Application of in-silico and in-vitro optogenetic tools to cardiac arrhythmia research

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CHAPTER 1

General Introduction and Outline of the Thesis

1 General Background

Cardiac arrhythmias, often colloquially referred as heart rhythm disorders , represent one of the main sources of mortal incidents in the modern industrialised world[1,2]. Arrhythmias negatively impact the cardiac mechanical function, which might cause inefficient delivery of blood to other organs with a cascade of negative outcomes. Despite their utmost importance, the fundamental mechanisms of cardiac arrhythmias remain insufficiently understood thus hindering further progress in improving clinical treatment of these disorders[3]. The main challenge in studying cardiac arrhythmias experimentally, both *in-vitro* and *in-vivo*, lies in the enormous number and complexity of biological parameters regulating the functioning of cardiac cells and the lack of convenient tools for spatiotemporal control of cardiac electrical activity. A decade ago, a technique called optogenetics was introduced[4], which provided a solution to the latter problem. Optogenetics allows to control specific biological function by light and leverage power of optical equipment to achieve precise spatiotemporal control.

Another way to curb the issue of complexity of cardiac systems is to utilize computational models in combination with mathematical theories of excitable media[3,5]. Modelling offers full flexibility to change parameters of a physiological system, thereby allowing to test theories in a time- and cost-effective manner without performing real experiments. Theory of excitable media can help to mathematically generalize results beyond the limitations of a particular model[5]. Such a simplification approach is particularly valuable since it allows to translate results across experimental species thus further defying biological complexity. As an additional step to deal with biological complexity[6], computational modelling, theory and optogenetic experiments can be combined to discover new mechanisms of cardiac arrhythmias. To sketch how optogenetic experiments can be combined with computational modelling and the theory of excitable media to enlarge the understanding of cardiac arrhythmias, the following paragraphs are dedicated to a general description of the heart in health and disease, an overview of cardiac modelling and the theory of excitable media and the latest relevant findings in cardiac optogenetics.

2 Mechanistic aspect of cardiac arrhythmias

The heart is an organ of immense complexity comprised of many different cell types, the most notable of which are ventricular and atrial cardiomyocytes, cardiac Purkinje and nodal cells, endothelial cells, vascular smooth muscle cells and cardiac fibroblasts. These cell types co-operate in highly organized structures to carry out the ultimate function of the heart — coordinated contraction for circulation of blood[7]. The electrical system of the heart plays a paramount role in this task by means of regulating electrical activation leading to synchronized contraction of first the atria and then the ventricles. The electrical activation of the heart starts at the sinoatrial node where pacemaker cells drive the rhythm of the heart by autonomously generating an action potential, which propagates to the rest of the heart. The action potential is a biophysical process governed by ion channels, exchangers and pumps located in the plasma membrane of the cardiomyocyte. Voltage-gated ion channels are major determinants of the action potential[8]. They are comprised of pore-forming proteins, which open or close upon a change in transmembrane potential (voltage) of the cardiomyocyte. The opening events allow selective passive flux of cations or anions following the electrochemical gradient. The action potential is usually described by 5 phases. First, initiating depolarization forces the membrane potential to elevate from its resting state and, after crossing the threshold of activation, results in activation of voltage-gated sodium channels causing an inward influx of sodium ions (I_{Na}) with rapid depolarization of the plasma membrane culminating in phase 0, the upstroke. The rapid depolarization causes opening and closing of several calcium and potassium voltage-gated ion channels. At the beginning, the synchronous inactivation of inward sodium current (I_{Na}) and activation of the transient outward potassium current (I_{to}) force the first repolarization, also designated as phase 1, the early repolarization. Next, the sum of inward currents (L-type I_{Ca} and late I_{Na}) and outward currents (I_{Kur} , I_{Kr}, I_{Ks}) produce the transient plateau phase (phase 2). Eventually, the delayed outward rectifying currents I_{Kr} and I_{Ks} together with the inward rectifying current I_{K1} and the inward current generated by the Na^+/Ca^{2+} exchanger force further repolarization (phase 3, late or rapid repolarization) thereby bringing the cardiomyocyte back to the resting state (phase 4, return to resting membrane potential). In a normal healthy sequence of electrical activation, an action potential travels from the sinoatrial node both to the left and right atrium exciting cardiomyocytes there. Then, the wave of excitation propagates through the atrioventricular node where it decelerates. This creates a delay between atrial and ventricular contraction allowing efficient transfer of blood from the atria to the ventricles. Finally, the wave of excitation spreads to the ventricles exciting ventricular cardiomyocytes from apex to base via the His-bundle network and Purkinje fibers. The resulting contraction of the left and right ventricle pumps the blood into the systemic and pulmonary circulation, respectively. The spread of action potentials between cardiomyocytes requires intercellular

