

Nantes Center Research project Cardiac diseases and sudden death

L'institut du thorax

The cardiac diseases and sudden death group gathers 5 different themes

THEME A: Clinical and molecular genetics (JJ Schott)

THEME B: Functional genomics and transcriptional control of ion channel remodeling (S Demolombe)

THEME C: Molecular and cellular physiology (I Baró)

THEME D: Experimental arrhythmias and transgenesis (F Charpentier)

THEME E: β -adrenoceptors (C. Gauthier)

Abstract

Sudden cardiac death (SCD) claims almost a million deaths annually in the USA and Europe, ventricular fibrillation being the most common underlying arrhythmia. For many years, the *institut* has been a key player in the field of **cardiac arrhythmias**. By elucidating the mechanisms responsible for **rare monogenic diseases**, our team has produced several breakthroughs, which have considerably improved our understanding of the molecular basis of normal and abnormal cardiac electrical activity. The efficiency of our research resulted from synergistic studies conducted in parallel in molecular and clinical genetics (theme Ia), molecular and cellular physiology (theme Ic), transgenic animals (theme Id) and genomics of ion channel genes (theme Ib). The *institut* is one of the very few places worldwide where all these abilities are gathered in the same laboratory.

A large number of our current projects deal with rare diseases with a special focus on **late onset diseases** such as progressive conduction defects and atrial fibrillation. Other projects also aim to address **genetic susceptibility to SCD in the general population**. Our hypothesis is that the risk for SCD is genetically modulated by key pathways and becomes manifest in the face of environmental triggers such as myocardial ischemia, drugs, or heart failure. Another major project is to elucidate the **mechanisms controlling ion channel transcription** based on our previous studies showing that regulations of ion channel expression play an important role in providing plasticity in response to genetic, pathological, or pharmacological insults. Studies conducted on transgenic animal or cellular models also identify important **new candidate genes** in the context of arrhythmia or conduction defect. Finally, because **β -adrenoceptors** play a key role in modulating cardiac function, we are evaluating their contribution in diseases that are known to alter ionic channel expression and predispose to cardiac arrhythmias such as heart failure (theme Ie)

For many years, the *institut* has been a key player in the field of **cardiac arrhythmias**. Researchers and clinicians at the *institut* have discovered important new concepts by elucidating the mechanisms responsible for rare monogenic diseases. Indeed, one key development driving our understanding of the molecular basis of normal and abnormal cardiac electrical behavior has been the identification of single mutations that greatly heighten the risk for arrhythmias and sudden cardiac death. In this field, the *institut* has produced several breakthroughs. This was due not only to the efficiency of its molecular and clinical genetics group (Prof. Hervé Le Marec, Drs Vincent Probst and Jean-Jacques Schott) but also because of the synergistic studies conducted in parallel on the mechanisms of cardiac arrhythmias, based on molecular and cellular physiology (Drs. Isabelle Baró and Gildas Lousouarn), cell biology (Dr. Jean Mérot), transgenic animals (Dr. Flavien Charpentier), and the genomics of ion channel genes (Drs. Sophie Demolombe and Françoise Le Bouffant). The *institut* is one of the very few places worldwide where all these abilities are gathered in the same laboratory.

This line of research has been central for our success in obtaining the label of '**Centre Thématique de Recherche et de Soins**'. An additional move has been the constitution of a transatlantic network of excellence sponsored at the level of 6 million dollars by the Leducq Foundation. The network (**Alliance Against Sudden Cardiac Death**) coordinated by Prof. D. Escande, includes the department of Pharmacology at Vanderbilt University (Prof. D. Roden), the department of Cardiology at Miami University (Prof. R. Myerburg), the department of Cardiology at Johns Hopkins University (Prof. E. Marbán) and the department of Cardiology at the University of Amsterdam (Prof. A. Wilde). At the national level, the *institut* is the only recognized **Expert Center for Hereditary Cardiac Arrhythmias**.

Sudden cardiac death (SCD) claims almost a million deaths annually in the USA and Western Europe (40,000 in France), and ventricular fibrillation is the most common underlying arrhythmia. Because of the magnitude of the problem, the opportunities for a real public health impact are enormous: even minor improvements (10-20%) in risk detection could reduce the number of victims by 100,000 or more. Device-based approaches have had a remarkable impact on SCD in patients at high risk. However, the fundamental public health problem of SCD is that the majority of victims do not fall into these high-risk groups. Our central hypothesis is that the risk of fatal arrhythmias is modulated by genetically determined variants in key pathways and that this risk becomes manifest in the face of environmental triggers such as myocardial ischemia, drugs, or heart failure.

At the *institut*, our research has focused for many years on **rare genetic model diseases (cardiac channelopathies)**. Still, a large number of our current projects deals with these rare genetic syndromes because of the unique opportunity we have of exploring the population of western France, which is an isolated population almost like Iceland with large pedigrees extending over centuries. More recently **our projects are moving to address genetic susceptibility to SCD in the general population**.

Cardiac channelopathies comprise a group of **rare genetic diseases** primarily affecting normal cardiac electrophysiology. This ensemble includes the long QT syndrome (LQTS), the Brugada syndrome (BS), the inherited Lenègre disease (also called Lev's disease), the inherited sick sinus syndrome, catecholaminergic polymorphic ventricular tachycardia and familial atrial fibrillation.

The long QT syndrome results from prolonged repolarization of the ventricular action potential. This anomaly is associated with a high risk of ventricular arrhythmias known as torsade-de-pointe arrhythmias. Prolonged cardiac repolarization can result from decreased outward (mainly K^+) currents or increased inward (Na^+ or Ca^{2+}) currents. In 1995, the group of Mark Keating¹ identified the first gene responsible for the long QT syndrome. Since then, at least 6 different genes have been identified as causative for LQTS including the *KCNQ1* gene (encoding for the voltage-dependent K^+ channel, *KCNQ1* also called *KvLQT1*) responsible for LQT1², *KCNH2* (encoding the human ether-à-gogo related K^+ channel, *HERG*) responsible for LQT2¹, *SCN5A* (encoding the major cardiac Na^+ channel) responsible for LQT3³, *ANK2*, encoding ankyrin B, responsible for LQT4⁴, *KCNE1* encoding for the K^+ channel subunit *KCNE1* (also called *Mink* or *IsK*) responsible for LQT5⁵ and the *KCNJ2* gene (encoding the inward rectifier K^+ channel *Kir2.1*) responsible for the Andersen syndrome⁶, which associates K^+ -sensitive periodic paralysis, ventricular arrhythmias, and dysmorphic features.

Our laboratory in conjunction with other groups in the USA has been instrumental in discovering the role of **ankyrin B** in LQTS. Ankyrins are a family of adaptor proteins that localize structurally-diverse ion channels and transporters to specialized membrane domains in various tissues. We have linked ankyrin B (AnkB) dysfunction with fatal cardiac arrhythmias in humans. The *ANK2* A4274G mutation leading to E1425G causes the "type 4" form of the congenital long QT syndrome. Affected family members display not only QT interval prolongation, stress- and/or exercise-induced polymorphic ventricular arrhythmia, syncope, and sudden cardiac death but also sinus node dysfunction (bradycardia or junctional escape rhythm), and episodes of atrial fibrillation. Subsequent evaluation of additional probands heterozygous for AnkB E1425G and identification and characterization of the phenotypes of probands with other AnkB loss-of-function variants demonstrated that the clinical phenotypes often extend beyond the typical long QT syndrome, leading to the label '**ankyrin-B-syndrome**': these phenotypes include bradycardia, sinus arrhythmia, delayed conduction/conduction block, idiopathic ventricular fibrillation, and catecholaminergic polymorphic ventricular tachycardia.

The **Brugada syndrome** is a new familial syndrome described in 1992 by the Brugada brothers. The syndrome associates a typical ECG pattern (ST segment elevation in the right precordial leads associated with an aspect of atypical right bundle branch block) and a risk of malignant ventricular arrhythmias such as polymorphic ventricular tachycardia or ventricular fibrillation responsible for syncope and sudden death. The diagnosis of the disease is often difficult due to potential confounding factor and the intermittent expression of the ECG abnormalities. In 1998, Chen *et al.* linked the syndrome with the α -subunit of the cardiac Na^+ channel (*Na_v1.5*) encoded by *SCN5A*⁷. Nearly seventy *SCN5A* mutations have been identified in patients over the past six years. Most are private mutations that induce a loss of function of the Na^+ channel through various mechanisms. However, in the Brugada syndrome only 20% of patients are carriers of a *SCN5A* mutation suggesting other culprit genes. In 2002, London and co-workers reported in a large family a new locus, BS2, close to *SCN5A* on chromosome 3 (3p22-25). In 2006, mutation in the *GPD1L* gene has been associated with BS2.

