Disruption of Gravin's Scaffolding Protects against Isoproterenol Induced Heart Failure in Mice

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A Dissertation Presented to the

Faculty of the Department of Pharmacological and Pharmaceutical Sciences

College of Pharmacy, University of Houston

In Partial Fulfillment of the

Requirements for the Degree of

Doctor of Philosophy

In

Pharmacology

By

Sonal Singh

August 2015

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ACKNOWLEDGEMENTS

First and foremost I would like to thank my advisor, Dr. Bradley McConnell without whom this entire project and my dissertation would not have been possible. He was a constant source of encouragement and guidance to me in both my academic and personal life. His biggest support was the very fact that he gave me the freedom to explore and make mistakes. Thank you for trusting me and being the best dissertation advisor that I could have hoped for. Next I would like to express my gratitude to my committee members. Special thanks to Dr. Richard Bond, for his constant guidance that helped my not only as a graduate student but has ingrained principles in me that will help me as a scientist as well. I want to thank Dr. Tahir Hussain, for his critical insights that helped me improve my dissertation. I also want to thank Dr. Robert Schwartz for guiding me to think beyond and look at the bigger picture. Also, I want to thank Dr. Ravi Birla, for his help.

I would like to express my sincere gratitude to Dr. Douglas Eikenburg for his guidance and support to me as a graduate student and inspiring me to do better. I also want to thank Dr. Samina Salim for her help and support during hard times. I am forever in their debt.

I cannot thank Santosh Suryavanshi enough for all his help during my PhD. He has been not only an excellent colleague but also a dear friend who has been a big support to me not only in my project but also in my personal life. I also want to thank Faisal Fa'ak for his help with the echocardiograph data on this project and for his constant encouragement and positive attitude. Also special thanks to Andrea Diaz Diaz for being such a good friend and teaching me so many things in the lab. Special thanks to the other members of the lab – Abeer Rabab'ah, Cori Wijaya, Qiying Fan, Peter Vu, Henry Vo, Pranav Shah, Prajakta Gadgil, Sumit Basu, Aisha Vadhariya for their help and for being such dear friends. I have been extremely lucky to meet such wonderful people during my PhD.

Finally, I want to thank my family for their support without whom I would not have come this far. I want to thank my parents for giving me the best life and teaching everything that has helped me establish myself. I want to thank my dad for all his support, for always teaching me to dream big and for all the hardships that he went through to help me achieve my dreams. I want to thank my mother, for her constant emotional support. Lastly, I want to express my deepest gratitude to my husband, Dhruvit for making my career dreams his priority and his never-ending care, concern and love. I can never thank him enough for his constant patience and support especially during the stressful times.

I am truly honored to have worked on this project.

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Abstract

Impairment in the β adrenergic pathway is one of the hallmark pathologies of heart failure. This vicious cycle starts with chronic stimulation of the βARs during heart failure causing their severe desensitization and down regulation. This causes further downstream molecular dysfunction leading to worsening of heart failure. Gravin is a scaffolding protein belonging to the family of A Kinase Anchoring Proteins and makes a very attractive target for heart failure. As an AKAP, gravin binds to multiple proteins such as PKA, PKC, β_2ARs, PDE4D3, PP2B etc. and is involved in the modulation of the desensitization and resensitization cycle of β_2 ARs indicating that disruption of gravin's scaffolding action might prove beneficial in heart failure.

In our studies we used isoproterenol to chronically stimulate the βARs to induce heart failure. We found that gravin t/t mice harboring the truncated non functional gravin leads to restriction of the extent of receptor desensitization during chronic βAR stimulation, due to loss in the assembly of the desensitization machinery mediated by the scaffolding of the gravin. The βAR density is significantly higher in the gravin t/t mice than the WT animals after 2 weeks of isoproterenol treatment. This decrease in the desensitization leads to the availability of more receptors on the heart to mediate the chronotropy, ionotropy and lusitropy, which

translated into the gravin t/t animals exhibiting enhanced cardiac function in face of the chronic stimulation. Major proteins such as cTnI, PLB, cMyBPC etc., which show aberration activation patterns during heart failure had normal phosphorylation levels in the gravin t/t mice, compared to the WT mice. Gravin t/t mice also displayed enhanced myofilaments sensitivity to calcium compared to the WT mice.

Our results indicate that gravin plays an important role in regulating the βAR pathway and disruption of its scaffolding augments cardiac function. Hence blocking of gravin's scaffolding can be further explored as a potential therapeutic target for heart failure.