channels known as gap junctions. These gap junctions, which are composed of two pore-forming assemblies of six connexin molecules present at the apposed plasma membranes of two neighboring cells, allow diffusion of ions and small molecules between cells ensuring electrical coupling[9]. Overall, in healthy conditions, action potentials arise from pacemaker cells in the sinus node and spread from the atria to the ventricles causing coordinated mechanical contraction. Under diseased conditions the generation and spreading of action potentials might be disturbed giving rise to cardiac arrhythmias[10]. Cardiac arrhythmias are broadly categorized into bradyarrhythmias and tachyarrhythmias, which refers to conditions with an abnormally low and high heart rate, respectively. Bradyarrhythmias are caused either by an abnormally slow pace of impulse generation at the sinus node or by impaired atrioventricular conduction. Tachyarrhythmias, among which are atrial and ventricular tachycardia and atrial and ventricular fibrillation, can originate from (i) ectopic activity or (ii) reentrant waves. Ectopic activity is a focal concentric activation of an excitation front, which originates from non-pacemaker cells outside the sinus node. Ectopic activity can occur due to abnormal automaticity or triggered activity. Abnormal automaticity might be caused by collective mechanism due to coupling between depolarized non-pacemaker cells (e.g. cardiac myofibroblasts) and healthy cardiomyocytes creating injury currents[11,12]. Such collective mechanisms are the focus of chapters III and IV of this thesis. At the single-cell level, abnormal automaticity might arise due to abnormal Ca_{2+} dynamics, inactivation and activation of delayed rectifier IK or disturbances in Na^+/Ca^{2+} exchanger activity[13]. During triggered activity, a new excitation pulse is initiated, which depends on the preceding action potential for its generation. This type of re-excitation is caused by a single-cell mechanism called afterdepolarization. Afterdepolarizations can originate early in the repolarizing phase manifesting so-called early-afterdepolarizations, EADs or after repolarization of the cells to the resting state demonstrating delayed-afterdepolarizations, DADs[14,15]. EADs occur during phase 2 or 3 of repolarization if an abnormal change in inward currents overpowers net repolarizing currents. Phase-2 EADs might be attributed to Na^+/Ca^{2+} exchanger, I_{Ca} and late I_{Na}. Phase-3 EADs are often connected to inactivation failure of sodium channels. DADs are often the consequence of calcium overload with the subsequent activation of the Na^+/Ca^{2+} exchanger and activation of chloride channels by calcium ions[16,17]. Reentrant arrhythmias arise if an excitation pulse re-excites cardiac tissue in a repetitive and self-sustained manner10. Such pulses of re-excitation can rotate around an inexcitable anatomical obstacle or a so-called functional core[3,10,18], representing anatomical and functional reentry, respectively. In case of an anatomical obstacle, its geometry together with the conduction velocity, refractory period and restitution characteristics of the tissue determine the pace of reentry as well as its initiation, stability and maintenance properties[3]. Functional reentry constitutes of rotating spiral wave, which circulates around an excitable core. The core of an spiral wave remains unexcited throughout rotations since the con-

vex curvature of the wavefront is maximum near the core making it impossible for the wavefront to propagate towards the core due to so-called sink-source relationships[3]. Overall, sink-source relationships play an important role in arrhythmia induction and maintenance as illustrated in chapter II and III of this thesis. Sink-source relationships are pertinent not only to cardiac systems but to excitable systems in general . The general description of an excitable medium can be viewed as a system comprised of interconnected elements, which can be either in a resting or an excited state. Most notable examples of such excitable elements are neurons, cardiac cells or units of far-from-equilibrium chemical reaction[19]. If an excitable element is stimulated above a certain threshold, it transitions from a resting to an excited state. After dwelling in the excited state for some time when no other stimulus can excite the element anew, it returns to the resting state and can become re-excited by a suprathreshold perturbation. An excitable element can trigger an excitation in neighbouring elements by means of spatial coupling, e.g. via diffusion in case of a chemical reaction or via gap junctions in case of cardiomyocytes. This can elicit a wave of excitation throughout an excitable medium thereby synchronizing the state of the elements. Propagating waves of excitation have been observed and studied in various physical, chemical and biological systems beyond the heart, among which are the Belousov-Zhabotinsky reaction[19], the oxidation of carbon monoxide on platinum surfaces[20], waves of cAMP in the social amoeba Dictyostelium discoideum[21], waves of electrical activity in the neocortex of the brain[22], waves of Rho activity and F-actin polymerization in the cell's cortical actin cytoskeleton[23]. The generality of qualitative behaviour between these completely different systems allows to formulate a generic theory of excitable systems using simple mathematical models.

