Optogenetic termination of ventricular arrhythmias in the whole heart: Towards biological cardiac rhythm management

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Abstract

Aims

Current treatments of ventricular arrhythmias rely on modulation of cardiac electrical function through drugs, ablation or electroshocks, which are all non-biological and rather unspecific, irreversible or traumatizing interventions. Optogenetics, however, is a novel, biological technique allowing electrical modulation in a specific, reversible and trauma-free manner using light-gated ion channels. The aim of our study was to investigate optogenetic termination of ventricular arrhythmias in the whole heart.

Methods and results

Systemic delivery of cardiotropic adeno-associated virus vectors, encoding the light-gated depolarizing ion channel red-activatable channelrhodopsin (ReaChR), resulted in global cardiomyocyte-restricted transgene expression in adult Wistar rat hearts allowing ReaChR-mediated depolarization and pacing. Next, ventricular tachyarrhythmias (VTs) were induced in the optogenetically modified hearts by burst pacing in a Langendorff setup, followed by programmed, local epicardial illumination. A single 470-nm light pulse (1000 ms, 2.97 mW/mm^2) terminated 97% of monomorphic and 57% of polymorphic VTs *vs* 0% without illumination, as assessed by electrocardiogram recordings. Optical mapping showed significant prolongation of voltage signals just before arrhythmia termination. Pharmacological action potential duration (APD) shortening almost fully inhibited light-induced arrhythmia termination indicating an important role for APD in this process.

Conclusion

Brief local epicardial illumination of the optogenetically modified adult rat heart allows contact- and shock-free termination of ventricular arrhythmias in an effective and repetitive manner after optogenetic modification. These findings could lay the basis for the

development of fundamentally new and biological options for cardiac arrhythmia management.

Introduction

Ventricular arrhythmias are a large and growing problem worldwide, with high annual mortality and morbidity rates.[1] Despite significant progress in therapeutic strategies, the current treatment options for ventricular arrhythmias remain suboptimal. In brief, drug treatment is rather ineffective, while catheter ablation may cause irreversible complications and generally has a modest long-term efficiency. Electroshock therapy, on the other hand, is effective in terminating ventricular arrhythmias and has shown to reduce mortality as represented by the implantable cardiac defibrillator (ICD). However, the high-voltage shocks delivered by these devices are traumatizing, especially when given inappropriately, as they are associated not only with severe pain, anxiety and depression[2], but also with myocardial tissue damage.[3]

In contrast, optogenetics is an emerging biological technique to electrically modulate cells, tissue and organs in a specific, reversible and shock-free manner using light-gated ion channels.[4] Thus, optogenetics may provide a new incentive for the development of biological and pain-free treatment options for cardiac arrhythmias. Recent studies have shown that cardiac optogenetics allows for optical pacing of the whole heart and light-induced arrhythmia termination in cell cultures.[5-8] The aim of our present study was to investigate whether optogenetic modification of the adult rat heart would enable light-mediated termination of ventricular arrhythmias.

Methods

Experimental procedures are described in detail in Supplementary material online.

Animal studies

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

Optogenetic pacing and arrhythmia termination

Four to six weeks after systemic injection of cardiotropic adeno-associated virus (AAV) vectors encoding red-activatable channelrhodopsin (ReaChR)[9], the hearts of anesthetized adult Wistar rats were rapidly excised, cannulated and subsequently perfused using a Langendorff-apparatus (n=8). Optical pacing was performed by exposure for 20 ms to 470-nm light-emitting diode (LED) light. Ventricular tachyarrhythmia's (VTs) were induced by irregular electrical burst pacing protocols with a cycle length varying between 20-80 ms. When the VTs lasted for at least 5 s, they were considered sustained and the hearts were subsequently exposed to a 1000-ms light pulse. VTs were considered to be optogenetically terminated when ventricular arrhythmias stopped within 2 s after the start of illumination. When optogenetic termination failed, bipolar electrical defibrillation was performed (20 ms, 80 V, 12 mA). To evaluate the effect of action potential duration (APD) shortening on optogenetic terminaton efficiency, 3 µL of 10 mM of the K_{ATP} channel opener P1075 was directly injected into the aortic cannula. Following an incubation period of 1 min, the hearts were subjected to the same arrhythmia induction and optogenetic termination protocol as used prior to P1075 infusion. P1075 was considered washed out after 15 min following incubation as APD was then normalized to standard values.

