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## Optogenetic investigation of cardiac arrhythmia mechanisms

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# Chapter

GENERAL INTRODUCTION AND  
OUTLINE OF THE THESIS

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## BACKGROUND

Heart rhythm disorders, also known as cardiac arrhythmias, are one of the major causes of morbidity and mortality in the world.<sup>1,2</sup> Such disorders affect the mechanical function of the heart, which could lead to a suboptimal distribution of oxygenated blood, nutrients, and regulatory factors to other organs. The clinical management of cardiac arrhythmias relies on several anti-arrhythmic therapies, *e.g.* drugs, ablation, and device implantation that, unfortunately, are often unspecific, irreversible and/or traumatizing, respectively.<sup>3-7</sup> Recently, to overcome these side effects, gene therapy has been introduced as an alternative option to treat cardiac arrhythmias. Its main advantage is, indeed, the possibility to modulate cardiac electrical function by specifically targeting the biological defect.<sup>8,9</sup> In this way, gene therapy can allow for a specific treatment that is based on the arrhythmic mechanism. However, gene therapy alone is lacking the possibility to gain precise spatiotemporal and quantitative control over a certain target. Interestingly, more recently, such precise control could be achieved with an innovative strategy, called optogenetics. Here, gene therapy is combined with optics allowing expression of light-activatable proteins that are specifically activate and deactivate by simply turning light on and off, respectively.<sup>10</sup> Among these proteins, there are, for instance, light-gated ion channels and light-gated pumps, which are normally expressed in algae and bacteria. Their transgene expression and light-activation may allow controlling of a specific biological function, like for instance the excitability of cardiac cells. In order to fully comprehend how optogenetics could be used to improve our understanding and thereby treatment and of cardiac arrhythmias, a concise description of the electrical function of the heart in healthy and diseased conditions is provided in the following paragraph.

### Electrical function in healthy and diseased conditions

The heart is a fascinating organ characterized by several cell types, *e.g.* atrial and ventricles cardiomyocytes, sinoatrial cells, atrioventricular cells, Purkinje fibers, fibroblasts, smooth muscle cells, and endothelial cells, that all together ensure normal cardiac function, *i.e.* electrical activation initiation and propagation, followed by synchronized mechanical activation.<sup>11</sup> In healthy condition, the electrical activation of the heart originates in the sinoatrial node, where so-called pacemaker cells spontaneously give rise to an action potential. This action potential is the result of a chain reaction based on the opening and closing of proteins that are located in the sarcolemma of the cardiac cells. In particular, voltage-gated ion channels are the ones that have a key role in shaping an action potential.<sup>12,13</sup> Those ion channels are pore-forming proteins that open and close in response to changes in the voltage and allow the selective passage of certain cations or anions according to their electrochemical gradient. This chain reaction is characterized by five phases. It starts with an initial depolarization that causes the physiological resting membrane potential to become less negative and activates the voltage-gated  $\text{Na}^+$  (sodium, inward current) channels if their threshold is overcome (phase 0, the upstroke). The consequent  $\text{Na}^+$  influx guides the rapid further depolarization of the sarcolemma that triggers the successive opening and closing of several  $\text{Ca}^{2+}$  (calcium, inward current) and  $\text{K}^+$  (potassium, outward