3 Computational modelling of cardiac tissue and theories

Computational modelling of the heart has achieved immense progress over the years and has become an important tool in guiding biological experiments. Cardiac electrophysiological models can be divided into two categories: detailed physiological cardiac models and general models of excitability. Detailed physiological models are typically based on Hodgkin and Huxley equations for the description of ionic currents[3,24] thereby limiting the system only to the reproduction of electrophysiological phenomena. The detailed physiological cardiac models can be further expanded to achieve a more fine-grained level of description of underlying biophysical processes. For example, some models implement physically more realistic Markov chain models based on stochastic switching of ionic subunits between conformation states instead of Hodgkin Huxley models[3], others reproduce the detailed shape of cells and mechanical forces during tissue formation[25] or mechanical feedback during cardiomyocyte excitation[26]. Physiologically detailed models allow to test multiple hypotheses

without performing costly *in-vitro* or *in-vivo* experiments. Detailed physiological cardiac models help to precisely determine the operational range of parameters to be tested *in-vitro* or *in-vivo* avoiding the waste of limited/expensive laboratory reagents and unnecessary use of laboratory animals . However, despite the large number of inbuilt details, these models cannot capture the full degree of biological complexity and simplifications are inevitably made. Also, building complex models requires huge amounts of experimental data and they always need to be experimentally validated. Also such models are computationally expensive and hard to study by analytical mathematical tools. On the contrary, general simple models of excitability, e.g. the FitzHugh-Nagumo model[27], are amenable to mathematical analysis. Given the generality of the dynamics of excitable systems, qualitative lessons learned from cardiac dynamics of one species can be applied to cardiac dynamics of another species. Also, simplified models allow application of the framework of nonlinear dynamics (chaos theory)[3,28], enabling a further generalization of the results of simulations and a faster prediction of the outcomes of experiments. Furthermore, such theories can help to distinguish between purely biological effects related to single-cell behaviour (such as remodelling of particular ion channels) versus collective emerging effects which cannot be attributed to single-cell dynamics and are a property of excitable systems in general. Such collective emerging effects can be pertinent to a wide variety of physiological parameters regardless of the precise biological details thus further improving the predictive power of a model[3]. The technique of optogenetics , first introduced in the context of neuroscience[4], leverages the joint power of genetic engineering and advanced optical techniques. This combination entails expression of light-sensitive proteins in either *in-vitro* or *in-vivo* systems, where such proteins can be activated by light at preselected locations within the sample, at a predetermined time and for a predefined duration while the degree of activation can be adjusted by changing the light intensity. In the field of optogenetics, depolarizing and hyperpolarizing optogenetic tools are the most heavily used with the goal to control transmembrane potential of excitable cells. The nonselective cation channel Channelrhodopsin-2 (ChR2) has been utilized for membrane depolarization, while Archearhodopsins (i.e. outward proton pumps) and Halorhodopsins (i.e. inward chloride ion pumps) as well as chloride channels were used for membrane repolarization[29–31] . Development of new ChR2 derivatives has been a major subject of improvement over the years yielding enhanced/altered ion selectivity, increased photocurrents, different spectral properties and kinetics[32,33] . However, a plethora of different types of, optogenetic tools have also emerged such as optogenetic reactive oxygen species generators[34] or cell signaling tools[35,36]to control cell physiology. In the field of cardiology, optogenetic studies can be divided into two categories: applied studies with the focus on cardiac pacing or termination of cardiac arrhythmias by optogenetics or fundamental biophysical research which seeks to use optogenetics as a research tool to gain new mechanistic insights[31]. Currently, optogenetic *in-vivo* and *ex-*