Results

Systemic delivery of cardiotropic AAV vectors encoding ReaChR resulted in widespread transduction of the cardiomyocytes in adult rat hearts with an average transduction rate of 93% (standard deviation (SD) 4%) (*Figure 1A*). Functionality of these light-gated depolarizing ion channels was confirmed by sharp-electrode measurements in cardiac tissue slices, revealing significant and sustained depolarization of the membrane potential during 470-nm LED illumination for 1000 ms *(Figure 1B*).

Upon excision and preparation of the hearts in the Langendorff setup for stimulation and readout (*Figure 1C*), part of the epicardial surface was exposed to short (*i.e.* 20-ms) 470-nm light pulses (0.97 mW/mm²). This allowed optical pacing up to 4.5 Hz, thereby producing QRS complexes similar to those induced by electrical stimulation (*Figure 1D*). Following the induction of sustained VTs by electrical burst pacing, a single 470-nm light pulse (1000 ms, 2.97 mW/mm²) was given illuminating circa 125 mm^2 of the ventricular surface. Such pulses led to an average successful arrhythmia termination rate of 97% for monomorphic VTs (corresponding to 26 VTs in 8 hearts) and 57% of polymorphic VTs (corresponding to 19 VTs in 6 hearts). (Supplementary material online, Video S1). Without illumination, none of these arrhythmias were terminated (n=6 for both mono- and polymorphic VTs) (*Figure 1E and F*).

Optical voltage mapping was performed in order to obtain more mechanistic insight into light-induced arrhythmia termination. Activation maps confirmed global high-frequency activation of the ventricles resembling reentrant activity (*Figure 1G).* Optical recordings showed that the 1000-ms light pulses resulted in arrhythmia termination within 420.9 ms (SD 189.7 ms, range 185-734 ms). Furthermore, based on the optical signals, significant prolongation of action potential duration (APD) was noted just before arrhythmia termination (58.6 ms (SD 7.3 ms) *vs* 72.3 ms (SD 16.3 ms) for APD_{80} before and during illumination,

p=0.021, n=7). (*Figure 1H and I).* As this finding indicated an important role for APD prolongation in optogenetic arrhythmia termination in these hearts, we next evaluated the effects of APD shortening on termination efficiency. For this purpose, the optogenetically modified hearts were treated with a single bolus of P1075, a K_{ATP} channel opener (n=2). As a result, APD_{80} during the arrhythmia was significantly shortened (62.9 ms (SD 11.8 ms) *vs* 44.0 ms (SD 13.7 ms) before and after P1075 administration, p=0.018). Using the same arrhythmia induction and optogenetic termination protocol, none of monomorphic VTs (0 out of 5) and only 1 out of 9 (11%) polymorphic VTs could be terminated. After a washout period of 15 min, and normalization of APD_{80}, optogenetic termination efficiency increased to 80% for monomorphic VTs (4 out of 5) and 71% for polymorphic VTs (5 out of 6) (*Figure 1 J).*

Discussion

Here, we demonstrate that forced expression of a light-gated depolarizing ion channel (ReaChR) in the adult rat heart allows contact- and shock-free termination of VTs through brief local illumination of the ventricular surface, *i.e.* without relying on conventional drugs, tissue ablation or electroshocks. Both mono- and polymorphic VTs could be terminated in an effective and repetitive manner by a light-induced electrical current driven by natural electrochemical gradients, providing proof-of-concept for biological arrhythmia termination.

The unique properties of optogenetics make it possible to modulate cardiac electrical activity by applying depolarizing or hyperpolarizing photocurrents with high specificity and unmatched spatiotemporal precision.[4] The finding that optogenetic arrhythmia termination is feasible in the intact heart could have an impact on the development of novel treatment options for cardiac arrhythmias, as there may be several advantages of biological anti-arrhythmic therapy over conventional therapy. These advantages could relate to the specificity of the intervention, which is superb for optogenetics and relatively poor for drugs.[10] In addition, optogenetic interventions allow temporary modulation of electrical function, as opposed to tissue ablation, which results in permanent alteration of the electrical properties of cardiac tissue.[11] Importantly, optogenetic arrhythmia termination obviates the use of high-voltage electroshocks. These shocks are not only known to be painful and to cause anxiety and stress in a significant percentage of patients wearing an ICD[2], but also to cause myocardial tissue damage.[3] In theory, these disadvantages could be avoided if the heart itself would be able to generate, on cue, the electrical current for arrhythmia termination, as with cardiac optogenetics. Obviously, many hurdles need to be overcome before clinical translation can be considered, but the concept of biological arrhythmia management seems worth further investigation considering the unique potential benefits.

