**Report for the ESC First Contact Initiative Grant**

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## Initiation of the Collaboration

I would like to thank the ESC Council on Basic Cardiovascular Science (CBCS) for selecting me as a winner of the *First Contact Initiative Grant*.

Thanks to this scholarship, I was able to visit the laboratory of Prof. Thomas Brand, Chair in Developmental Dynamics in the aforementioned host institute. This visit was divided in two parts. Initially, I visited the laboratory of Dr. Brand for three days in September 2015. During this visit, Dr. Brand showed me his laboratory and gave me an update on his line of research on the Popeye domain-containing (POPDC) proteins in determining the structure and function of cardiac and skeletal muscle. During this initial visit, I also had the opportunity to present my recent data on pacemaker activity of the sinoatrial node in sodium-calcium exchanger knockout mice in a talk organized at the Imperial Centre for Translational and Experimental Medicine (ICTEM). I also met with various researchers of the Section on Cardiovascular Function, which provided me with the opportunity to develop new contacts and to learn about the exciting research environment at this institution. After this initial contact, I planned together with Dr. Brand a second longer visit for a whole week that was finally set for October 2016. For this second visit, we planned several experiments, which focused on the role played by POPDC proteins in the control of sinoatrial node (SAN) pacemaking using the mouse *POPDC1* null mutant as a model. My results constitute an interesting extension of the initial data obtained by Dr. Brand and will be likely used for a grant application to the Medical Research Council and form the basis for a joint publication. Moreover, thanks to these initial contacts, we are now establishing a long-term collaboration between my current laboratory (Dr. Mangoni, Institute for Functional Genomics in Montpellier) and Dr. Brand's group, to merge my competence to study cardiac pacemaker activity and the wealth of knowledge of Dr. Brand in his functional studies of POPDC proteins.

## Results

**Introduction:** POPDC genes encode a family of membrane proteins abundantly expressed in cardiac and skeletal muscle (1). Three isoforms, POPDC1 (also known as BVES) POPDC2 and POPDC3 display differential and complementary expression domains in the atria, ventricles and the cardiac conduction system (2, 3). All three isoforms are present in the SAN and atrioventricular node (AVN), with Popdc2 presenting higher level of expression in the nodes compared to the surrounding atrial myocardium. POPDC proteins have three transmembrane domains. In the cytoplasmic part of the proteins, an evolutionary conserved Popeye domain is present, which functions as a cAMP-binding domain system (3). While the Popeye domain structurally resembles other cAMP-binding domains, the protein sequence is highly divergent in particular in the phosphate-binding cassette (4). Proteomic analysis to identify interaction partners yielded a large list of proteins able to bind to POPDC proteins, including Ankyrin B and G, SCN5A SCN4A, TREK1, NCX, NKA, CAV3, dystrophin, and dysferlin. Some of these proteins have been shown to be important for the pacemaker mechanism of the SAN (5, 6). It has been proposed that POPDC proteins controls membrane trafficking of electrogenic proteins in SAN myocytes (3). In agreement with this hypothesis, null mutants of *Popdc1* and *Popdcc2* develop a stress-induced bradycardia in an age-dependent manner. This phenotype in mice is similar to the sinus bradycardia and atrioventricular block discovered in patients carrying point mutations in *POPDC1* and *POPDC2* (7). Furthermore, knockdown of *popdc1, popdc2*, and *popdc3* genes in zebrafish caused different degrees of AV-block, sinus exit block, cardiac arrhythmia and heart failure (8).

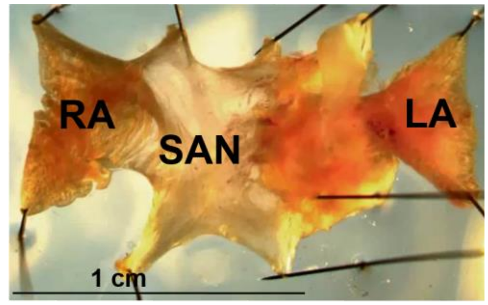


**Figure 1**: Anatomical structure of the sinoatrial preparation. SAN = Sinoatrial node, RA=right atrium, LA= Left atrium.

**Methods and results:** To directly measure the pacemaker rate in the intact SAN we used 2D-high-speed confocal microscopy. The sinoatrial preparation was dissected from the heart, and opened through the superior and inferior venae cavae. Subsequently, the tissue was gently pinned on a Sylgard-coated dish, to expose the SAN region (Fig. 1). The entire sinoatrial preparation was loaded for 45 min with the $Ca^{2+}$ dye Cal520-AM. $Ca^{2+}$-release was recorded under constant warm perfusion (35-36 C°), at 10x magnification to visualize a large part of the SAN. A sequence of $Ca^{2+}$ transients as a representative of the spontaneous rate of the SAN was obtained by averaging the fluorescence intensity derived from $Ca^{2+}$-release in the time series (Figs. 2, 3) These Ca transients occurred simultaneously throughout the area of tissue under observation, and their spatial average showed rapid upstrokes, consistent with transients elicited by depolarization and corresponding to action potentials (AP). Moreover, this technique also allowed the detection of variations of the diastolic $Ca^{2+}$-levels (see below Fig. 5).

