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Systemic gene transfer enables optogenetic pacing of mouse hearts

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Aims

Optogenetic pacing of the heart has been demonstrated in transgenic animals expressing channelrhodopsin-2 (ChR2). However, for the clinical use of optogenetics to treat cardiac arrhythmias, gene transfer to non-transgenic hearts is required. The aim of this study was to describe a reliable method for gene transfer of ChR2 into a sufficient percentage of cardiomyocytes to overcome the electrical sink of all the coupled non-expressing cardiomyocytes during optical pacing of the whole heart *in vivo*.

Methods and results

Adeno-associated virus (AAV) with cardiac tropism for expression of ChR2 in fusion with mCherry was systemically injected into wild-type mouse hearts. Bright mCherry fluorescence was detected in the whole heart 4–10 weeks later. Single-cell dissociation revealed that on average 58% cardiomyocytes were mCherry-positive. These showed light-induced inward currents, action potentials, and contractions. Pulsed illumination of the left ventricle induced ventricular pacing *in vivo* in 74% of mice, and higher light intensities were required for reduced pulse duration or size of illumination. Non-responding hearts showed low AAV expression, and the threshold for optical pacing was estimated to be 35–40% ChR2-expressing cardiomyocytes. Optical pacing *in vivo* was stable over extended periods without negative effects on normal sinus rhythm and ECG parameters after termination of stimulation indicating sufficient cardiac output during pacing.

Conclusions

Gene transfer generates sufficient ChR2 photocurrent for reliable optogenetic pacing *in vivo* and lays out the basis for future optogenetic pacemaker and pain-free defibrillation therapies.

Keywords

Channelrhodopsin • Adeno-associated virus • Pacemaker • Optogenetic

1. Introduction

Optogenetics is a recently developed method that combines the expression of light-sensitive proteins and illumination of cells and organs for cell-specific, artefact-free electrophysiological or biochemical stimulation.¹ Illumination of excitable cells expressing the light-gated cation channel channelrhodopsin-2 (ChR2) with blue light leads to depolarization and subsequent generation of free-running action potentials.^{2,3} Optogenetic pacing of the heart *in vivo* was demonstrated in transgenic embryonic zebrafish and adult mice with ChR2 expression in all cardiomyocytes,^{4,5} but for future applications, gene transfer of ChR2 into native hearts is mandatory. It is important to consider that gene transfer will, in contrast to the transgenic animals, result in a lower percentage of

positive cardiomyocytes. Therefore, the inward photocurrents of ChR2-expressing cardiomyocytes have to overcome the electrical sink of the gap junctional-coupled non-expressing cardiomyocytes before an action potential, and ventricular pacing can be generated.⁶

Although computer simulations *in silico* have suggested effective optogenetic cardiac pacing after gene transfer,⁷ *in vivo* experiments are lacking. Therefore, we have explored the effectiveness of adeno-associated virus (AAV) for ChR2 gene transfer in non-transgenic mouse hearts. These viral vectors were chosen because of their safe and effective cardiac transduction also in humans.⁸ Furthermore, subtypes of AAV vector capsids are known to have a high tropism towards specific cell types enabling the selective transduction of the tissue of interest even upon systemic injection of AAV.⁹

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2. Methods

An expanded Methods section is available in Supplementary material online.

2.1 Intravenous injection of AAV

About 2×10^{11} gc (genome copies) of AAV9-CAG-hChR2(H134R)-mCherry virus provided by Penn Vector Core (University of Pennsylvania) diluted in 100 μ L of PBS were slowly (~ 10 s) injected into the left jugular vein of anaesthetized 10-week-old female CD1 wild-type mice under general anaesthesia by ventilation with 1.5% isoflurane and O_2/N_2O (50%/50%).