Our data suggests that APD prolongation plays an important role in optogenetic arrhythmia termination. Such prolongation has been associated with destabilization of arrhythmic electrical activity, thereby favouring its termination.[12] However, as the exact mechanisms remain incompletely understood, further studies are needed in order to improve mechanistic insight, including evaluation of the minimum requirements for effective optogenetic termination, such as strength, area and location of illumination. Moreover, further development and optimization of optogenetic tools and light delivery would help to improve optogenetic termination efficiency and, together with advances in gene transfer technology, will potentially aid the translation to *in vivo* applications.

It is expected that potential *in vivo* applications are hindered by poor light penetration, and that therefore only a small fraction of the total number of light-gated ion channels would be activated.[13] An important finding of this study is that light-induced arrhythmia termination was already successful by illuminating only a small area of the epicardial surface. This finding at least suggests that it may not be necessary to illuminate the whole heart. Hence, the challenging aspect of illumination might be overcome by focusing the light on a relatively small, but in terms of arrhythmia maintenance, critical area. In addition, this finding also suggests that regional genetic modification by local delivery of viral vectors[14,15] or optogenetically modified cells into cardiac tissue may already be sufficient for effective optogenetic modulation, which would be of practical benefit. Furthermore, as heart size may play an important role in determining the efficiency of arrhythmia termination via optogenetics, further studies in larger hearts are needed.[16]

Taken together, our study demonstrates that the heart itself is able to produce an electrical current for arrhythmia termination upon forced expression of light-gated depolarizing ion channels and their activation by brief and local epicardial illumination. Such biological arrhythmia termination enables precise and shock-free control over disturbed cardiac rhythm,

allowing normal rhythm to regain. While further and more detailed studies are certainly needed, the findings presented in this brief communication do provide proof-of-principle for optogenetic termination of arrhythmias in the whole heart and may thereby pave the way for the design and development of fundamentally novel strategies of cardiac arrhythmia management.

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Conflict of Interest: none declared.

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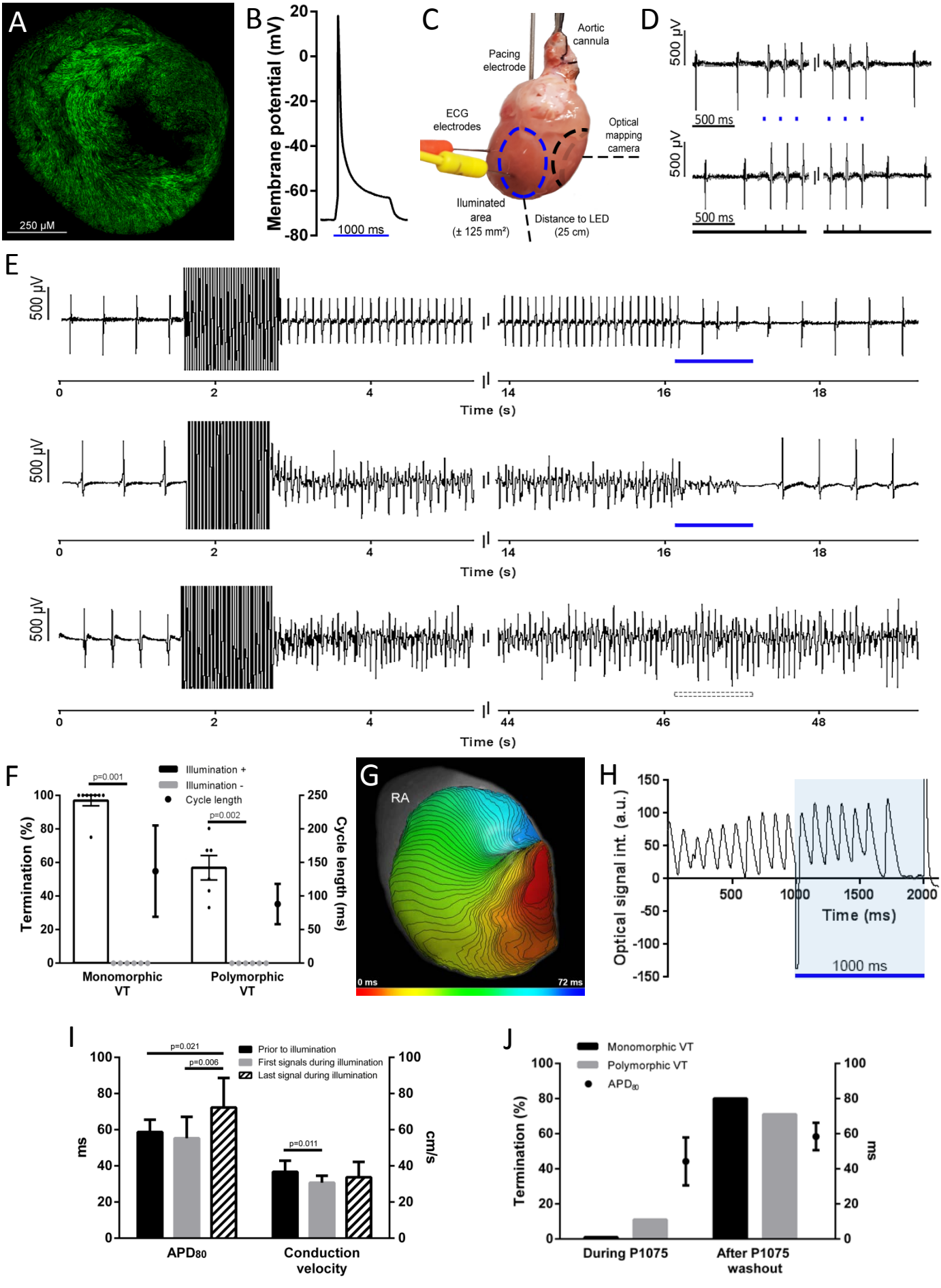
Legends

