval and pulse duration were fixed at 20 and 10 ms, respectively ($n = 6$). (C–E) Quantification of ERP when S1–L1 interval (C), light intensity (D), or pulse duration were varied (E) in ChR2-positive mouse hearts ($n = 6$). (C) S1–L1 interval was varied between 10 and 40 ms. Pulse width was fixed at 10 ms and light intensity at 6 mW/mm². (D) Light intensity was varied between 0 and 6 mW/mm². The S1–L1 interval was fixed at 20 ms and pulse duration at 10 ms. (E) Pulse duration was varied between 0 and 40 ms. The S1–L1 interval was fixed at 20 ms and light intensity at 6 mW/mm². * $p < 0.05$, † $p < 0.01$.

However, in intact hearts, in which neighboring cardiomyocytes are electrically coupled via gap junctions, source-sink relationships will determine to what extent an optogenetically invoked photocurrent is able to modify AP properties throughout the tissue. This study is the first to systematically analyze the potential role of optogenetic APD modulation in AT termination in intact mouse heart. Although our study is still observational study, we believe that our study provides a new perspective on the mechanisms of optogenetic arrhythmia termination and suggests that AP modulation combined with conduction block and filling of the excitable gap can provide robust arrhythmia termination. Interestingly, the degree of APD prolongation differed considerably depending on the time after AP initiation at which ChR2 was activated and was particularly enhanced during the repolarization phase from +30 to +40 ms, where ChR2 has inward rectification properties with a reversal potential of approximately 0 mV [10]. It is also possible that light stimulation in the repolarization phase could have produced a new action potential, creating an early after depolarization. Either way, we have shown that light stimulation performed during the repolarization phase, in spite of the refractory phase, causes dynamic changes in action potentials. Triggered irradiation may, therefore, be a highly efficient way to perform cardioversion. Future preclinical studies testing the possibility and efficacy of triggered and local optogenetic termination are warranted.

Our data showed that the probability of optogenetic termination was dependent on the intensity of light. Nonetheless, we did not find any relationship between the intensity of light and ERP prolongation, with intensities above 1 mW/mm² having no incremental effect. On the other hand, the intensity of irradiation was attenuated by approximately 60% through the atrial tissue (Supplemental Fig. 6); thus, a higher intensity of light increased the probability of termination by activating ChR2 molecules expressed deeper in the myocardium. Our observations were sensible [15] and also highlight the current technical challenges in translating optogenetic termination into clinical use. To deliver light into deeper tissue layers, the use of red light-activatable and more light-sensitive ChRs has been investigated [19,24,40]. Also, activation of ChRs by two-photon excitation provides a novel strategy for optogenetic modulation of deep tissue layers [41]. Furthermore, a variety of apparatus designs have been assessed for delivery of LEDs into deeper layers, including injectable cellular scale optoelectronics [42], LEDs in flexible biocompatible membranes [43], stiff multi-LED probes [44], or micro LED arrays [45]. The transmural delivery of light energy and the intricacies of optogenetic therapies must first be studied in more detail before this novel therapeutic approach for painless arrhythmia termination can be applied in clinical studies [8].

Our data also showed that continuous light stimulation did not suppress spontaneous atrial beats but induced atrial extrabeats. A similar phenomenon was shown in the early study by Bruegmann and colleagues, though they observed the generation of spontaneous ventricular extrabeats in transgenic mice ubiquitously expressing ChR2 [13]. On the contrary, in isolated cardiomyocytes, they and others have demonstrated a prolonged depolarized membrane potential during light exposure, causing the cells to be non-excitable during illumination [13,16,46]. Bruegmann et al. stated that uncoupling and block of activity was not achieved because of the low penetrance of light [13]. In our mouse model based on a Cx40-CreERT2 line as inducible Cre driver, the expression of ChR2 protein was not uniform and a histological

assessment showed some atrial myocytes not expressing ChR2 and heterogeneous ChR2 expression in individual cells. In atria, Cx43 and Cx40 are the predominant connexins. However, the composition of connexins in each gap junction is heterogeneous and, in addition, the expression level of Cx40 might not be homogeneous in atrial tissue [47]. Our data and the report by Bruegmann et al. suggest that heterogeneous expression of ChRs and/or insufficient illumination can cause differences in membrane potential between (groups of) cardiomyocytes due to leakage of the depolarization current to neighboring cardiomyocytes not expressing channelrhodopsin or not exposed to light. Long continuous illumination might lead to the unfavorable extrabeats under these conditions. As this issue has not been fully investigated and simulated, future studies are needed to investigate the potentially proarrhythmic side effects of optogenetic anti-arrhythmic therapy.