current) voltage-gated ion channels. First, the simultaneous inactivation of inward  $\text{Na}^+$  current ( $I_{\text{Na}}$ ) and activation of the transient outward  $\text{K}^+$  current ( $I_{\text{to}}$ ) guide the first repolarization of the membrane (phase 1, the early repolarization). Secondly, the balance between the inward currents ( $I_{\text{CaL}}$  and  $I_{\text{NaL}}$ ) and outward currents ( $I_{\text{Kur}}$ ,  $I_{\text{Kr}}$ , and  $I_{\text{Ks}}$ ) results in the transient plateau in membrane voltage (phase 2, the plateau). Finally, the delayed outward rectifying currents ( $I_{\text{Kr}}$  and  $I_{\text{Ks}}$ ), and the inward rectifying current ( $I_{\text{K1}}$ ) guide the repolarization and (phase 3, rapid repolarization) restore the cardiac myocytes resting state (phase 4, restoration on resting membrane potential). In this last phase, ionic pumps, like  $\text{Na}^+/\text{Ca}^{2+}$  exchanger also plays a role in the restoration of the resting state. An action potential propagates from the sinoatrial node to the cardiac myocytes of both atria.<sup>14</sup> Next, the electrical activation will propagate through the atrioventricular node, where it will slow down to create the critical delay between atrial and ventricular contraction, which is needed to allow blood emptying from the atria. Finally, the electrical impulse activates the cardiomyocytes of the ventricles, from apex to base, through a specialized conduction network consisting of the His-bundle, left and right bundle branches and the Purkinje network. Such propagation between cardiac cells relies on intercellular channels called gap junctions. Gap junctions consist of six hexamers of proteins called connexins that form transmembrane hemichannels (connexons) which connect to connexons of neighboring cells allowing the diffusion of small ions and small molecules.<sup>15,16</sup> Connexins can co-oligomerize with the same connexins and form homomeric connexons or with mixed connexins and form heteromeric connexons, although only certain combinations are permitted. The same is true for the connexon assembly.<sup>17</sup> Gap junction expression is tissue-specific and the ventricles mainly express connexin43 and connexin45, whereas connexin40, connexin43, and connexin45 are found in the atria and conduction system.<sup>18</sup>

In summary, in normal and healthy conditions, an action potential originates from the cells at the sinus node and propagates from the atria to the ventricles, followed by their mechanical activation.

In diseased conditions, *e.g.* ischemic heart disease, cardiomyopathy, coronary artery disease, the initiation and propagation of an action potential may become disturbed, thereby leading to cardiac arrhythmias.<sup>12</sup> Cardiac arrhythmias can be subdivided into bradyarrhythmias or tachyarrhythmias (on which this thesis is focused on), when the heart rate is either too low or too high, respectively. Bradyarrhythmias arise when impulse generation at the sinus node is abnormally slow or atrioventricular conduction is impaired. Tachyarrhythmias, such as atrial tachycardia, atrial fibrillation, ventricular tachycardia, and ventricular fibrillation, can originate from (i) abnormal impulse generation leading to automaticity or triggered activity and/or (ii) disrupted propagation that results in reentrant activation.<sup>19</sup>

Automaticity can be caused by enhanced normal automaticity or abnormal automaticity. Enhanced normal automaticity occurs when pacemaker cells, like those localized in the sinus node, give rise to an increased number of action potentials in time, due to an increased rate of spontaneous diastolic depolarization of the transmembrane potential. In those cells, the so-called funny current, also known as pacemaker current, is thought to have a major role in such depolarization.<sup>20</sup>

Abnormal automaticity, instead, originates from non-pacemaker cells when the resting potential is sufficient depolarized to induce spontaneous impulse initiation. Such automaticity can be caused by abnormal  $\text{Ca}^{2+}$  handling, activation, and inactivation of delayed rectifier  $I_k$ , and inward  $I_{\text{Na}}$  current through the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger.<sup>21</sup>

Triggered activity involves new impulse initiation that is caused by depolarizations that follow a preceding action potential. Such depolarizations are called afterdepolarization and can occur early during repolarization phase (early-afterdepolarizations, EADs), or late when the cells are back in their resting state (late-afterdepolarization, DADs).<sup>22,23</sup> EADs arise when the repolarization, during phase 2 or 3, is overcome by a transient shift of the net current towards an inward direction. Phase-2 EADs are mainly caused by  $I_{\text{Ca}}$ ,  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and late  $I_{\text{Na}}$ . Phase-3 EADs are, instead, caused by dysfunction of  $\text{Na}^+$  channels, *e.g.* failure to inactivate, increase of its narrow window current, and an increase in their recovery from inactivation. DADs are usually dependent on  $\text{Ca}^{2+}$  overload in the cytoplasm. Such increase activates the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger and the  $\text{Ca}^{2+}$  ions activate chloride channels that lead to depolarizing oscillation of the membrane potential that eventually will trigger a new action potential.