Figure 1 (A) Mid-ventricular transversal slice of an adult rat heart transduced with a cardiotropic adeno-associated virus (AAV) vector encoding red-activatable channelrhodopsin (ReaChR) fused to citrine showing global transgene expression 6 weeks after vector injection. **(B)** Confirmation, by sharp-electrode measurement, of light-induced and sustained depolarization in a ReaChR-expressing ventricular tissue slice. **(C)** Schematic overview of a ReaChR-expressing heart in the Langendorff setup equipped for electrical stimulation and recording, in which the area of LED illumination and optical mapping are indicated by the blue and black dotted line, respectively. **(D)** Optical (top panel) and electrical (bottom panel) pacing of a ReaChR-expressing heart showing 1:1 capture with similar electrocardiographic signals for both modes of stimulation. **(E)** Typical intra-cardiac electrogram readouts demonstrating successful termination of a monomorphic (top panel) and polymorphic ventricular tachyarrhythmia (VT) (middle panel) with a single 1000-ms local light pulse (blue line) onto the epicardial surface, while the arrhythmias are sustained without (bottom panel) such illumination (dotted black line). **(F)** Quantification of light-induced termination of mono- and polymorphic VTs expressed as a percentage of successful attempts averaged for all hearts (error bar represents one standard error of the mean) and of the average arrhythmia cycle length prior to illumination (error bar represents one standard deviation). **(G)** Electrical activation map of ReaChR-expressing heart, derived from voltage mapping, showing a reentrant conduction pattern. **(H)** Typical trace of optical voltage signals showing prolongation of the last voltage signal prior to VT termination by local epicardial illumination. **(I)** Quantification of APD_{80} and conduction velocity based on optical voltage signals at different stages of optogenetic arrhythmia termination (error bar represents one standard deviation). **(J)** The effect of the K_{ATP} channel opener P1075 on the APD_{80} and success of light-induced termination of mono- and polymorphic VTs, showing almost

complete failure of optogenetic termination of VTs upon APD shorting by P1075 and recovery of photocurrent-mediated VT termination ability upon P1075 washout (error bar represents one standard deviation).