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LIST OF ABBREVIATIONS

AC - adenylyl cyclase

AKAP - A-kinase anchoring protein

β-AR beta-adrenergic receptor

β_1-AR beta 1-adrenergic receptor

β_2-AR beta 2-adrenergic receptor

Ca^{2+}: calcium

CaMKII: Ca^{2+}/calmodulin-dependent protein kinase II

cAMP: 3'-5'-cyclic adenosine monophosphate

cMyBPC: cardiac myosin binding protein C

CO: cardiac output

EC-coupling: excitation-contraction coupling

EF: ejection fraction

FS: fractional shortening

G_i: inhibitory GTP-binding protein

G_s: stimulatory GTP-binding protein2

GRK2: G-protein coupled receptor kinase 2

HR: Heart rate

HF: Heart Failure

ISO: isoproterenol

IVSd: intra-ventricular septum at diastole

IVSs: intra-ventricular septum at systole

LV: left ventricular

LVPWd: left ventricular posterior wall diameter during diastole

LVPWs: left ventricular posterior wall diameter during systole

LVIDd: left ventricular inner diameter during diastole

LVIDs: left ventricular inner diameter during systole

NCX: sodium/calcium exchanger

PDE4D: phosphodiesterase type 4D

PDE4D3: Phosphodiesterase type 5D

PKA: protein kinase A

PKC: protein kinase C

PLB: phospholamban

PP2B: protein phosphatase 2B

RyR: ryanodine receptor

SERCA2a: sarcoplasmic reticulum Ca^{2+} ATPase

SR: sarcoplasmic reticulum

SV: stroke volume

TnI: troponin I

TnC: troponin C

WT: wild-type

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1. Introductions and Statement of Problem

American Heart Association defines heart failure (HF) as the condition wherein the heart is unable to pump enough oxygenated blood to meet the demands of the body. According to the American Heart Association's recently released statistics (Heart Disease and Stroke Update for the year 2015), HF is not only the leading cause of death in USA but also the most common cause for hospitalization. Around 5.7 million American's suffer from HF and it is alarming that by the year 2030 it is estimated that this prevalence will increase to around 8 million people. HF is a major health burden as in the year 2010 alone the direct and indirect cost of treating failure was estimated to be around $39.2 billion(Mozaffarian et al., 2015).

HF results in the reduction of the pumping efficiency of the heart, which is a result of reduced contractility. β-ARs are the primary mediators of contractility and during HF they undergo chronic stimulation to keep up the pumping efficacy of the heart. However, this chronic stimulation causes their desensitization and down regulation. During HF, the signaling mechanisms undergo severe dysfunction especially the downstream pathways that work subsequent to β-AR activation (Insel and Hammond, 1993; Katz and Lorell, 2000). PKA - a major effecter of the β-AR pathway undergoes significant posttranslational modifications, effects of which persist long after blockade of the chronic

stimulation of the β-ARs. PKA aberrantly phosphorylates its downstream targets such as TnI and MyBPC undergo hypophophorylation during HF whereas there is significant increase in the phosphorylation of the Ryanodine receptors. Other proteins such as GRK2, SERCA2a, Gi, and RII alpha subunit of PKA also show altered expression(Han et al., 2013; Wehrens and Marks, 2004). Altered protein expressions also alters the calcium handling in the cardiomyocytes which not only decreases the cardiac function but also reduces the responsiveness of the heart to stimulation and induces cardio toxicity resulting in apoptosis (Chakraborti et al., 2007). These changes lead to activation of pro-hypertrophic signaling mechanisms that further lead to cardiac remodeling and increase in the expression of hypertrophic markers. All these perturbations in the physiology, morphology and molecular mechanisms of the heart lead to severe cardiac dysfunction (Kehat and Molkentin, 2010a, b).

The current therapies are targeted towards increasing the heart function and alleviating the symptoms of HF either by rescuing the desensitization of the β-ARs (β-blockers), or by increasing the cAMP concentrations (phosphodiesterase inhibitors), or by reducing the stress inducers such as hypertension (angiotensin onverting enzyme inhibitors, angiotensin receptor blockers, diuretics) or by using agents that directly increase the ionotropy such as digoxin etc. Most of these therapies are targeted towards improving the

symptoms of HF and do not rescue the failing heart. So other than detrimental side effects these therapies are also plagued by the fact that they do not halt the progression of the disease. Hence in spite of developments in the medical therapy of the HF, there is a need for newer therapies that are targeted towards alleviating the root causes of HF without inducing significant amount if side effects (Cleland et al., 1999; Cohn, 1988; Lymperopoulos et al., 2013; Mudd and Kass, 2008; Packer et al., 2002).