4.1. Study limitations

The relationship between APD prolongation and AT termination was not directly examined in this study. For more robust proof, observations with varying time phases and regions of light stimulation in a simplified arrhythmia model are needed. Optical voltage mapping only allowed us to monitor cardiac electrical activity in the superficial layers of the atria. We were unable to observe whether AP modification occurred in the deeper layers of the myocardium and could not visualize the entire reentry circuit. A possible solution to observing APs in deeper layers could be mapping with multipolar electrodes [48]. However, this methodology could injure the atrial tissue and, more importantly, interrupt illumination. Even though ChR2(H134R) is poorly excited by green light [49] and the light for exciting RH237 was relatively weak, the 530-nm excitation light for optical voltage mapping might have caused a slight ChR2-mediated perturbation of electrical dynamics. We induced AT using a pharmacological agent that shortens the APD of atrial myocytes. In humans, the APD of atrial myocytes is trapezoidal and the action potential is above 0 mV for a longer time than in mice [50]. Therefore, it remains unclear whether pulsed light stimulation during ERP prolongs the APD in the human heart as in mice and whether it is equally effective when AF is caused by disease and/or aging in the human heart. Meanwhile, in clinical practice, AF is typically caused by a combination of APD shortening (much more triangular APD) [51] and conduction velocity slowing due to remodeling and interstitial fibrosis. A recent study showed efficient optogenetic termination of VTs in the pathologically remodeled heart using local illumination [52]. It will be of great interest to investigate whether APD prolongation can also be achieved by optogenetic means in fibrotic AF models and whether this might result in arrhythmia termination. Finally, the arrhythmia induced in the present mouse model was a macroreentrant atrial tachycardia. Hence, conditions may differ from those occurring during clinical atrial fibrillation.

5. Conclusion

Our results directly demonstrated that brief pulsed light stimulation prolonged the atrial refractory period depending on pulse duration in perfused mouse hearts and suggested that prolongation of the refractory period is one of the key mechanisms of optogenetic termination of AT.

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jmcc.2023.100000>.