¹ Curran ME, Splawski I, Timothy KW, *et al.* *Cell* 1995; 80:795-803.

² Wang Q, Curran ME, Splawski I, *et al.* *Nat Genet* 1996;12:17-23.

³ Wang Q, Shen J, Splawski I, *et al.* *Cell* 1995;80:805-11.

⁴ Mohler PJ, Schott JJ, Gramolini AO, *et al.* *Nature* 2003;421:634-9.

⁵ Duggal P, Vesely MR, Wattanasirichaigoon D, *et al.* *Circulation* 1998;97:142-6.

⁶ Plaster NM, Tawil R, Tristani-Firouzi M, *et al.* *Cell* 2001;105:511-9.

⁷ Chen Q, Kirsch GE, Zhang D, *et al.* *Nature* 1998;392:293-6.

Progressive cardiac conduction defect (PCCD) is characterized by an age-related alteration in the conduction of the cardiac impulse through the His-Purkinje system. PCCD, also called **Lenègre or Lev disease**, is a frequent affection that remains the predominant cause for pacemaker implantation. It is commonly thought that the disease issues from a slow process developing over decades, affecting the His bundle and its branches. Lenègre and Lev hypothesized that it was a primary degenerative disease or an exaggerated aging process of unknown origin with sclerosis affecting selectively the conducting tissue. As early as the 70's, familial investigation of patients with chronic bifascicular block or complete AV block suggested that genetic factors could be involved in the pathophysiology of the disease. Hereditary cases of bundle branch block have repetitively been reported. We reported a large French family in which cardiac conduction defect was transmitted as an autosomal dominant trait and was caused by a splice mutation in the *SCN5A* gene. *SCN5A* is the first identified gene responsible for hereditary Lenègre disease⁸.

Other cardiac channelopathies include catecholaminergic polymorphic ventricular tachycardia related with mutations in the ryanodine receptor gene (*RYR2*)⁹ and in the calsequestrin-2 gene (*CASQ2*)¹⁰. A *KCNQ1* mutation has been identified most recently in Chinese kindred with familial atrial fibrillation¹¹.

Heart failure is a classical situation where the risk of sudden cardiac death is increased. The failing human heart is characterized by sustained activation of the sympathetic nervous system. This increase in adrenergic drive is first beneficial but ultimately detrimental causing important myocardial damages. The increase in sympathetic stimulation is responsible for down-regulation of myocardial β_1 -adrenoceptors in association with alterations in β_2 -adrenoceptor coupling with its effector system whereas β_3 -adrenoceptors are up-regulated resulting in altered myocardial function. Concurrently, heart failure is associated with vascular β -adrenergic dysfunction, which has been less investigated. Until recently, the use of β -blockers was disregarded in the treatment of patients with congestive heart failure. The value of β -blockers is now firmly established even as a first-line treatment of congestive heart failure. The effects of chronic treatment with β -blockers are variable especially in heart failure of ischemic etiology and in the elderly. The cause for this variable clinical response is unknown.

The cardiac diseases and sudden death group gathers 5 different themes:

- A. Clinical and molecular genetics (Drs. **JJ. Schott** and V. Probst and Prof. H. Le Marec)
- B. Functional genomics and transcriptional control of ion channel remodeling (Drs. **S. Demolombe** and F. le Bouffant)
- C. Molecular and cellular physiology (Drs. **I. Baró**, G. Loussouarn and J. Mérot)
- D. Experimental arrhythmia and transgenesis (Dr. **F. Charpentier**)
- E. β -adrenoceptors (Dr. **C. Gauthier**)

THEME A: Clinical and molecular genetics (JJ Schott)

I- Previous activities

In the past ten years, we have identified new genes responsible for life threatening cardiovascular pathologies using **familial-based genetic** approaches. We have mainly focused our research on **cardiac arrhythmias** and more recently on late onset diseases such as **progressive cardiac conduction defects** and atrial fibrillation but also on valvular diseases such as mitral valve prolapse and calcific aortic stenosis using an **epidemiogenetic approach**. Our basic hypothesis is that there is, for most of these age-related pathologies, a genetic predisposition related to **a major gene**.

Dominant diseases are - by definition - caused by single gene variants. It is common knowledge among clinicians and human geneticists that these variants show the widest possible spectrum of disease severity ranging in principle from unaffected to severely impaired or dead carriers of the same mutation. Consequently major gene variants are a necessary but not a sufficient condition to develop the respective disease. Other causes for disease manifestation remain to be identified. These may be endogenous (modifier genes) or exogenous (environment, lifetime events, lifestyle choices, diet, etc.). The existence of modulating genes controlling the penetrance and the expressivity in each pathology constitutes another research orientation¹².

We have identified **new pathophysiological mechanisms for three diseases**. Elucidation of the molecular bases for these pathologies resulted from a close cooperation between clinicians, genealogists, geneticists and physiologists at the *institut*.

Our major achievements were to:

⁸ Schott JJ, Alshinawi C, Kyndt F, *et al.* *Nat Genet* 1999;23:20-1.

⁹ Priori SG, Napolitano C, Tiso N, *et al.* *Circulation* 2000;102:r49-53.

¹⁰ Lahat H, Pras E, Olender T, *et al.* *Am J Hum Genet* 2001;69:1378-84.

¹¹ Chen YH, Xu SJ, Bendahhou S, *et al.* *Science* 2003, 299:251-4.

¹² Kaab S, Schulze-Bahr E. *Cardiovasc Res* 2005, 67(3):397-413. (Review).

1- Identify a mutation in the *ANK2* gene leading to LQT4¹³. AnkB E1425G mutation induces not only QT interval prolongation, stress- and/or exercise-induced polymorphic ventricular arrhythmia, syncope, and sudden cardiac death but also sinus node dysfunction, and episodes of atrial fibrillation. Ankyrins are a family of adaptor proteins that localize ion channels and transporters to specialized membrane domains. This work constitutes the first demonstration that a cytoskeleton protein can lead to cardiac arrhythmias.

2- We identified a splice mutation (IVS.22+2 T→C) in the *SCN5A* gene encoding for the cardiac Na⁺ channel Na_v1.5 in a large family diagnosed with Lenègre disease.¹⁴ Functional analysis showed that haploinsufficiency was the disease causing mechanism¹⁵. *SCN5A* is the first identified gene responsible for hereditary Lenègre disease. Screening a second large family identified a second loss of function Na_v1.5 G1408R mutation in patients affected by Lenègre disease or Brugada syndrome. Interestingly, the two phenotypes were transmitted separately between different family branches and Brugada phenotype was predominantly diagnosed in males suggesting that gender and/or other modifiers genes affecting the expressivity of Na_v1.5 channels added to the complexity of *SCN5A* channelopathies¹⁶.

3- We also identified the first gene responsible for non-syndromic polyvalvular myxomatous dystrophy in a large family showing X-linked mode of inheritance¹⁷. Four distinct Filamin A mutations were identified¹⁸ inducing similar polyvalvular dystrophies. Filamin A gene encodes a ubiquitously expressed cytoskeleton protein with various signaling properties and structural functions. Although the precise disease causing mechanism still needs to be elucidated, this finding opens new insights in the genetics of valvular dystrophies where 3 loci for monogenic mitral valve prolapse have been identified.^{19,20,21}

4- We demonstrated a familial aggregation for calcific familial aortic stenosis (Monckeberg disease) in the western part of France, suggesting a monogenic origin in this family²².

The identification of these new molecular bases of common cardiac pathologies has shed new insight into pathophysiological mechanisms and urged us to reconsider our knowledge in the field of cardiac arrhythmias as well as valvular dystrophies.

II- Current Project

Our objectives are to pursue ongoing projects focused on rare familial cardiac arrhythmias but also to identify susceptibility genes for common cardiac diseases.

Strategies developed to achieve these goals are essentially based on **whole genome approaches** such as (i) **reverse genetics** for monogenic diseases; (ii) **epidemiogenetic strategies** for late onset disease; (iii) **association studies** for polygenic pathologies. Alternatively, **candidate gene approaches** are used when transgenic animal model show convincing similarities with human cardiac arrhythmias pathologies. These approaches require high throughput genotyping and sequencing technologies, which are available at our Génopôle OUEST platform.