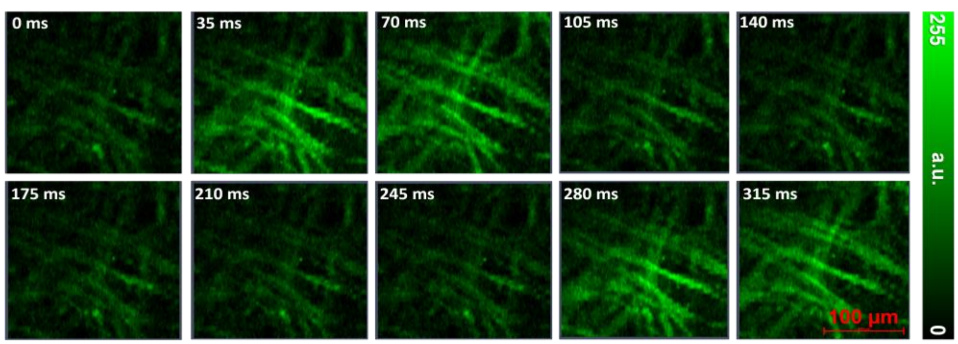


**Figure 2**: Time series of WT SAN tissue loaded with the $Ca^{2+}$ indicator Cal520 AM

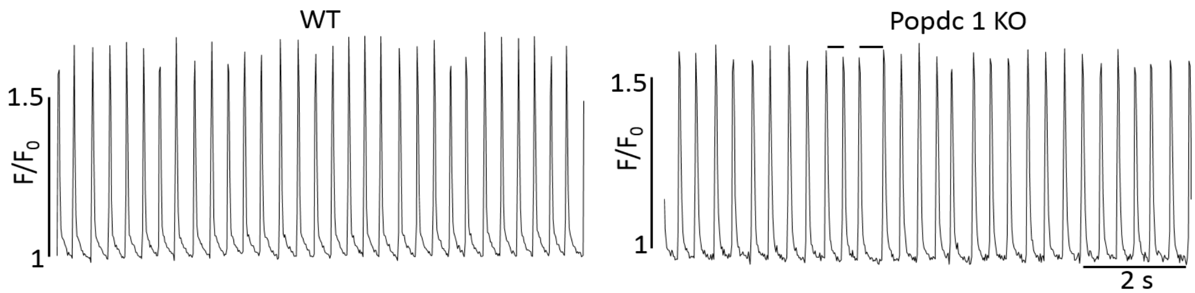


**Figure 3**: Examples of $Ca^{2+}$ transients recorded in WT and *Popdc1* KO SAN tissue under baseline conditions. Bars demarcate irregular pacemaker activity in mutants.

Previously, by electrocardiogram (ECG) recording on *Popdc1* and *Popdc2* null mutants has revealed sinus pauses to cause impaired heart activity after stress which developed in an age-dependent manner (3). We have now observed with the sinoatrial explants, pacemaker impairment even under baseline conditions (Tyrode's solution) in young adult *Popdc1* null mutants (KO). This difference in the timing and condition might be related to the increased sensitivity achieved in sinoatrial explants, which may not be detectable by ECG analysis. We started recording SAN activity in *Popdc* 1 KO mice and

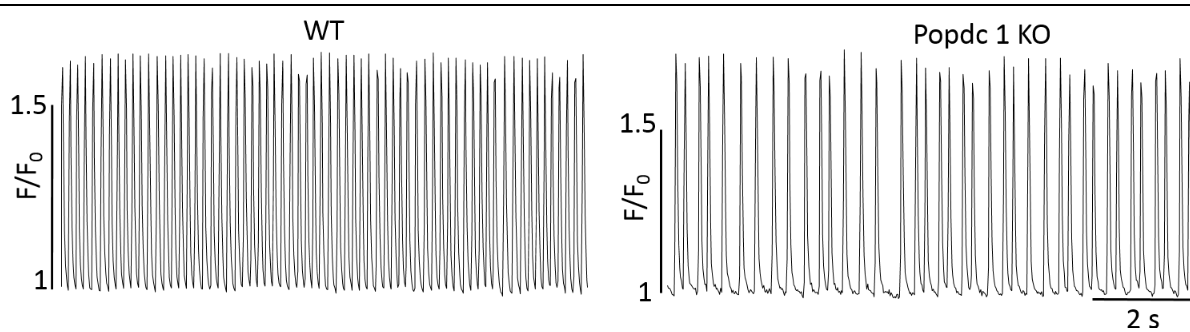


**Figure 4**: Examples of $Ca^{2+}$ transients recorded in WT and Popdc 1 KO SAN tissue under ISO 1 µM stimulation

WT littermates, aged between 4 and 5 months. As previously reported (9) WT sinoatrial explants (n=3) showed a very regular sequence of $Ca^{2+}$-transients (Fig. 3, left panel) with an average rate of 257±53 bpm. A similar rate of 235±44 bpm was recorded in *Popdc1* KO (n=4). Nevertheless, a slightly irregular pattern, consistent with the alternation between short and long inter-spike intervals, appeared in the latter mice (Fig. 3 right panel). Indeed, while no SAN pauses were recorded in *Popdc1* KO tissue, the coefficient of variability of their $Ca^{2+}$ transient frequency, tended to be higher than in WT (0.13±0.03 vs. 0.09±0.02).