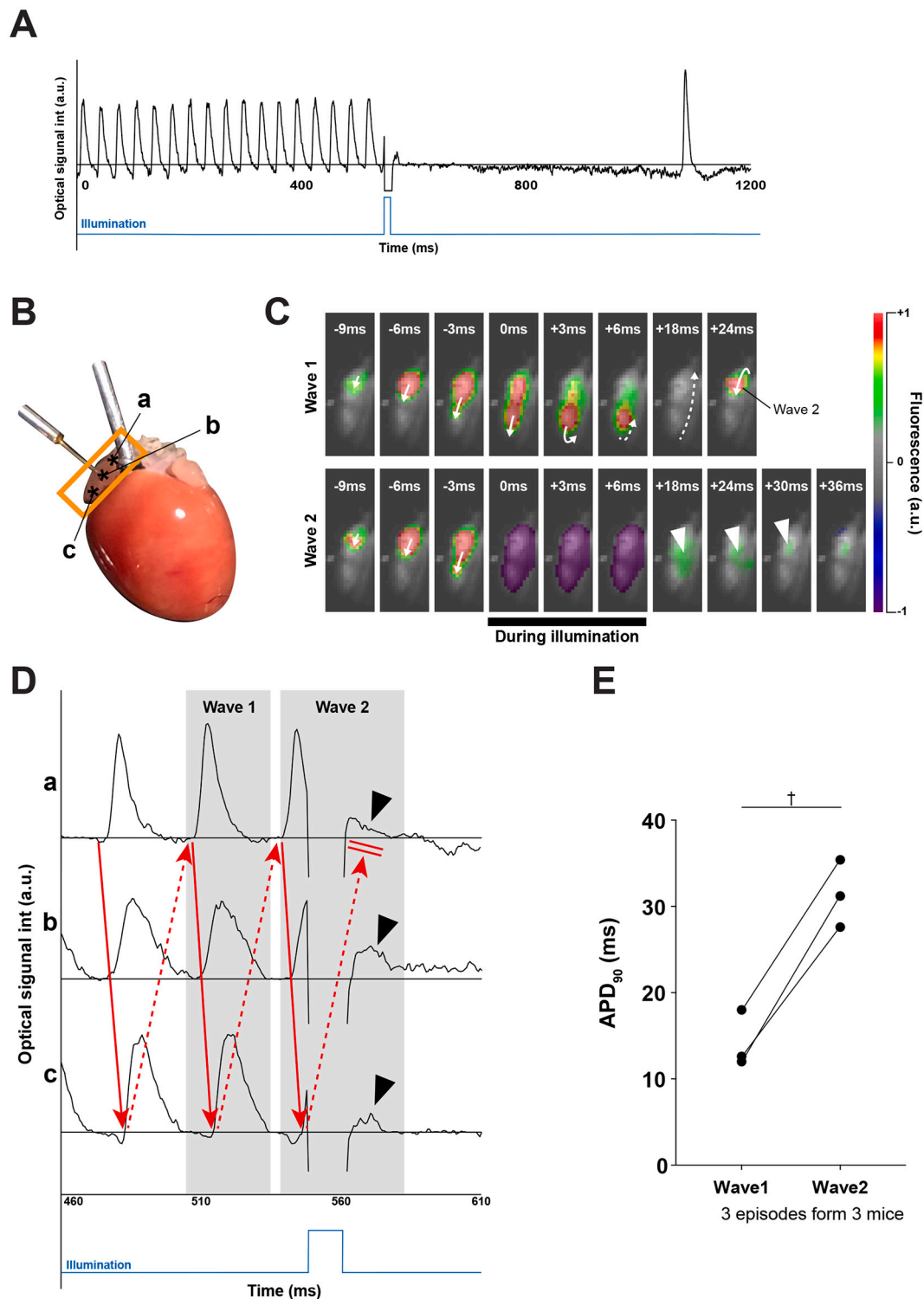


Fig. 6. Example of optogenetic AT termination in a ChR2(H134)-positive mouse heart. (A) Optical recording of AP during optogenetic AT termination by pulsed light stimulation (pulse width: 10 ms, light intensity: 10 mW/mm²). (B) A representative photograph of the right atrium used for optical recording. (C) Representative activation maps of the region in (B) demarcated by the yellow line. A reentrant wave disappeared after brief pulsed light stimulation. (D) Optical voltage traces from the points indicated in (B). “Depolarized optical fluorescence” (arrowheads) was observed at all three points even after illumination ended. (E) Statistical analysis of APD₉₀ of wave1 and wave2 indicated in (D). $\dagger p < 0.01$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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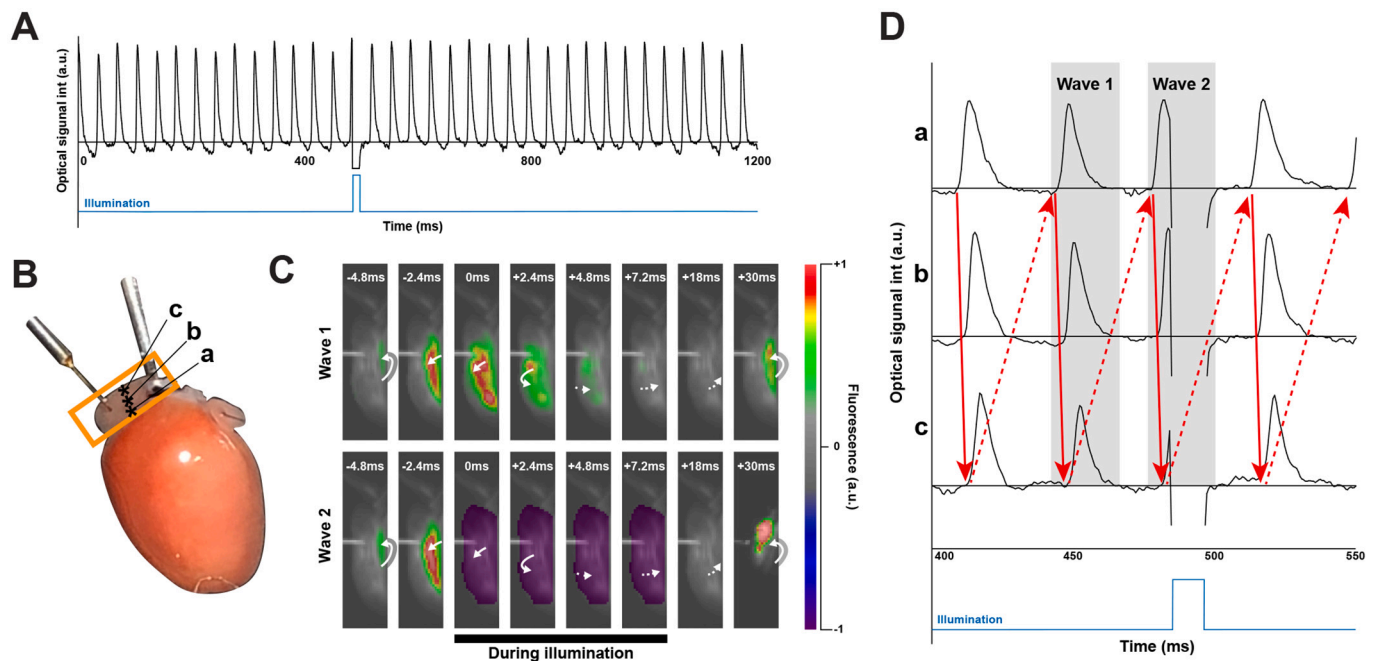


Fig. 7. Example of failed AT termination by brief pulsed light stimulation of a ChR2(H134)-negative mouse heart. (A) Optical recording of AP during brief pulsed light stimulation (pulse width: 10 ms, light intensity: 10 mW/mm²) (B) A representative photograph of the right atrium used for optical recording. (C) Representative activation maps of the region in (B) demarcated by the yellow line. (D) Optical voltage traces from the points indicated in (B). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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Author contribution statement

M.N., M.W. conceptualized the project. M.N., M.W. and L.M. performed experiments. M.N. and M.W. analyzed data. H.N., T.K., T.K., T.K., H.H., R.K., and T.T. interpreted the data. M.N. and M.W. wrote the manuscript. A.A.F.d.V. critically reviewed and edited the article. T.A. supervised and validated the revised article. All authors approved the final version of the article and agree to be accountable for all aspects of the work.

Declaration of Competing Interest

None declared.

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