Identifying genes responsible for late onset diseases will become crucial given the aging population. We plan to extend our strategy to identify **late onset monogenic diseases using an epidemiogenetic approach**. Families affected by late onset diseases are by definition difficult to identify. We plan to take advantage of the regional specificity of the population where families are generally large with limited geographical mobility creating series of isolates. We propose that the existence of geographical aggregates for a given disease reflects the existence of founder effects. A similar strategy applied **to the Icelandic population** representing a typical geographical isolate has shown **power** in human genetics^{23,24}.

In a later perspective, we plan to focus on identifying **susceptibility genes for sudden cardiac death**.

A- Monogenic diseases gene identification: new culprit genes for ventricular and atrial fibrillation

Brugada syndrome remains a true genetic challenge. The molecular data show that the implication of

¹³ Mohler PJ*, Schott JJ*, Gramolini AO, *et al. Nature* 2003, 421(6923):634-9. *Equivalent authors

¹⁴ Schott JJ, Alshinawi C, Kyndt F, *et al. Nat Genet* 1999, 23(1):20-1.

¹⁵ Probst V, Kyndt F, Potet F, *et al. J Am Coll Cardiol* 2003, 41(4):643-52

¹⁶ Kyndt F, Probst V, Potet F, *et al. Circulation* 2001, 104(25):3081-6.

¹⁷ Trochu JN, Kyndt F, Schott JJ, *et al. J Am Coll Cardiol* 2000, 35(7):1890-7

¹⁸ Kyndt F, Gueffet JP, Legendre A, *et al. Circulation* 2006, in press

¹⁹ Disse S, Abergel E, Berrebi A, *et al. Am J Hum Genet* 1999, 65(5):1242-51.

²⁰ Freed LA, Acierno JS Jr, Dai D, *et al. Am J Hum Genet* 2003, 72(6):1551-9.

²¹ Nesta F, Leyne M, Yosefy C, *et al. Circulation* 2005, 112(13):2022-30.

²² Probst V, Le Scouarnec S, Legendre A, *et al. Circulation* 2006, 113(6):856-60.

²³ Sveinbjornsdottir S, Hicks AA, Jonsson T, *et al. New Engl J Med* 2000, 343:1765-1770.

²⁴ Jonsson S, Thorsteinsdottir U, Gudbjartsson DF, *et al. JAMA* 2004, 292:2977-2983.

SCN5A gene is as low as 30% with a penetrance estimated at 11%²⁵. The difficulty to identify large informative families suggests that the penetrance of Brugada syndrome for the other as yet unidentified genes, is probably also very low. We have recruited and characterized new families, large enough for genome wide scan analysis.

We have also recruited smaller families, originating from the same geographical area. A large genealogical study showed that these families shared a common ancestor and thus represented a single large pedigree comprising numerous affected patients. This pedigree will also be investigated using a whole-genome SNP approach. Identification of novel genes involved in the Brugada syndrome will be a significant step toward a better understanding of the pathophysiology of this complex disease.

As for Brugada syndrome, familial AF remains a challenge for geneticists. Despite the identification of 4 loci²⁶, only *KCNQ1*²⁷ and *GJA5*²⁸ mutants have been identified. Using a classical family based approach, we have identified a new locus for early onset AF on chromosome 20. A common characteristic of the 8 AF patients was the occurrence of low P wave amplitude associated with mild dilation of the atrium. The identification of the disease-causing gene is currently in progress focusing on a 23 mega base interval.

In parallel, based on phenotypical evidence showing AF in a Cav1.3 Knock-In mice model, we are currently screening the human *CACNA1d* gene in a cohort of patients diagnosed with AF or sinus node dysfunction. Preliminary evidences have been obtained for potential disease-causing mutations. Functional analyses are ongoing.

B- Epidemiogenetic approaches for late onset pathologies

One of the major finding of this preliminary epidemiogenetic work was the identification of a large heterogeneity (from parish to parish) in the prevalence of several cardiovascular diseases.

Since our pioneer work, we have developed new approaches based on heterogeneous geographic distribution of pacemaker implantations for Lenègre disease. Several clusters with high implantation rates were identified. In a first approach, we searched for shared family names in areas with the highest implantation rate. We then screened for other family members affected by Lenègre disease. In one identified family, we mapped a new locus on chromosome 16. Genetic analyses for other families are also ongoing.

Similar approaches are used to map genes responsible for calcific aortic stenosis, mitral valve prolapse. In both conditions surgery remains the only efficient medical treatment.

C- Sudden Cardiac death in the community: susceptibility genes

Most often, sudden cardiac death (SCD) results from ventricular tachyarrhythmia mainly ventricular fibrillation complicating an unrecognized coronary heart disease. Most cases occur in asymptomatic individuals. Currently identified risk factors do not allow stratification of individual risk in the general population. An important challenge in molecular cardiology is **to identify high-risk individual who could benefit from preventive therapy**. Genetics could help to improve risk stratification in the general population and, thus, to establish preventive strategies guided by the individual risk. This research could have a tremendous impact owing to the numerical importance of SCD.

Our genetic hypothesis is based on three lines of evidence: (1) rare variants in genes encoding ion channels or regulators are associated with a high risk of sudden death; (2) gene-environment interactions can lead to a sudden death; and (3) family history of sudden death is an independent risk factor for sudden death. Rare genetic mutations identified to date only account for a very small part of SCD in the general population. **Most culprit genetic variants remain to be identified.**

Our project includes two complementary sections:

- to create a registry of out-of hospital SCD in the adult population of Loire-Atlantique, (1 134 000 inhabitants). Incidental cases will be exhaustively reported (estimate: around 600 cases/year).
- To establish a DNA bank with blood samples collected by the SAMU at the time of the cardiac arrest (estimate: 250 samples/year). This DNA collection will be used for a case-control genome-wide association study. A control group will be constituted with sudden-death-free subjects matched to cases in age, sex and ethnicity. All subjects will be genotyped using a high-resolution map of SNPs distributed throughout the genome. Positive SNPs will be confirmed in additional population samples obtained through our transatlantic collaborative network.

Our ultimate goal is to identify genetic variants associated with an increased risk of SCD and to develop a high-throughput low-cost screening test applicable to the general population for prevention guidance.

²⁵ Viswanathan PC, Balsler JR. *Trends Cardiovasc Med* 2004, 14(1):28-35. Review.

²⁶ Wiesfeld AC, Hemels ME, Van Tintelen JP, et al. *Cardiovasc Res* 2005, 67(3):414-8. Review.

²⁷ Chen YH, Xu SJ, Bendahhou S, et al. *Science* 2003, 299: 251-54.

²⁸ Gollob MH, Jones DL, Krahn AD, et al. *N Engl J Med* 2006, 354(25):2677-88.

THEME B: Functional genomics and transcriptional control of ion channel remodeling (S Demolombe)

I- Previous activities

Regulations of ion channel expression play an important role in maintaining a stable electrophysiological phenotype in cardiac myocytes as well as in providing plasticity in response to genetic, pathological, or pharmacological insults. Channel expression can be modified at several levels along the biosynthetic pathway, i.e. transcription, translation, trafficking and degradation. We hypothesized that the transcription plays a key role in the electrical plasticity of the heart because of its high specificity and accuracy. We validated this hypothesis by: 1/ evaluating ion channel expression at a genomic scale in response to changing physiological or pathological environment; 2/ by demonstrating that transcriptional modifications induce functional consequences.

A- Physiological genomics of ion channel genes

We investigated the regional distribution of ion channel expression in the mouse and non-diseased human hearts.²⁹ Expression of ion channels is heterogeneous within the heart muscle and this heterogeneity is key to specialization of cardiac areas. In a mouse study, we demonstrated that the remarkable signature of the nodal tissues results from increased expression of a large panel of ion channel genes rather than decreased expression. In a human study, we showed that atria, Purkinje fibers and ventricular tissues show specific patterns, whereas inter-tissue differences between epicardium and endocardium, and left and right-sided cavities are subtle. Our data point to significant differences in regionally-determined ion-channel expression, with important potential implications for understanding regional electrophysiology, arrhythmia mechanisms, and responses to ion-channel blocking drugs.

We also verified that the expression profile of ion channel genes in the normal mouse heart is dependent on the genetic background,³⁰ and may account for the previously reported differences in ECG characteristics and susceptibility to programmed electrical stimulation-induced ventricular arrhythmias.³¹

The molecular mechanisms underlying gender-related differences in arrhythmic risks are poorly understood.³² We investigated at a genome-scale the ion-channel expression in non-diseased epicardium and endocardium samples from either sex.³³ We demonstrated that transcript expression differences between samples from men and women are subtle but involve key genes implicated in conduction and repolarization. The lower expression in women of several K⁺-channel α - and β -subunits provides a potential molecular basis for female lower repolarization reserve and poor penetrance of the Brugada syndrome.