2.2 Optical pacing of the heart

Four to eight weeks after AAV injection, mice were anaesthetized by ventilation with 1.5–2% isoflurane and O_2/N_2O (50%/50%) and intubated; the ECG was recorded and light from a LED was focused with a macrocope (MVX 10, Olympus) through the open chest onto the left ventricular epicardium. Pulsed illumination was performed at frequencies 30–50 bpm above the intrinsic heart rate with various light pulse durations (2, 5, 10, 20, and 50 ms). The size of illumination was adjusted to 25.3 and 10.2 mm^2 using the zoom function of the macrocope, which yielded a maximal light intensity of 1.5 and 2.7 $mW\ mm^{-2}$, respectively. For each pulse duration and illumination size, the lowest light intensity was determined for reliable pacing with 1:1 coupling of light pulses to ventricular pacing in the ECG.

Individual rheobase and chronaxie values were obtained by fitting the strength–duration curve from each individual experiment with the Lapicque–Hill equation.¹⁰ Anatomical characterization of pacing thresholds in atria and ventricles were tested in Langendorff-perfused hearts. Some hearts were enzymatically dissociated and used to determine the percentage of ChR2-expressing cells. Therefore, brightfield and mCherry fluorescence images were taken with an AxioZoom.V16 macrocope (Zeiss), and mCherry-positive cells were counted and normalized to the number of all cardiomyocytes.

2.3 Single cardiomyocyte analysis

For single-cell dissociation, mice were sacrificed by cervical dislocation and hearts were enzymatically dissociated as reported earlier.⁵ Optical stimulation of single cardiomyocytes was performed with blue light (470 nm) from a temperature-controlled LED module (LEDMOD LAB 470 nm, Omicron Laserage), which was coupled with an optical fibre to the epifluorescence port of the microscope. Patch-clamp experiments were performed in the whole-cell configuration as reported earlier.⁵ Action potentials were elicited by 1 ms light pulses with an intensity of 5 $mW\ mm^{-2}$. Photocurrents were induced by 1 s long illumination with 5 $mW\ mm^{-2}$ and measured at a holding potential of -52.1 mV. Videos of beating isolated cardiomyocytes were recorded through a 20x Fluor objective (NA 0.75, Zeiss) at 20 frames per second during suprathreshold pulsed illumination (1 Hz, 1 ms pulse duration, 8 $mW\ mm^{-2}$).

2.4 Fluorescence quantification, histology, and IF staining

To determine the brightness of epicardial mCherry fluorescence, pictures with brief exposure time (8 ms) were taken in the diastole. Average pixel intensity was quantified in arbitrary fluorescence units (a.u.) with the ImageJ software (National Institutes of Health) and the ROI/Multi Measure plugin. Immunohistochemical staining of histological slices (Figure 1) was performed according to standard protocols as reported earlier⁵ and described in detail in Supplementary material online.

2.5 Statistical analysis

All data are shown as mean \pm SEM. Statistical analysis was performed using paired (Figures 2C, 3B, 4B and C, and 5B and C) and unpaired (Figure 3C) two tailed Student's *t*-test or two-way ANOVA multiple comparison test

(Figure 4A) and one-way ANOVA multiple comparison test (Figure 4D) both with the Bonferroni *post hoc* analysis, and values of $P < 0.05$ were considered statistically significant. The success rate of optogenetic pacing after a short (4–6 weeks) and long (6–10 weeks) incubation period was analysed with Fisher's exact test with a CI of 0.95.

3. Results

For gene transfer of ChR2, we have used AAVs that express ChR2 with the H134R mutation in fusion with the mCherry fluorescence protein under the control of the chicken- β -actin promoter. AAVs pseudotyped with viral capsids from serotype 9, which were proved to have a strong tropism towards cardiomyocytes in rodents,^{9,11,12} were systemically injected through the external jugular vein of CD1 wild-type mice.