vivo termination of cardiac arrhythmias is well-established for rodent models and has been performed in a variety of experimental settings[37,38]. For both applied and fundamental studies light-emitting diodes (LEDs) are the most frequently used light source due to their cost-effectiveness and the ease with which their light output can be controlled. For detailed *in-vitro* studies, patterned illumination is often used allowing precise computer-programmed spatiotemporal control. *In-vitro* studies are often complemented by modelling studies with incorporation of channelrhodopsin properties into the electrophysiological cell model[6], where precise spatiotemporal control is simply programmed into the parameters of the model. Such combination of *in-vitro* and *in-silico* studies allowed to directly confirm old theories and formulate new hypotheses. Most importantly, known facts about spiral wave dynamics were reasserted with a higher degree of precision and control by optogenetics. First, it was proven clearly that the area of conduction block must connect the core of a spiral wave to the border of the domain in order to terminate reentrant electrical activity[39]. Another study demonstrated an attract-anchor-drag strategy to steer the tip of one or more spiral waves along a predefined path[40] thereby confirming the hypothesis of spiral wave steerability only by impacting its core[41]. In addition, a recent study[42] confirmed a decades-old theory of spiral wave drift[43] along the border of areas with different excitability by imposing regional subthreshold illumination. Also various new hypotheses were tested. Evidence was provided that increases in cardiac alternans are responsible for termination of arrhythmias under subthreshold optogenetic illumination conditions[44]. On the other hand, it was shown that global low-energy (i.e. subthreshold) optogenetic stimulation can induce arrhythmias[45]. Also with the aid of optogenetic pacing, the geometry of multicellular domains was proven to directly influence single-cell dynamics causing arrhythmic conditions only in some geometries even though all geometric configurations consisted of the same cellular substrate[46]. More indirect use of optogenetics was made by performing experiments demonstrating general properties of biological electrical tissue. So-called topological action potentials were shown at the interface of non-excitable tissues[47], biologically controlled oscillators have been proposed to be used as information storages devices[48] and formation of the domains with different transmembrane potentials was detected in bioelectric tissue[49].

4 Aims of the thesis and summary

The aim of this thesis is to discover new fundamental biophysical properties of excitable media, in particular cardiac tissue, using a combination of *in-vitro* optogenetic experiments, tissue engineering, numerical modelling and analytic mathematical tools, namely nonlinear dynamics and bifurcation theory. **Chapter I** presents an overview of cardiac arrhythmias, *in-vitro* optogenetic and computational approaches for studying cardiac arrhythmias. In **Chapter II**, the novel phenomenon of cardiac arrhythmic activity originating from

”dormant” reentrant sources is described. The arrhythmic activity from these ”dormant” sources is released once the properties of the medium are changed. The general biophysical plausibility of this phenomenon is proven *in-vitro* in optogenetic experiments with neonatal rat atrial cardiomyocytes and corroborated by 2D computational modelling. The possible translational implications of the findings are illustrated *in-silico* in a detailed 3D anatomical model of human atria. While the underlying biophysical principles are well-known and the outcome of their combination is fully predictable by the general theory of excitable media, the possibility that local reentrant circuits can serve as a source of global arrhythmias represents a novel mechanism of cardiac arrhythmogenesis. **Chapter III** describes the fundamentally novel biophysical phenomenon of ectopic wave ignition from high-curvature boundaries of depolarized zones in optogenetics experiments with monolayers of neonatal rat ventricular myocytes (NRVMs). This phenomenon contradicts the so-called general sink-source mismatch theory, which is valid for a wide range of excitable systems beyond cardiac tissue. As such, it represent a novel nonlinear mechanism in generic reaction-diffusion systems. To explain this counterintuitive phenomenon, numerical simulations were performed in generic and electrophysiologically detailed models of excitable media. On top of this, mathematical tools from quantum mechanics were used to analyze and explained the results. In **Chapter IV** another novel generic and biophysical phenomenon is discovered, namely the occurrence of resonance during the induction of ectopic activity. We demonstrate this phenomenon in *in-vitro* optogenetic experiments with NRVMs expressing the depolarizing optogenetic actuator CheRiff. To gain mechanistic insights, experiments were corroborated by computational modelling and the results of both were analyzed by techniques of nonlinear dynamics. This analysis proved the generality of the results, which allowed us to extend the applicability of the phenomenon to various situations of ectopy generation in cardiology and to resonance phenomena in other systems, e.g. in neuronal networks. Finally, in **Chapter V** the generic properties of NRVMs as an excitable medium on tissue-engineered recombinant spidroin constructs are investigated. We clearly show that such constructs allow growth of confluent NRVM layers, which support uniform propagation of excitation waves. This proves that such spidroin constructs can be used as fully biological matrices for cardiac tissue engineering.

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