B- Pharmacological genomics of ion channel genes

We also investigated the remodeling of ion channels as a possible mechanism of drugs effects. In this context, we thought that amiodarone might be prototypic. Amiodarone has a remarkable antiarrhythmic efficacy and the pharmacological profile of the drug is complex including direct modulation of ion channel function. This drug is also known to modify thyroid function mostly due to its iodinated nature.³⁴ The question arose as to whether the long-term effects of amiodarone might stem from its molecular interaction with thyroid hormone receptors or others mechanisms including modulation of gene expression in addition to its direct effect on channels.³⁵ The effects of the drug on cardiac ion channel transcripts of treated adult mice are compatible with the pharmacological profile of the drug, which associates decreased conduction and prolonged repolarization, and in nature comparable to that induced by thyroid hormone as we previously analyzed.^{36,37} Most importantly, remodeling of ion channel expression induced by amiodarone is dose-dependent and correlates with the dose-dependent effects of the drug on the ECG.

C- Clinical genomics of ion channel genes

Our group has investigated the remodeling induced by chronic atrial fibrillation (AF) in patients undergoing open-heart surgery.³⁸ Limitations of previous studies of ion channel expression changes in patients with AF include the arbitrary selection of subunits for study and inadequate disease-matched controls. We compared a group of patients with chronic AF and underlying valvular heart disease with

²⁹ Marionneau C, Couette B, Liu J *et al. J Physiol* 2005;562:223-34.

³⁰ Demolombe S, Marionneau C, Le Bouter S, *et al. Cardiovasc Res* 2005;67:438-47.

³¹ Maguire CT, Wakimoto H, Patel VV, *et al. Physiol Genomics* 2003;15:84-91.

³² James AF, Choisy SC, Hancox JC. *Prog Biophys Mol Biol* 2005 (in press).

³³ Gaborit N, Varro A, Le Bouter S, *et al.* 2006 (submitted).

³⁴ Kennedy RL, Griffiths H, Gray TA. *Clin Chem* 1989;35:1882-87.

³⁵ Drvota V, Blange I, Haggblad J, *et al. J Cardiovasc Pharmacol* 1998;32:654-61.

³⁶ Le Bouter S, El Harchi A, Marionneau C, *et al. Circulation* 2004;110:3028-35.

³⁷ Le Bouter S, Demolombe S, Chambellan A, *et al. Circ Res* 2003;92:234-42.

³⁸ Gaborit N, Steenman M, Lamirault G, *et al. Circulation* 2005;112:471-81.

a group of patients with valvular heart disease in sinus rhythm. We conclude that the transcriptional profile of genes involved in ion transport is profoundly affected by valvular heart disease. However, AF is associated with a specific pattern of expression, but whether these are caused by AF or characterize valvular heart disease patients more likely to develop AF is still unknown.

A simplistic view of cardiac channelopathies would be that a genetic defect selectively alters the function of a key ion channel leading to abnormal electrogenesis and arrhythmias. However, cardiac cells adapt to the defect by remodeling gene expression. We therefore hypothesized that Brugada syndrome could remodel ion channel gene expression in the myocardium as a result of compensatory molecular mechanisms. Novel methods to accurately investigate the expression of hundreds of transcripts in very small size biological samples have recently been developed in our *institut*.²⁹ We used these methods to obtain full profiling of ion channel expression in right ventricular septal endomyocardial biopsies from patients with Brugada syndrome.³⁹ The ion channel signature demonstrates a complete discrimination between endomyocardial tissues from patients with Brugada syndrome and those from two groups of control subjects (donor hearts and recently transplanted hearts). We observed that patients with Brugada syndrome share a common molecular signature irrespective of the culprit gene. We are now envisaging a larger biopsy collection from patients receiving an implantable cardiac defibrillator for various indications. Results from this new investigation should conclude whether molecular profiling could reliably diagnose ventricular arrhythmias.

II- Current project

Our projects are dedicated to elucidate the mechanisms controlling ion channel transcription. This project includes two aspects: 1/ to identify the transcription factors (TFs) involved in ion channel transcription; 2/ to identify the feedback pathways linking the electrophysiological phenotype of the cardiomyocytes and the transcription machinery to control phenotype alteration. As a first step, our investigations will focus on the transcriptional regulation of the cardiac voltage-gated Na⁺ channel (Na_v1.5) and the Kv channel-interacting protein 2 (KChIP2) involved in the transient outward potassium current I_{to}. Both I_{Na} and I_{to} currents play key role in the physiology and pathophysiology of the heart.^{40,41} Both are also linked to the pathophysiology of the Brugada syndrome.⁴²

The role of transcription factors has been extensively studied in cardiac embryogenesis.⁴³ In comparison, much less is known about their role in the adult heart. Our knowledge of transcriptional regulation of ion channel gene expression is even sparser.⁴⁴ This is certainly an open and important avenue for new research.

Our strategy will be as follows:

Aim #1: to correlate ion channel profiling with transcription factor profiling in different conditions. To investigate the complex transcriptional regulation of ion channel genes, global transcription can be measured using high throughput real-time RT-PCR, and computational analysis of this information can lead to a deeper understanding of the system as a whole. These 'system approaches' have been successfully used by Gilchrist et al. in the context of immune regulation.⁴⁵ We have already mapped 164 TF RNA species in different regions of the adult mouse and non-diseased human hearts. We will also investigate the expression dynamics of TFs in relation to progressive remodeling of cardiac ion channel transcripts as induced by hypertrophy, using a mouse model developed at the *institut* by Flavien Charpentier. A collection of human samples from hypertrophic hearts is also available in our laboratory. However, unlike in mice, dynamic information with progressing hypertrophy cannot be obtained in human.

Aim #2: to correlate gene profiling with abnormal intracellular Ca²⁺ handling. Ca²⁺ ions modulate many cellular processes including gene expression through transcription factors, such as members of the CREB, NFAT, and MEF2 families,^{46,47} it is assumed that Ca²⁺ channels represent the most important feedback link between electrical activity and regulation of ion channel gene expression. For example, it has been recently shown that [Ca²⁺]_i controls the functional expression of I_{to} in cardiac myocytes.⁴⁸ Our goal is to correlate dynamic changes in ion channel expression and transcription regulators with intracellular Ca²⁺ anomalies in the mouse model of cardiac hypertrophy.

Aim #3: bioinformatics. We ambition to identify *in silico* the promoter region for every gene encoding ion channel subunits and Ca²⁺ regulators expressed in the heart. Promoter analysis will provide: 1/ a

³⁹ Wichter T, Gaborit N, Varro A, et al. *Circulation* 2006 (in revision).

⁴⁰ Balsler JR. *J Mol Cell Cardiol* 2001;33:599-613.

⁴¹ Oudit GY, Kassiri Z, Sah R, et al. *J Mol Cell Cardiol* 2001;33:851-72.

⁴² Di Diego JM, Cordeiro JM, Goodrow RJ, et al. *Circulation* 2002;106:2004-11.

⁴³ Bruneau BG. *Circ Res* 2002;90:509-19.

⁴⁴ Rosati B, McKinnon D. *Circ Res* 2004;94:874-83.

⁴⁵ Gilchrist M, Thorsson V, Li B, et al. *Nature* 2006;441:173-8.

⁴⁶ Dolmetsch R. *Sci STKE* 2003, P E4

⁴⁷ Wilkins BJ, Molkenin JD. *Biochem Biophys Res Commun* 2004;322:1178-91.

⁴⁸ Rossow CF, Dilly KW, Santana LF. *Circ Res*. 2006, 98:1306-13.

number of putative transcription factor binding sites located in predicted promoter regions, 2/ shared organization of co-regulated ion channels. This information will be correlated with our biological data.

Aim #4: ChIP on chips approach. Our ChIP on chips approach will be conducted in collaboration with Dr. Remi Houlgatte.

Aim #5: functional correlate. Depending on the outcome of the preceding aims, different mouse models invalidated for key ion channels and transcription factor genes will be generated or obtained through collaborative agreements.

THEME C: Molecular and cellular physiology (I Baró)

Our projects are mainly focused on the gene-function relationship of K⁺ and Na⁺ channels, which are key elements of the cardiac action potential. These **ion channels are now rather regarded as macromolecular complexes** made of different proteins having various functions: channel *per se*, chaperone, regulatory or anchoring protein. However, many molecular mechanisms underlying channel behavior, and many of the actors of the channel-complexes are still unknown. The genotype-phenotype relationships in hereditary channelopathies often remain difficult to establish.

Our objectives are 1/ to describe the structural events leading to channel activity and 2/ to identify the channel-complexes actors that may turn out to be new therapeutic targets for cardiac diseases handling.