Regarding disturbed propagation, reentrant arrhythmias occur when an impulse propagates and re-excite cardiac tissue, giving rise to repetitive cycles.<sup>24</sup> Reentrant activity can propagate around an anatomical obstacle or a functional core defining in this way anatomical or functional reentry, respectively.<sup>24,25</sup> In an anatomically determined circuit, the wave-front and the wave-tail are separated by a zone of excitable tissue called, excitable gap. The inexcitable anatomical obstacles delineate a pathway that is fixed in length and location, giving rise to tachyarrhythmias that are characterized by a monomorphic electrocardiographic pattern. The initiation and maintenance of anatomical reentry rely on conduction velocity and refractory period, indicating that its extinction appears when the excitation wave-front encounters tissue that is not yet recovered. Functional reentry can acquire the form of a rotor that forms a dynamical organizing center supporting spiraling waves around an excitable, yet inexcited core. An important characteristic of such spiral waves is the phase singularity, where the wave-front and wave-tail meet each other. The wave-front is characterized by a curvature that progressively increases toward the core. At the phase singularity, the convex curvature reaches a critical value that makes it impossible for the activity to invade the core. A spiral wave can be stable or drift, meander and break-up giving rise to monomorphic or polymorphic electrograms, respectively. Such latter behavior can appear in the presence of heterogeneities, such as gradients in excitability and refractoriness.<sup>26,27</sup> Furthermore, a reentrant wave can make alternate transition between functional and anatomical reentry by pinning to or unpinning from an anatomical obstruction.<sup>28-30</sup> Such phenomenon, which depends on several factors (*e.g.* size of the obstacle and tissue excitability),<sup>29,30</sup> highlights the difficulty in identifying which type of reentry drives cardiac arrhythmia at a given time and space.

### Treatment of cardiac arrhythmias via gene therapy

As already mentioned, the available treatments for cardiac arrhythmias are far from optimal, often characterized by side effects such as limited specificity, pain, and permanent tissue

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damage.<sup>3-7</sup> Such limitations may be overcome by an alternative strategy, *i.e.* gene therapy. This strategy relies on three main strategies: gene transfer, gene silencing, and gene editing. Gene transfer aims to i) replace a missing or not functioning protein or ii) introduce a protein that is normally not expressed. Gene silencing, instead, is used to reduce the expression of a certain protein. Finally, gene editing aims to repair a DNA sequence to reestablish its functionality.<sup>8,9</sup> All gene therapy strategies need the selection of a vector and a delivery system. The vectors are needed to translocate genetic material into the targeted cells. Those vectors can be nonviral or viral. The nonviral vectors include plasmid DNA, which often forms complexes with other molecules that will improve the intracellular translocation.<sup>31</sup> Several studies have indicated how the percentage of genetically modified cells would increase when instead viral vectors were used.<sup>32</sup> Those viral vectors are viruses that have been mutated to inhibit their reproduction and the pathology associated with them. For the *in vivo* application adeno-associated viruses (AAV) seem to be the first choice.<sup>33,34</sup> These vectors, indeed, allow long-term gene expression with limited immune reactions. Furthermore, their small diameter allows easier penetration through the cardiac tissue. However, they cannot be always used since they cannot incorporate a transgene higher than 4.6 kb. For the *in vitro* application, the choice often goes towards the application of lentiviral (LV) vectors. Those vectors are characterized by a positive sense single strand linear RNA molecules that are reverse transcribed into cDNA that will allow integration of the transgene into the host genomic material allowing long-term gene expression. However, this latter property might cause insertional mutagenesis.<sup>35,36</sup>

The use of viral particles allowed genetic modification of large mammalian heart via myocardial injection, intracoronary perfusion, and atrial epicardial gene painting. Genetic interventions have been used, for instance, to suppress atrial fibrillation in pigs by kv11.1 channel inactivation, *i.e.* by prolonging the action potential duration and the effective refractory period. Such modification was achieved in two independent studies by epicardial gene painting or direct atrial injection, respectively.<sup>37,38</sup> In another study, AF was suppressed by increasing the expression of connexins 40 and 43, therefore increasing the conduction velocity across the atria.<sup>39,40</sup> Ventricular arrhythmias have been suppressed by gene therapy in rat and pig ischemic heart models. An ischemic event leads to a decreased level of ATP and, as a consequence, to a decreased activity of the ATP-dependent calcium pump, SERCA, leading to an increased  $\text{Ca}^{2+}$  intracellular concentration, which, as mentioned in the previous paragraph, can induce EADs. In these studies, the overexpression of such pump was able to reduce ventricular arrhythmias.<sup>41,42</sup>

However, such genetic modifications are lacking the full control over their quantitative activation in space and time. Such control might be achieved thanks to optogenetics that combines optics and gene therapy.