A
250 µM
B
Membrane potential (mV)
20
0
-20
-40
-60
-80
1000 ms
C
Aortic cannula
Pacing electrode
ECG electrodes
Optical mapping camera
Illuminated area (± 125 mm²)
Distance to LED (25 cm)
D
500 µV
500 ms
E
500 µV
Time (s)
F
Illumination +
Illumination -
Cycle length
p=0.001
p=0.002
Termination (%)
Cycle length (ms)
Monomorphic VT
Polymorphic VT
G
RA
0 ms
72 ms
H
Optical signal int. (a.u.)
Time (ms)
1000 ms
I
Prior to illumination
First signals during illumination
Last signal during illumination
p=0.021
p=0.006
p=0.011
ms
cm/s
APD80
Conduction velocity
J
Monomorphic VT
Polymorphic VT
APD80
Termination (%)
ms
During P1075
After P1075 washout

Supplementary methods

Animal studies

All animal experiments were approved by the Animal Experiments Committee of the Leiden University Medical Center and conformed to the Guide for the Care and Use of Laboratory Animals as stated by the US National Institutes of Health.

Construction of adeno-associated virus vector (AAVV) shuttle plasmid

Molecular cloning was performed with enzymes from Fermentas (Thermo Fisher Scientific, Breda, the Netherlands) or from New England Biolabs (Bioke´, Leiden, the Netherlands) and with oligodeoxyribonucleotides from Sigma-Aldrich (St. Louis, MO) using established procedures or following the instructions provided with specific reagents.

The AAVV shuttle construct pDD.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA (Figure S1) was assembled from DNA fragments of the AAVV shuttle plasmids pDD4[1]; AAVV backbone and simian virus 40 polyadenylation signal) and AcTnTeGFP[2]; *Gallus gallus Tnnt2* promoter) and the lentiviral vector shuttle plasmid pLenti-ReaChR-Citrine[3]; coding sequence of the ReaChR~citrine fusion protein and woodchuck hepatitis virus posttranscriptional regulatory element).

AAVV production

To restrict transgene expression to cardiac muscle tissue, the AAVV genomes encoded by pDD.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA were packaged in liver-detargeted AAV serotype 9 capsids.[4] The resulting AAV2/9.45.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA particles were produced by transfecting subconfluent monolayers of 293FT cells (Thermo Fisher Scientific) with the packaging plasmids pHelper (Agilent Technologies, Santa Clara,

CA), pXR9.45 (National Gene Vector Biorepository, Indianapolis, IN) and the shuttle plasmid pDD.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA at equimolar ratios. For transfection, 293FT cells were cultured in 143-cm^2 cell culture dishes (Greiner Bio-one, Alphen aan den Rijn, the Netherlands) in 15 ml of high-glucose Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific; catalogue number: 41966) containing 5% fetal bovine serum (FBS), 25 mM HEPES-NaOH (pH 7.4) and 1 µg/ml G418 (Santa Cruz Biotechnology, Dallas, TX). At ±70% confluency, the cells were transfected by directly adding a mixture containing 40 µg of plasmid DNA and 120 µg of polyethyleneimine (PEI MAX 40000; Polysciences Europe, Eppelheim, Germany) in 2 mL of 150 mM NaCl to the medium in each cell culture dish. Sixteen h later, the transfection medium in each dish was replaced by 15 ml of high-glucose DMEM supplemented with 1% FBS and 10 mM HEPES-NaOH (pH 7.4). At 68-72 hours after the start of the transfection procedure, both culture medium and cells were harvested. Cells were separated from the medium by centrifugation for 10 min at room temperature (RT) and 3,000×*g*. The supernatant was poured off and centrifuged once more for 10 min at 10,000×*g* after which it was stored at 4°C until further use. The cells were suspended in 1 ml of lysis buffer (50 mM Tris-HCl [pH 8.0], 100 mM NaCl, 2 mM $MgCl_2$) per cell culture dish and subjected to three freeze-thaw cycles. Next, 100 U of OmniCleave endonuclease (Epicentre, Madison, WI) per cell culture dish was added followed by a 60-min incubation period at 37°C. Finally, 50 µL of 10% w/v sodium deoxycholate was added to each cell culture dish. After incubation for 15 min at RT, the cell lysate was cleared by centrifugation for 10 min at 3,000×*g* and filtration through a 0.45-µm pore-sized, 33-mm diameter polyethersulfone Millex-HP syringe filter (Millipore, Amsterdam, the Netherlands). The processed culture medium and cell lysate were then combined in glass bottles, supplemented with NaCl and polyethylene glycol 8000 (VWR International, Amsterdam, the Netherlands) up to final concentrations of 0.5 M and 8%,