I- Previous activities

A- Molecular pathophysiology of the KCNQ1-related channelopathies

The pathophysiological sequence that leads to the congenital LQTS sometimes appears as a simple correlation between the mutation and the patient phenotype. In the past, we dedicated ourselves to understand the molecular physiology of the KCNQ1 protein⁴⁹, and to determine the functional consequences of mutations in its gene in the context of LQTS^{50,51}. Indeed, *KCNQ1* gene encodes for a channel protein, which associates with the membrane protein, KCNE1, to constitute a channel-complex responsible for the repolarizing K⁺ current (I_{Ks}) of the myocardium^{52,53}. In LQTS, mutations in the *KCNQ1* gene are the most frequent. In previous studies, we and others have shown that mutations leading to a *loss of function* of the channel are associated with the disease.

We have also shown that a KCNQ1 mutation may be associated with another cardiac arrhythmia: the short QT syndrome⁵⁴. In a patient resuscitated after a sudden death and presenting a QT interval shortening, we detected a mutation inducing a *gain of function* of the K⁺ channel.

Our most recent discoveries were focused on:

1) Molecular mechanisms for loss-of-function

Mutations may alter the K⁺ current in different ways, from no expression, impaired trafficking to altered channel activity in the sarcolemma. Most recently, studying LQT1-associated KCNQ1 mutations, we unveiled 1/ **a new role of the N-terminus of KCNQ1 channel in its trafficking** and its implication in severe forms of LQTS⁵⁵, 2/ we identified **PIP₂ and MgATP as regulators of KCNQ1** and showed that at least three KCNQ1 mutations are linked to a decrease in channel-PIP₂ affinity^{56,57}.

2) KCNQ1 channel-complex

Even if, most of the time, the genotype-phenotype relationship may be direct, it remains that the ratio of mutation carriers presenting the disease over the total number of carriers in a family, i.e. its penetrance, may be highly variable. Our hypothesis was that other partners of the complex may contribute to the channel dysfunction and thus explain part of the variable penetrance. Therefore, we initiated a systematic analysis of potential components of KCNQ1 channel-complex.

The study of the KCNQ1/KCNE1 complex in a recombinant system identified A kinase anchor proteins (AKAP) as key component in the adrenergic regulatory transduction pathway for KCNQ1 in cardiomyocytes⁵⁸. Very recently, in collaboration with Hugues Abriel (Lausanne, Switzerland) we also showed that **Nedd4/Nedd4-like proteins associate to KCNQ1 and regulate stability and internalization of KCNQ1** through ubiquitylation processes⁵⁹.

⁴⁹ Demolombe S, Baró I, Péréon Y, *et al.* *J Biol Chem* 1998, 273:6837-6843

⁵⁰ Mohammad-Panah R, Demolombe S, Neyroud N, *et al.* *Am J Hum Genet* 1999, 64:1015-1023

⁵¹ Gouas L, Bellocq C, Berthet M, *et al.* *Cardiovasc Res* 2004, 63:60-68

⁵² Barhanin J, Lesage F, Guillemare E, *et al.* *Nature* 1996, 384:78-80

⁵³ Sanguinetti MC, Curran, ME, Spector PS, *et al.* *Nature* 1996, 384:80-83

⁵⁴ Bellocq C, van Ginneken AC, Bezzina CR, *et al.* *Circulation*. 2004, 109:2394-2397

⁵⁵ Dahimène S, Alcoléa S, Escande D, *et al.* *Circ Res* in revision

⁵⁶ Loussouarn G, Park KH, Bellocq C, *et al.* *EMBO J* 2003, 22:5412-5421

⁵⁷ Park KH, Piron J, Dahimène S, *et al.* *Circ Res* 2005, 96:730-739

⁵⁸ Potet F, Scott JD, Mohammad-Panah R, *et al.* *Am J Physiol* 2001, 280:H2038-H2045

⁵⁹ Jespersen T, Membrez M, Nicolas C, *et al.* submitted

B- Molecular pathophysiology of the SCN5A-related channelopathies

The *SCN5A* gene coding for the cardiac Na⁺ channel Na_v1.5, is also implicated in channelopathies. The functional study of these *SCN5A* mutations explained the phenotypical impact. Na_v1.5 channel generates the Na⁺ current responsible for the fast depolarization of the action potential (AP). The *loss of function* of the mutated Na⁺ channel induces a heterogeneous shortening of the ventricular AP that contributes to ventricular fibrillation associated to BS^{60,61}. In collaboration with the team of Jean-Jacques Schott at the *institut* we unveiled similar *loss of function* of Na⁺ channel for *SCN5A* mutations in two families presenting with Lenègre disease^{62,63}. However, in Lenègre disease, the *loss of function* of the Na⁺ channel induces a conduction defect, a very different phenotype compared to BS.

By analogy with *KCNQ1* and *KCNE1*, we suspected that the phenotype of the *SCN5A* mutation carriers may be determined by polymorphism of Na_v1.5 partners. In a search for new Na_v1.5 partners, we have recently identified 14-3-3 protein as a component of Na_v1.5 complex. We showed that **14-3-3 regulates Na⁺ current availability** during the AP⁶⁴.

The identification of new ion-channel protein partners and characterization of their mechanisms of interaction at the molecular level may have major implications: 1/ understanding the channel physiology and the pathophysiology of the congenital cardiac arrhythmias and conduction diseases; 2/ **identification of new candidate genes** leading to detection of asymptomatic carriers with elevated risk of sudden death; 3/ ion channel partners may also represent **potential targets for new pharmacological agents**.

II- Current project

In a continuum with our work, our current projects concern the mechanisms implicated in the regulation of the cardiac ion channels *KCNQ1* and Na_v1.5 in the context of cardiac arrhythmia and conduction defect.

A- Partners of channel-complexes

Previously, we used a 'candidate partner' approach. More recently, we have launched a more systematic screening method.

1) *KCNQ1* and b-tubulin

Françoise Le Bouffant detected different proteins interacting with Na_v1.5⁶⁴ or *KCNQ1*, using a yeast two-hybrid screen of a mouse cDNA library. Among them, β-tubulin interacts with the N-terminus part of *KCNQ1*. After validation in mammalian expression systems and in cardiomyocytes of the binding between the full-length channel and the candidate protein by co-immunoprecipitation, we colocalized both proteins in transfected cells and cardiomyocytes at the level of the intercalated discs and sarcolemma. We are currently evaluating the regulation of *KCNQ1-KCNE1* complex by microtubules in both models. Our preliminary results show **a regulation of *KCNQ1-KCNE1* sensitivity to PKA-dependent stimulation by the microtubules**. A second candidate protein interacting with *KCNQ1* C-terminus is also currently under investigation.

2) Proteomics approaches

In order to further detect potential partners of *KCNQ1* or Na_v1.5, we are also using conventional (pull-down, immuno-precipitation) and new (Blue native/SDS-PAGE adapted for membrane proteins⁶⁵) proteomics approaches coupled to mass spectrometry analysis (MS). Protein sequencing is performed in the Laboratory of Proteomics of the RIO Platform (INRA) in Nantes who has established the appropriated procedures⁶⁶. Once identified, channel-partners interactions will be analyzed in expression systems (co-immunoprecipitation and co-localization) and in cardiomyocytes. The following steps will be to investigate the role of the newly identified binding proteins on channel maturation (folding and trafficking) and function (channel activity) in cell lines and in adult cardiomyocytes transfected with cDNA and/or siRNA.

B- Molecular physiology of *KCNQ1*

We are convinced that **a better knowledge of the intimate molecular mechanisms of the channel maturation and activity is required**.

1) Folding, maturation and trafficking of *KCNQ1*

Our recent works identified impaired trafficking as the cellular mechanism involved in severe forms of Romano Ward LQT1 syndrome⁵⁵. Our ongoing analysis further highlighted endoplasmic reticulum (ER) retention and protein quality control as key stages in *KCNQ1* mis-trafficking. These data point to

⁶⁰ Dumaine R, Towbin JA, Brugada P, et al. *Circ Res* 1999, 85:803-809

⁶¹ Wan X, Chen S, Sadeghpour A, et al. *Am. J. Physiol* 2001, 280:H354-H360

⁶² Schott JJ, Alshinawi C, Kyndt F, et al. *Nat Genet* 1999, 23:20-21

⁶³ Probst V, Kyndt F, Potet F, et al. *J Am Coll Cardiol* 2003, 41:643-652

⁶⁴ Allouis M, Le Bouffant F, Wilders R, et al. *Circ Res* 2006, 98:1538-1546

⁶⁵ Brouillard F, Bensalem N, Hinzpeter A, et al. *Mol Cell Proteomics* 2005, 4:1762-1775

⁶⁶ Tamaskovic R, Bichsel SJ, Rogniaux H, et al. *J Biol Chem* 2003, 278:6710-6718

early steps in channel biogenesis as targeted by the mutations. Our objectives are to go one step further and to analyze at the molecular level 1/ the molecular chaperons that participate in the channel biogenesis, 2/ the impact of the mutations on the interactions with these chaperons and the fate of the protein in the ER (retro-translocation - degradation), 3/ the impact of the mutations on the topogenesis (insertion and orientation of the transmembrane domains) of the channel.