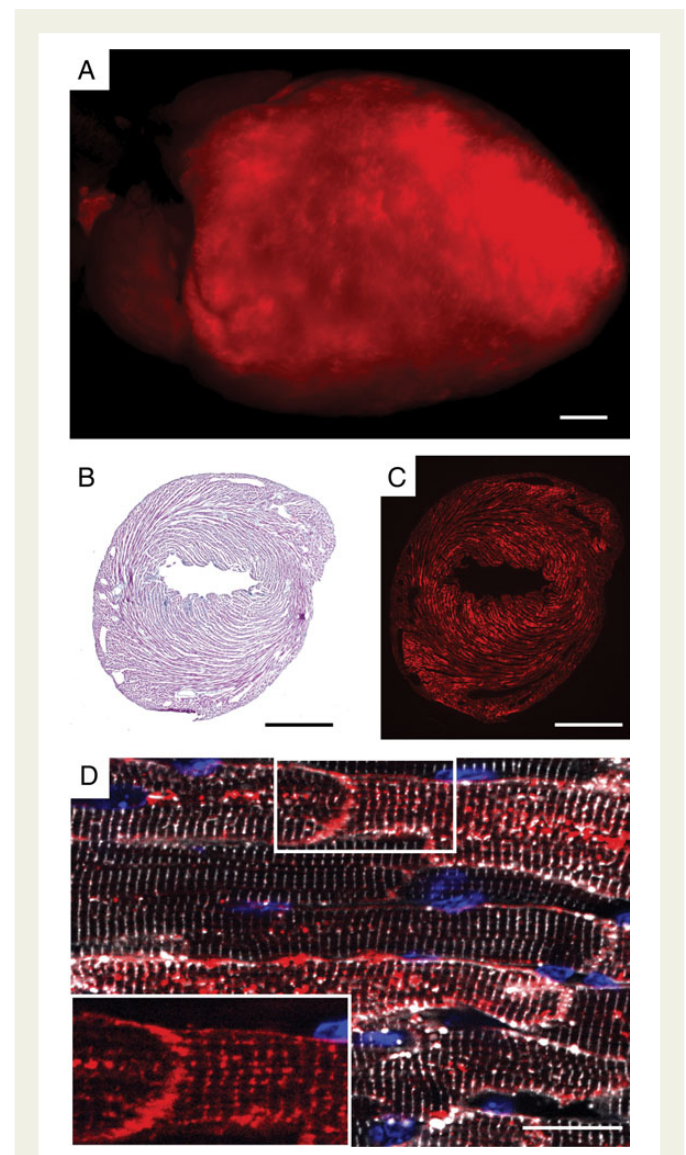


Figure 1 ChR2–mCherry expression in wild-type mouse hearts. (A) Epicardial view of the left ventricle with mCherry fluorescence. (B and C) Consecutive sections through the left ventricle with haematoxylin and eosin staining (B) and mCherry expression (C). (D) Membrane-bound ChR2–mCherry signals (red) in α -actinin positive (white) cardiomyocytes (nuclear staining in blue). Scale bars: 1 mm (A–C) and 20 μ m (D).