respectively and slowly stirred at 4°C for >12 h. Next, the samples were transferred to polypropylene centrifuge tubes and centrifuged at 6,500×*g* for 30 min at 4°C. The supernatants were carefully removed and the cell pellets suspended in phosphate-buffered saline (PBS) supplemented with 1 mM $MgCl_2$ and 2.5 mM KCl (PBS-MK; 5 mL/24 cell culture dishes). Subsequently, 400 U of Omnicleave was added per 24 cell culture dishes and incubated for 1 h in a 37°C. The AAVV particles were purified and concentrated by iodixanol step gradient ultracentrifugation essentially as described by Choi and colleagues[5] except for the use of 4, 9, 9 and 5 ml of 15, 25, 40 and 60% of OptiPrep (Axis-Shield, Olso, Norway), respectively. Moreover, centrifugation at 16°C in the 70Ti rotor (Beckman Coulter Nederland, Woerden, the Netherlands) was for 90 min at 69,000 revolutions per min (rpm) instead of 1 h at 70,000 rpm. AAVV particles at the 40%-60% iodixanol interface were collected with a 19-gauge needle. Next, the samples were diluted 1:3 with PBS-MK, applied to Amicon Ultra-15 centrifugal filter units (nominal molecular weight limit: 100 kDa; Millipore) and concentrated by centrifugation at 5,000×*g* for 30 min at RT. PBS-MK buffer was exchanged with PBS-MK+5% sucrose by two additional centrifugation steps using the Amicon Ultra-15 tubes. The concentrated viral vectors were stored in 300-µl at -80°C until use.

Titration of AAVV preparations by quantitative polymerase chain reaction (qPCR) amplification

Twenty µL of purified AAVV vector suspension were mixed with 2 µL DNase I buffer and 1 µL (1 U) of amplification grade DNase I (Thermo Fisher Scientific) and incubated for 15 min at RT. Enzyme activity was stopped by addition of 2 µL of 50 mM EDTA and capsids were subsequently denatured by a 10-min incubation at 80°C. Ten µL of the sample was then mixed with 0.5 µL of 20 U/µL SmaI, 2 µL of 10× CutSmart buffer (both from New England

Biolabs) and 7.5 µL water and incubated at 37°C for ≥3 h. The endonuclease was inactivated by a 20-min incubation at 65°C and the concentration of vector genomes in the resulting sample was determined by an established qPCR procedure targeting the AAV inverted terminal repeats.[6]

Intravenous AAVV injection

Six-week-old female Wistar rats (Charles River, The Netherlands) were anesthetized by inhaling 1.8% isoflurane and 0.8 L O_2/min. Next, 2.3-3.5×10^{14} genome copies of AAV2/9.45.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA particles diluted in 400 µL of PBS-MK+5% sucrose were slowly injected in the tail vein using a 25-gauge needle attached on an 1-ml syringe (both from Becton Dickinson, Breda, the Netherlands).

Optical pacing and arrhythmia termination

Four to 6 weeks following intravenous AAV injections, the animals were anesthetized, intubated and mechanically ventilated with 1.8% isoflurane and 0.8 L O_2/min. Adequate anaesthesia was confirmed by absence of reflexes. A thoracotomy was performed and hearts were rapidly excised and immediately placed in ice-cold Tyrode solution (composition in mM: NaCl 143, KCl 5.4, $MgCl_2$ 0.5, CaCl 1.8, NaH_2PO_4 0.33, glucose 5.5, BDM 20). supplemented with 20 mM 2,3-butanedione monoxime (BDM; Sigma-Aldrich). The aorta was cannulated and immediately flushed with ice-cold BDM supplemented Tyrode's solution in order to rinse out blood from the coronary vasculature. The hearts were subsequently perfused (constant flow of 10.8 mL/min by peristaltic pump) using a Langendorff apparatus (AD Instruments, Oxford, United Kingdom) with oxygenized and BDM supplemented Tyrode's solution. Bipolar ECG electrodes were placed in the ventricles and recordings were interfaced by a 8-channel PowerLab data acquisition device and recorded and analysed using