As expected stimulation of the β-adrenergic pathway with the agonist isoproterenol (ISO, 1 µM) increased the rate in WT and induced further impairment of the SAN activity in *Popdc1* KO explants (Fig. 4). Notably, under higher doses of ISO (10 µM) the SAN preparation of *Popdc1* KO mice generated burst of $Ca^{2+}$ transients characterized by rise of diastolic $Ca^{2+}$ and following decrease of $Ca^{2+}$-transient frequency (Fig. 5). Moreover, when the inter-spike interval between two $Ca^{2+}$-transients was longer than in WT, SAN cells within the entire tissue generate intracellular $Ca^{2+}$-waves indicative of $Ca^{2+}$-overload (Fig. 6).

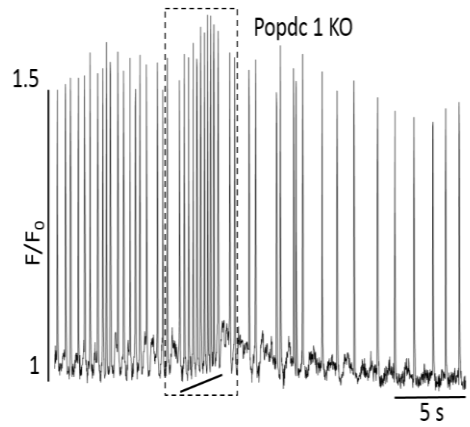


Figure 5: Examples of $Ca^{2+}$-transients recorded in Popdc 1 KO sinoatrial explants after addition of 10 µm ISO.

## Conclusions

*Popdc1* is highly expressed in SAN cells and seems to play an important role in membrane trafficking of several proteins as well as in their anchoring to the plasma membrane. Nevertheless, the evidence collected until now in the laboratory of Dr. Brand did not relate the absence of *Popdc1* to an impairment of the mechanism of depolarization in pacemaker cells. Our result suggests accumulation of $Ca^{2+}$ in SAN cells stimulated with ISO. Therefore, an interesting hypothesis is that *Popdc1* could be necessary for the proper $Ca^{2+}$-handling in SAN cells. This is a likely explanation, given that POPDC1 interacts with several anchoring proteins (ankyrinB and G). These proteins are necessary for the correct membrane localization of two of the most important proteins for $Ca^{2+}$-handling in SAN cells: the L-type $Ca^{2+}$ channel $Ca_V1.3$ and the $Na^+/Ca^{2+}$ exchanger (NCX) (5, 6). Indeed, $Ca_V1.3$ drives the main influx of $Ca^{2+}$ in SAN cells, while NCX is necessary for the normal $Ca^{2+}$-extrusion during SAN repolarization. Moreover, POPDC1 has been related to CAV3 localization, which seems to be essential to create micro-domains of $Ca^{2+}$ close to the plasma membrane, where the activity of several channels involved in pacemaking is regulated. Therefore, in the absence of POPDC1, the abnormal localization of the aforementioned proteins could disrupt the normal $Ca^{2+}$-influx/efflux mechanism in SAN cells as well as their compartmentalization, necessary to handle $Ca^{2+}$. These conditions could lead to aberrant spontaneous activity accentuated by β-adrenergic stimulation as we reported here.

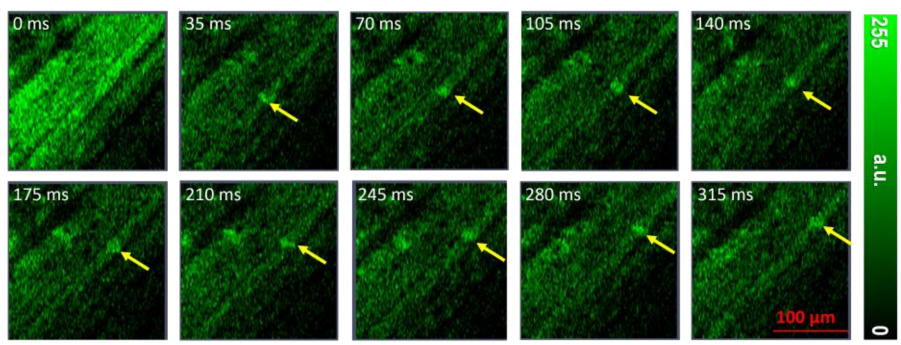


Figure 6: Time series highlighting the presence of intracellular $Ca^{2+}$ waves in Popdc 1 KO sinoatrial explants after addition of 10 µm ISO. First image (0 ms) correspond to the coordinated release of $Ca^{2+}$ ($Ca^{2+}$ transient). Yellow arrows point the intracellular conduction of a $Ca^{2+}$ waves along the time.

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