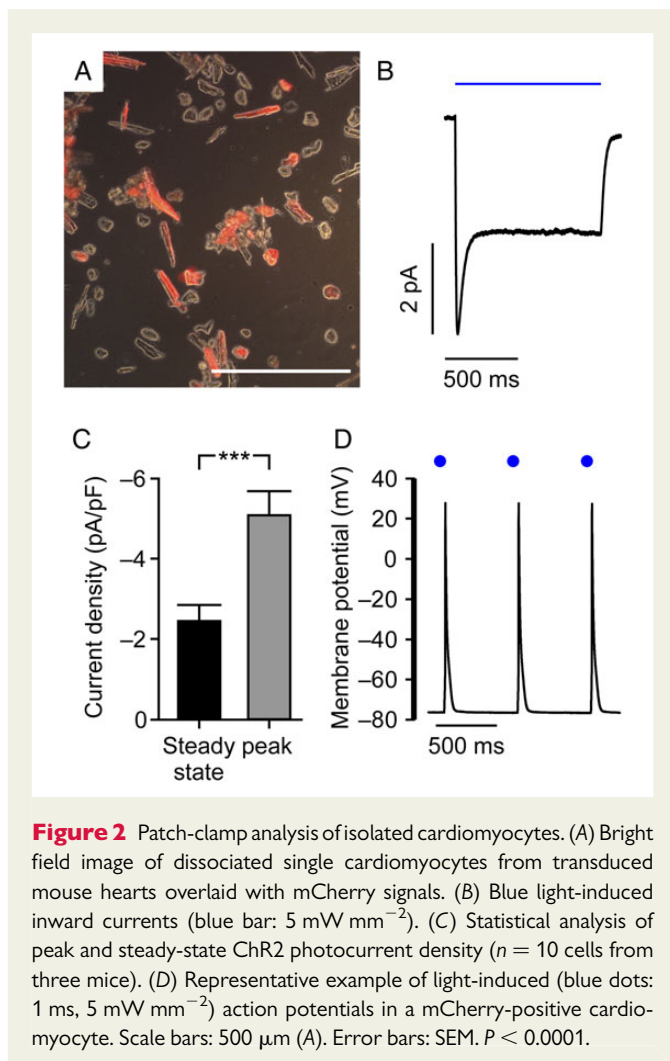


Figure 2 Patch-clamp analysis of isolated cardiomyocytes. (A) Bright field image of dissociated single cardiomyocytes from transduced mouse hearts overlaid with mCherry signals. (B) Blue light-induced inward currents (blue bar: 5 mW mm⁻²). (C) Statistical analysis of peak and steady-state ChR2 photocurrent density ($n = 10$ cells from three mice). (D) Representative example of light-induced (blue dots: 1 ms, 5 mW mm⁻²) action potentials in a mCherry-positive cardiomyocyte. Scale bars: 500 μ m (A). Error bars: SEM. $P < 0.0001$.

Four weeks later, epicardial images showed strong mCherry fluorescence in the whole heart (Figure 1A). The mCherry expression was stable over time because quantification of epicardial mCherry signals showed similar ($P = 0.49$) average pixel intensities after short (4–6 weeks: 1966 ± 338 a.u., $n = 9$) and long (6–10 weeks: 2352 ± 419 a.u., $n = 10$) incubation periods. In histological sections, no lymphocytic infiltration indicative of adverse immune response could be observed (Figure 1B), and mCherry fluorescence was uniformly distributed throughout the ventricular wall (Figure 1C) presumably enabling optical stimulation either from the endocardial or the epicardial side. Larger magnification showed mCherry signals in the plasma membrane of cardiomyocytes including the invaginations of the T-tubules (Figure 1D, insert). Neither vimentin-expressing fibroblasts nor vessel-like structures were mCherry-positive, proving cardiomyocyte-specific expression of ChR2 using the AAV serotype 9 (see Supplementary material online, Figure S1A). Screening of other organs revealed several mCherry-positive muscle fibres within the diaphragm and very few mCherry-expressing cells in the liver, but no expression in the lung, kidney, spleen, or brain (see Supplementary material online, Figure S1B–H).

Dissociation of hearts showed single cardiomyocytes with mCherry expression (Figure 2A) that could be paced with short light pulses (see Supplementary material online, Video S1). Patch-clamp analysis revealed a resting potential of -69.2 ± 2.6 mV ($n = 5$) and typical ChR2 photocurrents upon 1 s long illumination with peak and desensitization to

steady-state current (Figure 2B and C). Action potentials could be evoked by 1 ms short light pulses (Figure 2D).

To test whether the inward photocurrents in the ChR2-expressing cardiomyocytes are large enough to overcome the electrical sink of gap junctional-coupled ChR2-non-expressing cardiomyocytes, we analysed optogenetic pacing of the ventricle *in vivo* after gene transfer in anaesthetized and ventilated mice. This approach mimics the clinical setting much better than isolated hearts, because it also includes attenuation of the blue light by haemoglobin in red blood cells and hormonal (counter-) regulations.