LabChart Pro software version 7.3(all from AD Instruments). Action potential (AP) propagation was visualized by loading hearts with 12.5 µM of the voltage-sensitive dye di-4-ANDDQBS (AAT Bioquest, Sunnyvale, CA). Optical images were captured using a MiCAM ULTIMA-L imaging system (SciMedia, Costa Mesa, CA). Hearts were stimulated either electrically using an bipolar concentric electrode (FHC, Bowdoin, ME) with suprathreshold electrical stimuli (pulse duration of 10 ms, puls strength of 2500 mV) or optically with light pulses from a mounted 470-nm light-emitting diode (LED) with adjustable collimation lens (M470L3-C4, Thorlabs, Munich, Germany) positioned at approximately 25 cm from the heart and powered by a 1200-mA LED driver in trigger mode (T-Cube LED driver, Thorlabs). Irradiance was measured using a PM100D optical power and energy meter (Thorlabs) equipped with a S130C slim dual range sensor with the range set at 5 nW-5 mW and the wavelength set at 470 nm. A specialized stimulus generator (STG 2004) with corresponding software (MC Stimulus II; both from Multichannel Systems, Reutlingen, Germany) was used to perform both electrical and optical stimulation. Hearts were allowed to stabilize for 15 min before the start of the experiment. Optical pacing was performed by exposure for 20 ms to 470-nm LED light at 0.97 mW/mm^2. Ventricular tachyarrhythmias were induced by irregular electrical burst pacing protocols with a cycle length varying between 20-80 ms. When the arrhythmias lasted for more than 5 s, they were considered sustained and the hearts were subsequently exposed to a 470-nm light pulse (1000 ms, 2.97 mW/mm^2, illuminating approximately 125 mm^2 of the ventricular surface). VTs were considered to be optogenetically terminated when ventricular arrhythmias stopped within 2 s after the start of illumination. When optogenetic termination failed, bipolar electrical defibrillation was performed (20 ms, 80 V, 12 mA). To evaluate the effect of AP duration shortening on optogenetic terminaton efficiency, 3 µL of 10 mM of the K_{ATP} channel opener P1075 (Tocris Bioscience, Bristol, United Kingdom) was directly injected into the aortic cannula following

decoupling of the afferent peristaltic tube from the cannula. Immediately thereafter, the peristaltic tube was re-attached and perfusion with oxygenized Tyrode's solution was continued. Following an incubation period of 1 min, the hearts were subjected to the same arrhythmia induction and optogenetic termination protocol as used prior to P1075 infusion. P1075 was considered washed out after 15 min following incubation as APD_{80} of was then normalized to standard values. The experiments typically lasted for approximately 90 min, or were discontinued once the amplitude of the APs on the ECG showed a substantial decrease. Specialized software was used for data analysis and construction of activation maps (BrainVision Analyzer 1101; Brainvision, Tokyo, Japan). For baseline shift adjustment during LED light exposure, several filters were applied allowing data interpretation during the LED-on period.

Transversal slicing of living hearts

Slices of living optogenetically modified hearts were made for microelectrode recordings and evaluation of transgene expression. Before slicing, hearts were embedded in 4% low melting point agarose (type VII-A) dissolved in oxygenized Tyrode's solution at 37°C and subsequently fixed to the sample holder of the vibratome (VT1200S; Leica Microsystems, Rijswijk, the Netherlands) using Histoacryl (B. Braun Medical, Oss, the Netherlands). The sample was then quickly submerged in ice-cold and pre-oxygenated Tyrode's solution and cut into 200- or 300-µm thick tissue slices using Derby extra super stainless double edge razor blades. These slices were stored on ice until processed for further experiments.

Sharp electrode recordings

To assess the effects of 470-nm light stimulation on resting membrane potential, microelectrode recordings of cardiac slices were made at 28°C in oxygenized and BDM

supplemented Tyrode's solution (n=3). Cells were impaled with the tip of a quartz glass micro-electrode (5–20 MΩ, filled with 3 M KCl), connected to an Axon Geneclamp 500B amplifier (Molecular Devices, Sunnyvale, CA) for signal amplification and filtering (10 kHz low pass). The signal was digitized at 10 kHz sampling frequency and stored on a personal computer's hard disk using an Axon Digidata 1550 digitizer and the Clampex 10.4 data-acquisition program (both from Molecular Devices). For viewing and off-line analysis of signals we used the Clampfit 10.4 program (Molecular Devices). APs were evoked by a similar optical stimulation protocol as used for optogenetic arrhythmia termination (470 nm, 1000 ms, 2.55 mW/mm^2).