These new aspects of KCNQ1 maturation and trafficking will be analyzed with different experimental approaches already mastered in the laboratory including immunoprecipitation, western blotting, metabolic radiolabeling with ³⁵S-methionin and *in vitro* translation reaction.

2) Molecular regulations of KCNQ1 activity

KCNQ1 intrinsic regulation. The mechanisms for voltage-dependent channel activation still need clarification. In this context, we hypothesized that a 6 transmembrane domains (TMDs) Kv channel may be both structurally and functionally equivalent to a voltage sensor, comprising the four initial TMDs (S1-S4), merged to a two-TMDs K⁺ channel comprising TMDs S5-S6 and the pore. One consequence of such a model would be that the **S4-S5 linker behaves like a voltage-dependent ligand able to bind to the C-terminal part of S6 (CterS6)**. Preliminary data show that overexpression of a peptide corresponding to the S4-S5 linker indeed decreases the open state stability of KCNQ1 whereas a peptide corresponding to the CterS6 increases the open state stability. These data confirm our initial hypothesis and suggest that complementation of abnormal voltage dependence by specific peptides could be used *in vivo* to correct mutant channels.

KCNQ1 osmoregulation and PIP₂. During ischemia and reperfusion, cardiac cells have been shown to undergo significant swelling, which has marked effects on cardiac electrophysiology by modulating the function of several sarcolemmal ions channels and transporters⁶⁷. In particular, it has been shown that KCNQ1 is sensitive to osmolarity, both in presence and absence of KCNE1⁶⁸. We have recently shown that several KCNQ1 mutants associated with LQTS1 are characterized by a decrease in PIP₂ sensitivity⁵⁷. One of them is almost insensitive to a decrease in PIP₂. This mutant is also poorly sensitive to change in osmolarity, suggesting **a role of PIP₂ in the osmoregulation of the channel activity**. Using several mutations with various PIP₂ affinities, we are currently characterizing the role of PIP₂ in KCNQ1 osmoregulation.

THEME D: Experimental arrhythmias and transgenesis (F Charpentier)

I- Previous activities

A- Pathophysiology of cardiac conduction defects

In the past years, we characterized the phenotype of a mouse model invalidated, at the heterozygous state, for *Scn5a* gene.^{69,70} We demonstrated that the *Scn5a*^{+/-} mouse is a good model for Lenègre disease with similar age-related deterioration in conduction defects. The age effects are due to progressive occurrence of extensive ventricular fibrosis and remodeling of connexin expression. Our study provides the **first univocal demonstration that a monogenic ion channel defect can lead with age-related myocardial structural anomalies**. It also supports Lenègre's original hypothesis that progressive conduction defects were due to a slow fibrotic process. Finally, it offers an opportunity to develop a **preventive therapy of fibrosis and progressive conduction defects**.

In a subsequent study, we demonstrated that: (i) the expressivity of the conduction anomalies is variable among *Scn5a*^{+/-} mice, as also observed in patients with inherited Lenègre disease; (ii) mice with a strong phenotype exhibit more myocardial rearrangements with aging than mice with a mild phenotype; (iii) old mice with a strong but not with a mild phenotype have a reduced conduction reserve and exhibit spontaneous ventricular arrhythmias as also observed in the **Brugada syndrome**; (iv) the expressivity of the conduction deficit correlates with the ability of the normal *Scn5a* allele to produce larger amount of functional channel proteins. Our data suggest that variable expressivity of the disease in *Scn5a*^{+/-} mice results from **variable compensatory mechanisms governing the expression of wild-type Na⁺ channel proteins**. *Scn5a*^{+/-} mice can be used to investigate for the mechanisms of variable expressivity of *SCN5A* loss-of-function mutations in man.

Finally, *Scn5a*^{+/-} mice present a moderate bradycardia due to abnormal conduction from the sinus node to surrounding atrial myocytes and sino-atrial blocks.⁷¹ Our results provide **new insights into the understanding of the pathophysiology of SCN5A-linked sinus node dysfunction**.⁷²

B- Development of a biological pacemaker

⁶⁷ Vandenberg, J.I., Rees, S.A., Wright, A.R. *et al.* *Cardiovasc Res.* 1996, 32:85-97

⁶⁸ Kubota T, Horie M, Takano M, *et al.* *Jpn J Physiol* 2002, 52:31-39.

⁶⁹ Royer A, van Veen TA, Le Bouter S, *et al.* *Circulation* 2005, 111:1738-46.

⁷⁰ van Veen TA, Stein M, Royer A, *et al.* *Circulation* 2005, 112:1927-35.

⁷¹ Lei M, Goddard C, Liu J, *et al.* *J Physiol* 2005, 567:387-400.

⁷² Benson DW, Wang DW, Dymment M, *et al.* *J Clin Invest* 2003, 112:1019-28.

In France, about 180,000 patients are equipped with an electronic cardiac pacemaker and the implantation rate is continuously increasing with population aging. Although electronic pacemakers are an efficient medical therapy, they do have limitations including lack of responsiveness to autonomic neurohumoral regulation, lead- and procedure-related complications and the need for periodic battery replacement that led to the attempt of developing **biological pacemakers by *in situ* gene transfer**.

All studies performed so far used viruses as gene vectors. As an alternative gene delivery system, we used the tetrionic 304, a poloxamine block copolymer developed by Bruno Pitard's team,⁷³ to transfect locally ventricular myocytes *in situ* with genes encoding the hyperpolarization-activated pacemaker channel HCN2 and the β_2 -adrenergic receptor. With this method, we generated **long-lasting biological pacemakers** that are **regulated by β -adrenergic stimulation** and that **improve life expectancy** in mice in chronic atrioventricular block.

II- Current project

A- Pathophysiology of cardiac conduction defects

Characterization of the *Scn5a*^{+/-} mouse model will continue. Our objectives are now:

- 1- to identify the mechanisms for variable expressivity of the disease;
- 2- to identify the gene transcription regulatory pathway(s) responsible for increased fibrosis and deterioration of conduction with age;
- 3- to investigate the mechanisms of cardiac arrhythmias;
- 4- to evaluate the therapeutic potential of drugs that prevent myocardial fibrosis in the context of Lenègre disease

Variable expressivity of the conduction defects between mice with a mild phenotype and mice with a strong phenotype can be explained either by the presence of modifier genes (polymorphisms), an hypothesis that will be tested with Affymetrix GeneChips® microarrays (with Jean-Jacques Schott, theme IA), or by differential adaptive gene expression remodeling. Differentially-expressed genes between the two groups of mice will be identified at the mRNA level using Applied Biosystems Mouse Genome Survey Microarrays® in collaboration with Marja Steenman (team III). Finally, the mechanisms for higher expression of Na_v1.5 protein in mice with a mild phenotype will be investigated with Jean Mérot (theme IC) and Hughes Abriel (Lausanne, Switzerland).

The gene transcription regulatory pathway that links moderately impaired ventricular activation in young mice with increased fibrosis with aging will be identified with Sophie Demolombe (theme IB) using high-throughput quantitative RT-PCR (MicroFluidic Cards, Applied Biosystems). In this context, we will determine if the identified pathway is triggered by the conduction slowing *per se* or by the impaired pattern of activation as previously observed in a canine model of ventricular pacing.⁷⁴

Other channelopathies, such as the Brugada syndrome, have been linked to loss-of-function mutations in *SCN5A*.⁷⁵ The Brugada syndrome is usually attributed to a disequilibrium between a large transient K⁺ current in the right ventricular subepicardium and a decreased Na⁺ current.⁷⁶ But altered right ventricular conduction could also lead to the disease.⁷⁷ Indeed, the frontier between the Brugada syndrome and isolated cardiac conduction disease remains unclear and in the same family the same mutation can induce both diseases.⁷⁸ We have shown that *Scn5a*^{+/-} mice exhibit an earlier and larger conduction delay in the right ventricle and that mice with a severe phenotype have a 50% incidence of ventricular tachycardia. We thus propose to explore further the conduction in the right ventricle by recording epicardial monophasic and/or transmembrane action potentials.