Optogenetic pacing was performed at 30–50 bpm above the intrinsic heart rate and evoked ventricular stimulation identified by broad QRS complexes without preceding p-waves (Figure 3A and B) in the ECG in 73.7% of the tested hearts ($n = 19$). Quantification of epicardial mCherry expression in ventricles yielded significantly lower mCherry signals in the non-responding compared with the excitable hearts (Figure 3C), explaining the failed stimulation by reduced expression of ChR2. These variations could be due to pre-existent innate immunity against AAV, which is known to affect the efficacy of gene transfer in human and mice.^{9,13} Dissociation of excitable hearts and cell counting revealed an average of $58.2 \pm 6.5\%$ ($n = 5$) of mCherry-expressing cardiomyocytes (Figure 3C). Because one heart with low epicardial mCherry signals had 39% ChR2-positive cardiomyocytes and non-responding hearts showed only slightly weaker mCherry signals (individual data added in Figure 3C), the minimal percentage for optogenetic pacing is estimated to be in the range of 30–40% expressing cells. Importantly, a temporal decay of ChR2 expression can be excluded because the success rate of optogenetic pacing was similarly high after short (4–6 weeks: 66.7%, $n = 9$) and long (6–10 weeks: 80.0%, $n = 10$, $P = 0.63$) incubation periods.

To quantify the light intensity thresholds for stable optical pacing *in vivo*, we determined the minimal light intensity required for stable ventricular pacing at different pulse durations. When illuminating an area of 25.3 mm² with 20 or 50 ms long pulses, light intensities above 1.46 mW mm⁻² were sufficient for stable optical pacing in all excitable hearts (Figure 3D). Shorter light pulses or lower light intensities reduced the number of excitable hearts in a dose-dependent manner (Figure 3D), and overall we found that higher intensities were required when using shorter pulse durations (Figure 4A). Furthermore, when lowering the size of the illuminated area to 10.2 mm², significantly higher light intensities were required at all pulse durations (Figure 4A), indicating a larger influence of the electrical sink generated by the non-illuminated surrounding cardiomyocytes. The strength–duration curve from each individual heart was fitted with the Lapicque–Hill equation to determine rheobase (minimal light intensity for infinite pulse duration), which was significantly higher for the smaller area (Figure 4B) and to calculate the chronaxie (minimal pulse duration at double rheobase strength, Figure 4C). To compare the pacing thresholds in different anatomical locations, some hearts were explanted, perfused in Langendorff configuration, and stimulated at the right and left atrium, and at the left, septal, and right ventricle. Ventricular stimulation showed no significant differences in pacing thresholds (Figure 4D). The right atrium could be stimulated in three of four cases with similar pacing thresholds and stimulation of the left atria failed (Figure 4D).

To prove efficiency of long-term optogenetic pacing and exclude desensitization, we performed 10-min long suprathreshold stimulations, and found reliable pacing without single dropouts (Figure 5A). Importantly, after termination of stimulation hearts immediately returned to physiological sinus rhythm with unaltered heart rate, QRS