Immunohistochemistry and transgene quantification:

Following the end of the Langendorff experiments, hearts were cut transversely in two equal parts and fixed by overnight incubation at 4°C in 4% paraformaldehyde. Next, hearts were embedded in paraffin. Transverse sections of 5 µm were cut at basal, mid-ventricular and apical levels. Sections were deparaffinized and dehydrated in UltraClear (Klinipath, Duiven, the Netherlands) and a graded ethanol series. Antigen retrieval was performed by incubation with a 0.05% trypsin solution (Sigma-Aldrich) supplemented with 0.1% calcium chloride (Merck, Darmstadt, Germany) for 20 minutes at RT. Sections were stained with antibodies directed against cardiac troponin I (mouse IgG1, clone 6F9; Hytest) and green fluorescent protein (GFP; rabbit IgG, Thermo Fisher Scientific, catalogue number: A-11122). Incubation with primary antibodies (1:200 dilution in PBS/2% donkey serum (Thermo Fisher Scientific) and corresponding Alexa Fluor 488/568-conjugated secondary antibodies (Thermo Fisher Scientific; 1:200 dilution in PBS/2% donkey serum) was done at RT. Nuclear counterstaining was performed at RT with 1 µg/mL Hoechst 33342 (Thermo Fisher Scientific). Sections were subsequently mounted in Vectashield mounting medium (Vector Laboratories,

Burlingame, CA). Images were acquired with a TCS SP8X WLL confocal microscope and a 25× NA 0.75 oil immersion objective (both from Leica, Wetzlar, Germany) and a Nikon Eclipse 80i fluorescence microscope (Nikon Instruments Europe, Amstelveen, the Netherlands). In order to determine the transduction rate, a minimum of three fields from the interventricular septum and the left and right ventricular free walls from basal, mid-ventricular and apical sections (n=4 hearts). at 20× magnification were analyzed. The transduction rate was calculated per field by dividing the number of citrine-positive cardiomyocytes by the total number of cardiomyocytes.

Statistical analysis

Statistical analysis were performed using SPSS Statistics v23.0 (IBM Corporation, Armonk, NY). As the successful arrhythmia termination rates were unequally distributed, the Mann-Whitney U test was performed for the comparison of illumination and no illumination groups. Since optical mapping data were normally distributed ,comparisons were made by using the Student's T-test for unpaired data and the paired Student's T-test for paired data. Data were expressed as mean ± standard deviation, unless stated otherwise. Differences were considered statistically significant at $P < 0.05$.

Supplemental figure

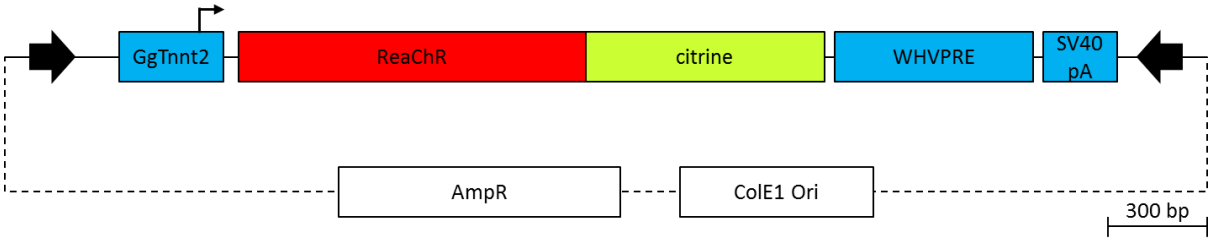


Figure S1 Map of the AAVV shuttle construct pDD.GgTnnt2.ReaChR~citrine.WHVPRE.SV40pA. Black arrows, AAV inverted terminal repeats. GgTnnt2, *Gallus gallus Tnnt2* promoter. ReaChR, coding sequence of red-activatable channelrhodopsin. Citrine, coding sequence of improved *Aequorea victoria* yellow fluorescent protein. WHVPRE, woodchuck hepatitis virus posttranscriptional regulatory element. SV40 pA, simian virus 40 polyadenylation signal. ColE1 Ori, bacterial origin of replication. AmpR, Escherichia coli ß-lactamase gene.

References of supplementary methods

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