Finally, our previous results provide an experimental background to support evaluation of the therapeutic potential of drugs that prevent myocardial fibrosis in the context of Lenègre disease. Many studies have shown that angiotensin converting enzyme (ACE) inhibitors, angiotensin receptors antagonists and aldosterone blockers can prevent or even reverse fibrosis and ventricular remodeling in different cardiac pathologies. We are currently designing a blinded study in the *Scn5a*^{+/-} mouse model aimed at evaluating the beneficial effects of long lasting administration of ACE inhibitors on conduction parameters, ventricular remodeling and fibrosis.

Our project should result in the identification of **prognostic markers of the progression of the disease** in patients with Lenègre disease or Brugada syndrome and of **new therapeutic targets**. If positive, our study with ACE inhibitors will be followed by a **clinical study** performed in the Cardiology Department of the *institut du thorax* in collaboration with the "Centre d'Investigation Clinique Inserm" of Nantes. It could provide the **first preventive therapy** for the Lenègre disease.

B- Development of a biological pacemaker

⁷³ Pitard B, Bello-Roufai M, Lambert O, *et al. Nucleic Acids Res* 2004, 32:e159.

⁷⁴ van Oosterhout MF, Prinzen FW, Arts T, *et al. Circulation* 1998, 98:588-95.

⁷⁵ Chen Q, Kirsch GE, Zhang D, *et al. Nature* 1998, 392:293-6.

⁷⁶ Antzelevitch C, Brugada P, Brugada J, *et al. J Am. Coll. Cardiol* 2003, 41:1665-71.

⁷⁷ Merigalli PG, Wilde AA, Tan HL. *Cardiovasc Res* 2005, 67:367-78.

⁷⁸ Kyndt F, Probst V, Potet F, *et al. Circulation* 2001, 104:3081-6.

After proving the feasibility of generating biological pacemakers with non-viral gene transfer, we now plan i) to improve our pacemakers and ii) to validate in dogs the strategies identified in mice.

Further molecular developments to increase the firing rate and the longevity of our engineered pacemakers include the evaluation of different promoters and investigation of the efficacy of mutated HCN2 channels in the mouse model. Longer-term follow-up (> 1 year) of the pacemaker activity is also needed once the most adequate promoter and transgenes will be chosen.

Because the mouse cardiac electrical activity differs markedly from the human one, engineered pacemakers need further evaluation in larger mammals such as dogs. Whether or not they will lead to the same firing rate in dogs as in mice will be addressed by these studies. Dogs will also be useful for developing tools and procedures for proper intra-cardiac targeting of gene delivery in man. We will also ensure that the engineered pacemaker can express itself in the atrium, an issue that cannot be solved in the mouse. This part of the project will be performed in collaboration with Marc Vos (Utrecht University Medical Center, NL) who developed a model of dog in chronic atrioventricular block.

The expected results of this project are to **develop a biological alternative to electronic pacemakers**. We hope that a **clinical trial** will be ultimately performed in the *institut du thorax*. Finally, this project opens **new perspectives for the gene therapy of other cardiac diseases**.

C- Pathophysiology of atrial fibrillation

In the atrium and sinus node, $I_{Ca,L}$ is carried by $Ca_v1.2$ (a_{1C}) and $Ca_v1.3$ (a_{1D}) subunits. Because previous studies had shown that $Ca_v1.3$ knockout mice ($Cav1.3^{-/-}$) exhibit sinus node dysfunction,^{79,80} we further characterized them *in vivo* and demonstrated that they also present spontaneous episodes of atrial fibrillation (AF). The phenotype of $Cav1.3^{-/-}$ mice is thus reminiscent to the brady-tachycardia syndrome ("maladie rythmique de l'oreillette"), which associates sick sinus syndrome, episodes of AF and atrioventricular conduction disturbances. We will use the $Cav1.3^{-/-}$ mouse model to elucidate the pathophysiology of this syndrome. More precisely, we will investigate whether susceptibility to AF is the result of chronic sinus node dysfunction, secondary to the hypertrophy produced by chronic bradycardia or directly related the loss of $Ca_v1.3$ -related $I_{Ca,L}$ component in atrial myocytes.

In parallel, we will characterize a transgenic mouse model expressing, in a cardiac specific manner, the S140G-mutated KCNQ1 channel that is responsible for a familial form of idiopathic AF.⁸¹ Our preliminary results show that these mice exhibit spontaneous atrial extrasystoles and are more prone to develop AF under programmed electrical stimulation. Our objective is to identify the mechanisms linking the gain of function of the mutated KCNQ1 channel and the occurrence of AF.

These studies should provide new insights in understanding the pathophysiology of AF, which not only is the **most common arrhythmia** but also represents a **challenge for the clinician** who lacks efficient therapies. Our results on the $Cav1.3^{-/-}$ mouse prompted Jean-Jacques Schott (theme IA) to screen for mutations in the $Ca_v1.3$ gene in patients affected by sick sinus syndrome or lone AF. This ongoing study has already yielded mutants demonstrating the pertinence of the mouse model.

THEME E: β -adrenoceptors (C. Gauthier)

I- Previous activities

Our team has characterized the expression and function of β_3 -adrenoceptors (β_3 -ARs) in the cardiovascular system and their putative involvement in cardiovascular diseases such as heart failure (HF) and hypertension.

In human endomyocardial biopsies, we have demonstrated that β_3 -AR stimulation, in marked contrast with that of β_1 - and β_2 -ARs, induces a decrease in contractility through activation of $G_{i/o}$ proteins⁸² and stimulation of the nitric oxide (NO) pathway leading to an intracellular cGMP increase⁸³. Similar results have been obtained in dog. However, β_3 -ARs are not endogenously expressed in hearts of small animals as rats or mice. We thought to explore whether rabbits could represent a relevant alternative to dogs. In rabbit cardiomyocytes, β_3 -AR stimulation induces negative inotropic and positive lusitropic effects associated with inhibition of L-type Ca^{2+} current and activation of the delayed rectifier K^+ current (I_{Ks}) through $G_{i/o}$ -NOS pathway. These results suggest that β_3 -ARs could be relevant target for the treatment of arrhythmias by decreasing Ca^{2+} overload and preventing prolongation of repolarization.

At end stages of human HF, β_3 -ARs are upregulated although the negative inotropic effect induced by a β_3 -AR agonist is blunted⁸⁴. At earlier stages, the β_3 -AR upregulation may be a compensatory mechanism preventing further cardiomyocyte damage. Indeed, in transgenic mice overexpressing

⁷⁹ Zhang Z, He Y, Tuteja D, Xu D, *et al. Circ Res* 2002, 90:981-7.

⁸⁰ Mangoni ME, Couette B, Bourinet E, *et al. Proc Natl Acad Sci USA* 2003, 100:5543-8.

⁸¹ Chen YH, Xu SJ, Bendahhou S, *et al. Science* 2003, 299:251-4.

⁸² Gauthier C, Tavernier G, Charpentier F, *et al. J Clin Invest* 1996, 98, 556-562

⁸³ Gauthier C., Leblais V., Kobzick L, *et al. J Clin Invest* 1998, 102:1377-1384.

⁸⁴ Moniotte S., Kobzick L., Feron O, *et al. Circulation* 2001, 103(12):1649-1655.

human β_3 -AR in cardiomyocytes⁸⁵, no histological evidence of myocyte hypertrophy or fibrogenesis is observed, suggesting that β_3 -AR activation is not responsible for cardiac damages during HF.

In rat thoracic aorta, the stimulation of endothelial β_3 -ARs produces slowly developing relaxation^{86,87} leading to NO pathway activation and subsequent increase in intracellular cGMP levels. Several K^+ channels: B_{KCa} , K_{ATP} and K_v , are activated. We obtained similar results in human internal mammary arteries⁸⁸. Our work highlights the role of β_3 -AR in vasomotor control of internal mammary arteries and opens new fields of investigation in coronary bypass graft management.

In hypertension, there are only few data regarding the potential role of vascular β_3 -ARs. Using a canine peri-nephritic hypertension model, we suggested that β_3 -AR stimulation might be beneficial by normalizing the blood pressure⁸⁹. In spontaneously hypertensive rats, we have described a β_3 -AR overexpression, although this was not associated with an increased β_3 -AR-dependent vasorelaxation⁹⁰.