## Optogenetics

The term optogenetics, introduced for the first time by Deisseroth, refers to the combination of optical and genetic techniques. Such combination allows the expression of light-activatable proteins, named microbial rhodopsins, which activation can be precisely controlled in space,

time and quantity by light.<sup>10</sup> Bacteriorhodopsins, halorhodopsins, and channelrhodopsins are the microbial rhodopsins that have been intensively used in the optogenetic field. These proteins are usually expressed in organisms such as archaeobacteria and algae, where they exploit different functions. These functions range from the conversion of light into chemical energy or the motile reorientation away or towards a light source.<sup>43</sup> Structurally, these proteins are characterized by seven transmembrane  $\alpha$ -helices with the N- and C-terminus facing the extracellular or intracellular milieu, respectively. In all microbial rhodopsin a chromophore, *i.e.* all-trans-retinal, is attached by a Schiff base linkage to the  $\epsilon$ -amino group of a Lysine side chain in the middle of the helix 7. The retinal Schiff base is usually protonated, thereby determining the protein absorption into the visible light spectrum. When instead the Schiff base is unprotonated the absorption is shifted in the UV region. Beside the Schiff base protonation, the absorption spectrum is also determined by the chromophore-protein interactions such as electrostatic interaction with charged and polar amino acids.<sup>44</sup> Functionally, these proteins are activated by light of a specific wavelength that will isomerize the retinal from all-trans to 13-cis. Such isomerization will change the conformation of the proteins and allow the preferential passage of certain ions. Upon a light pulse, each of these proteins is characterized by an initial current,  $I_{\text{Peak}}$ , which decays to a steady-state current,  $I_{\text{Steady-state}}$ . Relaxation from  $I_{\text{Peak}}$  to  $I_{\text{Steady-state}}$  is commonly called desensitization. Finally, the off-kinetics are determined by the rate of channels closure at the end of the light pulse.<sup>45</sup> With the aim to control the membrane potential of excitable cells these proteins have been expressed in heterologous living cells and tissue. Channelrhodopsin-2 (Chr2) have been used to depolarize the membrane of cells while Halorhodopsins and Arceorhodopsins have been used to inhibit excitation by producing a hyperpolarizing current, based on chloride ions that are pumped in or protons that are pumped out, respectively.<sup>46-48</sup> Over the years several Chr2 mutants have been generated with the aim to improve biophysical properties of the channels, like the ion selectivity, kinetics, spectrum response properties.<sup>48</sup> This now opens new and unique possibilities for cardiac arrhythmias research based on optical modulation of excitability with superb spatiotemporal resolution.

## AIM OF THE THESIS AND SUMMARY

Therefore, the aim of this thesis is to employ the unique features of optogenetic for investigating the underlying mechanism of arrhythmia initiation, maintenance, and termination in order to identify novel biological anti-arrhythmic strategies. In **Chapter I** of this thesis a comprehensive description of rhythmic disturbances, gene therapy, and optogenetics is presented. In **Chapter II**, all the steps needed to successfully optogenetically modify atrial cardiomyocytes (aCMCs) are described, going from the isolation of native aCMCs, to the production of the LV particles, the use of the particle to optogenetically modify the aCMCs and finally the functional assay showing the possibility to pace those monolayers by light. In **Chapter III**, monolayers of aCMCs expressing the depolarizing tool CatCh (calcium translocating channelrhodopsin)<sup>48</sup> were used to investigate rotor termination by optogenetically blocking electrical activation at or near the rotor core region. The block at the rotor core region would mimic a new ablation strategy, that recently has been adopted in clinical settings, *i.e.* rotor guided ablation, and allow

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to explore its mechanism of termination. In **Chapter IV**, the CatCh-expressing monolayers were used to investigate the possibility to optogenetically control the spatial and temporal dynamics of a spiral wave. In this study *in vitro* experiments were implemented by *in silico* experiments. In **Chapter V**, CatCh was expressed in neonatal ventricular tissue slices to investigate optogenetic termination of anatomical reentry. Finally, in **Chapter VI** we exploited a different optogenetic tool in combination with patterned illumination to quantitatively exert spatial and temporal control over the production of reactive oxygen species (ROS) in monolayers of neonatal rat ventricular myocytes (NRVMs). This combination allowed to assess ROS effects on arrhythmogenicity.

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