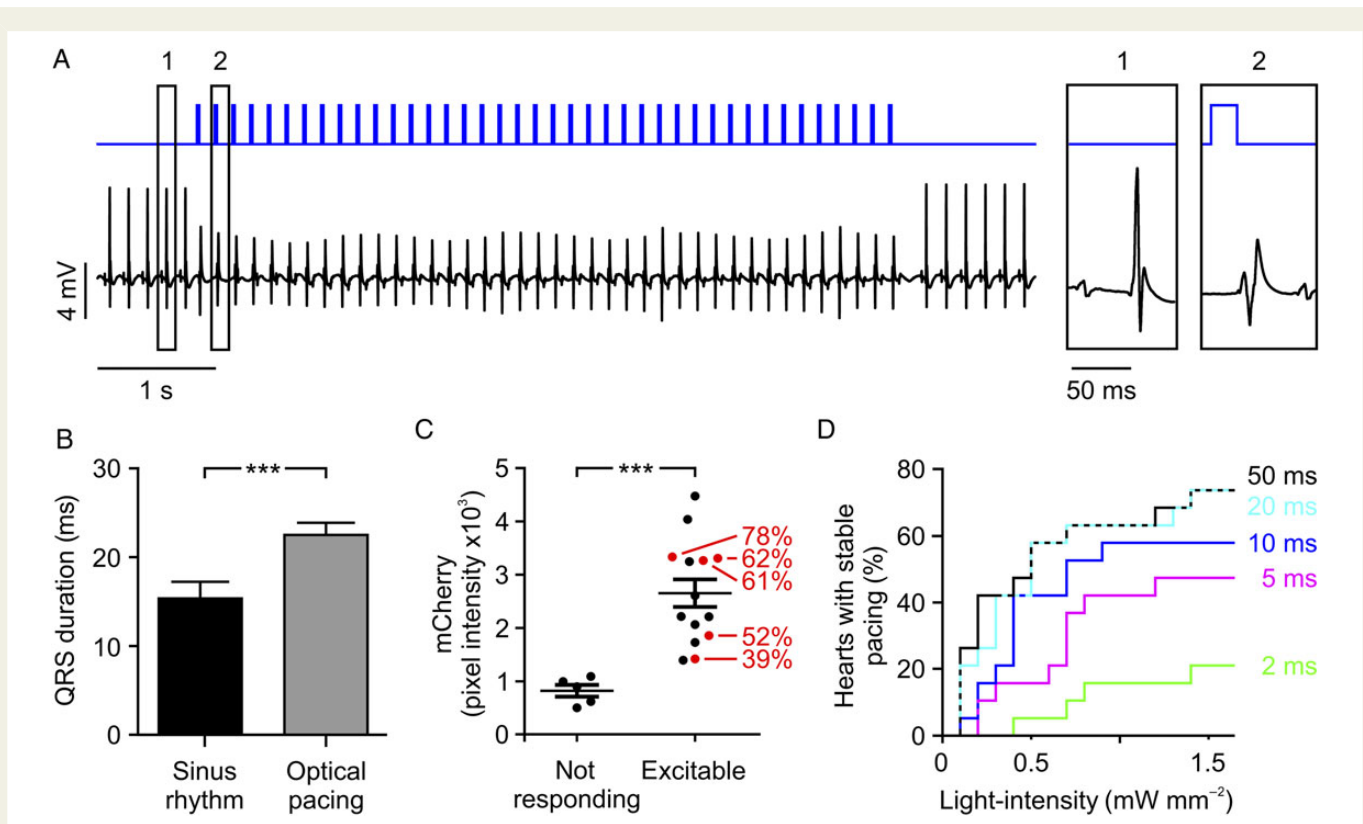


Figure 3 Optical pacing of hearts *in vivo*. (A) Pulsed illumination of the left ventricle (blue line: illumination area 25.3 mm², 0.52 mW mm⁻², 20 ms) and parallel recording of the ECG (black). Magnifications of the black boxes are shown on the right. (B) QRS duration in sinus rhythm and during optical pacing (20 ms, 25.3 mm², $n = 14$ mice, $P = 0.0002$). (C) Dot plot of epicardial mCherry average pixel intensities in excitable ($n = 14$) and non-responding ($n = 5$) hearts ($P = 0.0007$) with the percentage of mCherry-expressing cardiomyocytes (red). (D) Stimulation-response diagram showing the percentages of hearts with a 1:1 light-pulse to ventricular extra-beat coupling at the indicated light intensity and pulse duration ($n = 14$ mice, illumination area 25.3 mm²). Error bars: SEM.

durations, and PR intervals as before stimulation (Figure 5B and C). In addition, there were neither arrhythmias nor signs of cardiac infarcts, indicating that prolonged optogenetic stimulation generates sufficient cardiac output and intact oxygen supply to the sinus node and cardiomyocytes.