The relevance of β -blockers in the treatment of HF is now firmly established even as a first-line treatment of congestive HF. Whether vasodilating properties of β -blockers may contribute to the clinical effects is debatable. A recent clinical trial demonstrated that nebivolol is an effective and well-tolerated treatment for HF in the elderly⁹¹. However, its target is not clearly identified. We have recently shown that in rat aorta, nebivolol-induced relaxation results from activation of β_3 -ARs. In addition, we have confirmed the involvement of endothelium and NO pathway in the vascular response⁹². We have also described a β_3 -AR activation by nebivolol in human biopsies.

II- Current projects

Identification of β_3 -ARs in human heart has changed the classically admitted paradigm on the regulation of heart function by the β -adrenergic system. β_3 -AR activation induces negative inotropic effects, associated with acceleration of relaxation and decreased intracellular calcium levels⁹³. Regarding the role of β_3 -AR on the heart rate, only a few studies have been performed with no formal conclusion.

β_3 -ARs stimulation may offer protection against excessive catecholaminergic β_1 -AR stimulation. It is well established that β_1 -ARs are down-regulated in HF and several studies reported an upregulation of β_3 -AR. Current clinical uses of β -blockers include treatment of primary and secondary prevention of myocardial infarction in patients with coronary artery disease, atrial and ventricular arrhythmias, arterial hypertension, and, most recently, HF. However, β -blockers are still considered underused in current clinical practice. In addition, elevated heart rate is a well-established independent predictor of coronary artery disease and cardiovascular morbidity and mortality. One possible mechanism of cardioprotection elicited by β -blockers is bradycardia.

Thus, inversely to β_1 - or β_2 -ARs, β_3 -ARs inhibit cardiac contractility. We hypothesized that β_3 -ARs may have a buffer or 'rescue' function in front of excess catecholamines; such as occur in hyperadrenergic states including heart failure. Until now, only dogs were suitable as a model to analyze cardiac β_3 -ARs. Our preliminary results indicate that rabbit could be an appropriate alternative, which reproduces the contractile effects obtained in human heart.

A- β_3 -adrenoceptors in the heart

1) Function

At present, the precise and specific physiological role of cardiac β_3 -ARs is not fully understood, leading to conflicting results in *in vitro* and *in vivo* studies. We will evaluate the consequences of β_3 -AR stimulation in comparison with β_1 - and /or β_2 -AR stimulation in rabbit isolated cardiomyocytes (video-imaging), papillary muscles, whole heart (Langendorff) and *in vivo* (pressure-volume loops). Our preliminary results obtained in isolated rabbit cardiomyocytes show that β_3 -AR stimulation produces negative inotropic and positive lusitropic effects associated with a reduction in I_{CaL} . as a result from $G_{i/o}$ -NO pathway activation. Surprisingly, these effects are not seen in the whole heart. Additional experiments are needed to explain such discrepancy.

It is admitted that β -AR stimulation increases the heart rate. However, the β -AR subtype involved in this effect is not yet fully identified. In clinical practice, administration of dobutamine, a β_1 -AR agonist, is arrhythmogenic. Salbutamol, a β_2 -AR agonist, increases heart rate but also prolongs the QT interval. One study suggests that the β_3 -AR stimulation produces opposite effects on the heart rate depending

⁸⁵ Tavernier G, Toumaniantz G, Erfanian M, *et al. Cardiovasc Res* 2003, 59: 288-296.

⁸⁶ Trochu J.N., Leblais V., Rautureau Y, *et al. Br J Pharmacol* 1999, 128: 69-76.

⁸⁷ Rautureau Y., Toumaniantz G., Serpillon S, *et al. Br J Pharmacol* 2002, 137(2), 153-161.

⁸⁸ Rozec B., Serpillon S., Toumaniantz G, *et al. J Am Coll Cardiol* 2005, 46: 351-359.

⁸⁹ Donckier J.E., Massart P.E., Van Mechelen H, *et al. Eur J Clin Invest* 2001, 31(8):681-689.

⁹⁰ Mallem Y., Toumaniantz G, Serpillon S, *et al. Br J Pharmacol* 2004, 143: 599-605.

⁹¹ Flather, M.D., Shibata, M.C., Coats, A.J, *et al. Eur Heart J* 2005, 26: 215-225.

⁹² Rozec B., Tran Quang T., Noireaud J, *et al. Br J Pharmacol* 2006, 147: 699-706.

⁹³ Rozec B., Gauthier C. *Pharmacol Therap* 2006,111: 652-673.

on the activated signaling pathway⁹⁴. We will compare the effects of β_1 , β_2 and β_3 -AR stimulation on rabbit cardiac electrical activity (K^+ currents and action potential) and the respective activated signaling pathway will be determined. This project will be carried out in association with F. Charpentier.

2) Localization

To complete the functional studies, we plan to evaluate β_3 -AR expression and cellular localization in the different cardiac chambers as well as in the conductive tissue of the rabbit heart. We have successfully tested molecular and biochemical tools used in rats. In addition, in human atria and ventricles, β_3 -ARs have been identified, but their stimulation does not lead to the same contractile effect. Indeed, in atria, β_3 -AR stimulation does not influence contractility⁹⁵. Thus, the roles of β_3 -ARs in atria remain to be clarified.

3) Signaling pathways

We have demonstrated that β_3 -ARs are linked to $G_{i/o}$ -NO-cGMP pathway in the human ventricle. The cAMP pathway seems to be involved in the regulation of heart rate. Thus, it is necessary to evaluate cAMP and cGMP respective levels and downstream pathways after β_3 -AR stimulation on heart rate and contractility. It has been shown that cardiac β_1 and β_2 -AR stimulations elicit distinct subsarcolemmal cAMP signals, which are regulated by different phosphodiesterases (PDEs). In collaboration with R. Fischmeister (Inserm U769), we will determine the PDE isoforms involved in β_3 -AR effects in rabbit cardiomyocytes and their putative cross-talk with the signaling pathways of β_1 and β_2 -ARs. The β_3 -AR effects will also be evaluated on the Ca^{2+} cycle (collaboration with D. Potreau CNRS UMR 6187, Poitiers).

B- β -adrenoceptors subtypes and heart failure

In failing heart, the increased adrenergic tone and the impaired global β -adrenergic response are well known, but the relative contribution of each β -AR subtype and their cross-talks have received little or no attention. Different data obtained both in normal and failing hearts support the hypothesis that upregulation and/or stimulation of β_3 -AR-mediated pathway may prevent myocardial damage under catecholamine excess, particularly in early stages of the disease. Several animal models could be used to investigate the consequences of cardiac ischemia. In the rabbit, we will evaluate the expression of each β -AR subtypes after an infarct (collaboration with B. Ghaleh, Inserm U660, Créteil). Another suitable model is the ischemic rat model of HF. This model will permit investigations of either "basal" remodeling of β -adrenergic system or after treatment with different β -blockers. We will compare the expression level and localization of each cardiac β -AR subtypes as well as the electrophysiological and contractile responses at different stages of HF in ischemic rats.

As β_3 -ARs are overexpressed at end stage of HF and β_3 -AR are overexpressed both in endothelial cells and cardiomyocytes, we have developed a transgenic rat model (Tg β_3) overexpressing human β_3 -AR in endothelial cells. Preliminary results show that endothelial β_3 -AR overexpression is associated with β_1 -AR decrease in cardiomyocytes whereas β_2 -ARs are unchanged. In addition, the isoproterenol-induced positive inotropy is also altered. These results demonstrate for the first time that endothelial β_3 -ARs regulate cardiac contractility and β -AR expression in cardiomyocytes. It remains to determine the cellular mechanisms involved in the cardiac remodeling.

Concurrently, and in collaboration with Pr J.L. Balligand (Brussels, Belgium), we will use replication-deficient adenovirus encoding human β_3 -AR to evaluate on cardiac expression remodeling and electrical and contractile activities, the effects of β_3 -AR overexpression by delivering the adenovirus either into the myocardium bordering the ischemic area, in the whole heart, or in the coronary system. The pro-angiogenic effect of β_3 -AR overexpression will be evaluated.

Finally, we will determine whether β_3 -AR agonist or antagonist properties of different β -blockers in accurate therapeutic windows could improve outcome, which underlying mechanisms are implied and the putative cross-talks with other pathways involved. We will compare the effects of several β -blockers on electrophysiological and contractile responses. This project should lead to determine the different β -AR targets of β -blockers in order to optimize their use for a given patient.

⁹⁴ Sterin-Borda L, Bernabeo G, Ganzinelli S, *et al.* *J Mol Cell Cardiol* 2006, 40: 580-588.

⁹⁵ Pott, C., Brixius, K., Bundkirchen, A, *et al.* *Br J Pharmacol* 2003, 138 : 521-529.