4. Discussion

Taken together, systemic injection of AAV induces sufficient ChR2 expression in native mouse hearts to achieve stable optical pacing *in vivo*. This AAV-based gene transfer of optogenetic tools will help to investigate basic physiological principles, regulation, and cell-cell interactions in the intact heart *in vivo*. Importantly, in contrast to transgenic animals, this approach provides a cheap, effective, and fast method that can easily be applied in larger animals like pigs or dogs with more similar cardiac physiology to humans. Furthermore, AAV-based gene transfer would enable optogenetic pacing in patients to treat cardiac arrhythmia, because clinical trials have shown safe, effective, and over 2 years long protein expression from AAV in the human heart.⁸

Compared with local gene transfer by using gene painting or direct intramyocardial injection of AAV, which requires risky thoracotomy or ultrasonic-guided injection, we believe that the intravascular systemic injection of AAV might be the safest and simplest approach, especially when reducing extra-cardiac expression using a cardiac-specific promoter.¹²

However, further technical developments are required for the clinical use of optogenetics in hearts of patients. Implantable light sources have to be used with sufficient light intensities. Similar to conventional electrical pacemakers, this could be achieved by coupling a LED to a light guide that can be placed in the endocardium through the venous system. Calculations predict less power consumption using optical pacing than electrical stimulation, because of the high efficiency of LEDs.¹⁴ This would prolong the life span of batteries reducing the need of pacemaker replacement surgery and could even enable piezo-electric energy harvester for self-powered cardiac pacemaking.¹⁵ In the future, the efficiency of optical pacing could be increased illuminating with longer wavelengths and the use of ChR2 variants with red-shifted excitation spectra^{16,17} leading to deeper tissue penetrance and reduced absorption by haemoglobin. Furthermore, injectable cellular scale optoelectronics that have been reported to generate sufficient ChR2 photocurrents in neurons¹⁸ could be injected directly into several regions of the myocardium. Alternatively, flexible and biocompatible integumentary membranes with several integrated LEDs¹⁹ could be wrapped around the heart. Both approaches would enable multisite pacing for cardiac resynchronization therapy, which is highly beneficial for patients with heart failure with wide QRS complexes.²⁰ In addition, multisite optogenetic depolarization could also be used for defibrillation of the heart, because continuous activation of ChR2 blocks electrical activity in the illuminated region *in vitro*.⁵ In contrast to the electrical shock of an implanted defibrillator, selective optogenetic

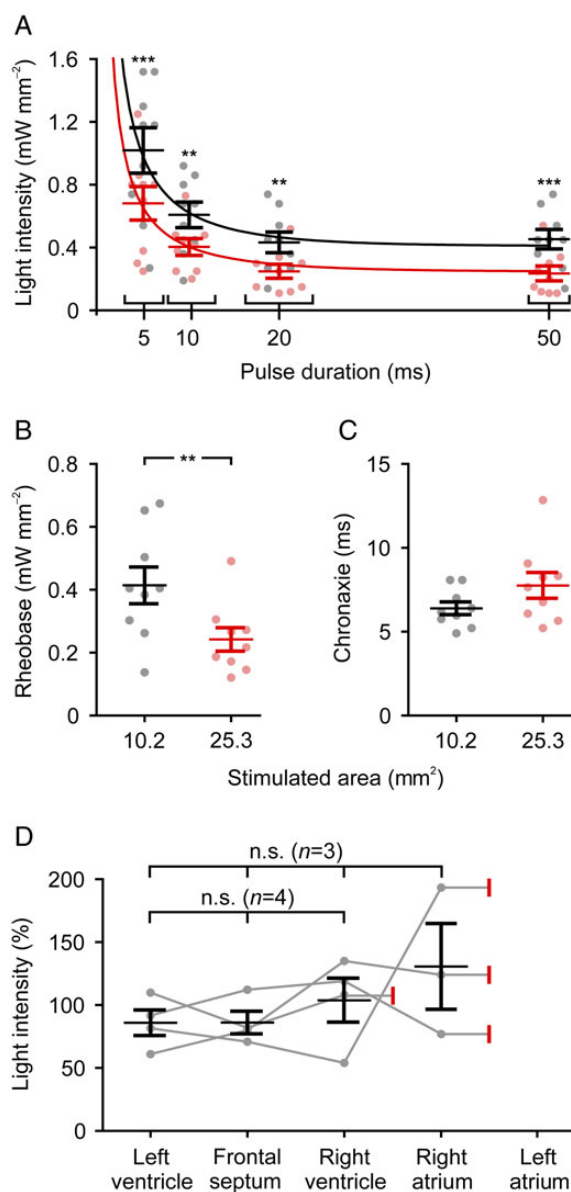


Figure 4 Strength–duration analysis of optical pacing thresholds. (A) Individual values, mean, and theoretical Lapicque–Hill fit of thresholds for stable ventricular pacing for two sizes of illumination (25.3 mm² in red, 10.2 mm² in black, $n = 9$ mice, $P \leq 0.005$). (B and C) Rheobase and chronaxie values from individual experiments. (D) Normalized pacing thresholds for 10 ms light pulses at 10.2 mm² in different anatomical regions of the heart ($n = 4$, $P > 0.05$). Note that one heart could not be paced from the right atrium and no heart could be paced from the left atrium (indicated by red dash). Error bars: SEM.

stimulation of cardiomyocytes would not affect sensory neurons or skeletal muscles, and thereby enable pain-free defibrillation. This would be a major step forward for the treatment of patients with therapy-resistant chronic forms of atrial fibrillation or ventricular tachycardia.

In summary, our study demonstrates that the high efficiency of systemic AAV injection results in sufficient ChR2 expression to allow the investigation of basic physiological principles in the non-transgenic heart *in vivo*. As a future perspective, our findings suggest that cell-specific expression of ChR2 in cardiomyocytes in combination with

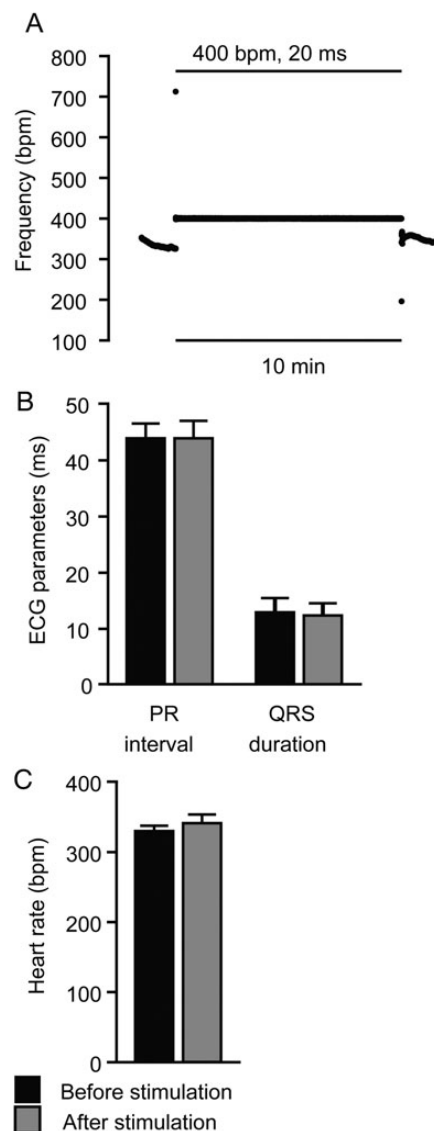


Figure 5 Effects of long-term optical pacing. (A) Frequency analysis during a 10-min long pulsed suprathreshold illumination (bar: illuminated area 10.2 mm², 1.5 mW mm⁻², 20 ms). (B and C) Statistical analysis of PR interval, QRS duration (B), and heart rate (C) in sinus rhythm before and after a 10-min long suprathreshold optical pacing ($n = 3$ mice, n.s.). Error bars: SEM.

the high efficiency of light stimulation may decrease side-effects during cardiac pacemaker therapy and even enable pain-free optogenetic defibrillation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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Conflict of interest: none declared.

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