Gravin which is an A Kinase Anchoring Protein (AKAP) is a very attractive target for HF. It is involved in modulating very important signaling pathways involved in the cardiac function. It scaffolds key proteins such as β_2-AR, PKA, PKC etc. involved in maintenance of cardiac function. Also, gravin is required for the complex formation of GRK and β-arrestin with β-ARs during desensitization and internalization (Lin et al., 2000a; Tao et al., 2003). Gravin thus modulates the cross talk between several signaling pathways important for mediating contractility. Moreover absence of gravin increases the myofilament sensitivity to calcium, which can prove very beneficial as it can circumvent the cardio toxicity and side effects that come hand in hand with the current treatment strategies that are aimed towards increasing the intracellular calcium concentration in the myocytes (Guillory et al., 2013).

2. Review of the literature

HF is a complex syndrome, which can be a result of various kinds of heart diseases affecting the pumping efficiency of the heart. A normal healthy heart is deemed as the workhorse of the body, which pumps about 7200 liters of blood daily through an individual to enable the transport of nutrients and excretion products to and from the tissues and organs. Various cardiovascular diseases such as myocardial infarction, hypertension, valvular heart disease etc. can adversely affect the contractile function of the heart due to damage to the heart muscle. Also other conditions such as hypertension, thyroid disease, birth defects etc. can severely over work the heart resulting in the impairment of the contractile function of the heart. HF is the culmination and the result of this progressive contractile dysfunction (Hilfiker-Kleiner et al., 2006).

2.1 Contractility of the Heart

Heart is a very unique organ compared to other striated tissue on account of its myogenic ability to contract without any external signal. The heart has the ability to generate its own stimulus that is carried down a complex conduction system that ensures effective contractility via diastole and systole. The beating of a heart consists of cascade of synchronized events that adjust the amount of blood pumped by the heart in response to changing heart rate, afterload, preload etc (Li and Atlas, 2015; Poole-Wilson, 1982). This maintenance of stroke volume

is essential for the oxygenated blood to reach all the organs of the body. Also changes in pH, concentration of calcium, increased oxygen demand etc also can alter the contractility of the heart (Poole-Wilson, 1982; Veksler et al., 1985).

2.2 Excitation-Contraction Coupling:

Excitation contraction coupling is a series of events that initiates with action potential mediated electrical excitation of cardiac myocyte and culminates with the contraction of the heart. Calcium is the important ionophore that is essential for the modulation of the cardiac action potential currents (Balke and Goldman, 2003). Cardiac troponin C (cTnC) and cardiac troponin I (cTnI) are two major sarcomeric proteins that interact with calcium and modulate the actin-myosin cross bridging during excitation-contraction coupling of cardiac fibers (Sharma et al., 2004). It starts with an action potential depolarizing the cardiac myocyte, which causes an influx of extracellular calcium into the cytosol via the activation of the voltage gated L-type calcium channels on the sarcolemma. Calcium is a direct regulator of cardiac contractility via its modulation of the myofilaments (Hobai and Levi, 1999). Influx of calcium in the cytosol further triggers the calcium release from the sarcoplasmic reticulum (SR) via the activation of the ryanodine receptors. This phenomenon is known as calcium induced calcium release (CICR). This causes significant increase in the intracellular concentration of calcium. Binding of this calcium to the cTnC causes

a conformational change in the TnI in a way that it exposes the actin which then binds with the myosin leading to the cross bridging of actin-myosin. The myosin heads upon binding to actin causes them to slide past each other thereby causing the contraction because of the sarcomere shortening. The calcium concentration then reduces in the cytosol, as the SR reuptakes calcium with the help of a pump known as SERCA (Sarcoplasmic Endoplasmic Reticulum Calcium ATPase). Other than SERCA, NCX (sodium calcium exchange) pump located on the sarcolemma is also involved in the efflux of the calcium from the cytosol. These two mechanisms remove major fraction of the calcium from the cytosol. The remaining calcium is effluxed out via the mitochondrial calcium uniport and sarcolemmel calcium ATPase. The exact ratio of the amount of calcium effluxed by each of these mechanisms is dependent on the species. This reduction in the cytosplasmic calcium concentration again induces conformational changes in the troponin complex leading the cTnI to inhibit the actin again resulting in the relaxation of the sarcomere. (Bers, 2002)

2.3 Adrenergic Regulation of Cardiac Contractility:

Adrenergic system is one of the most important systems, which contributes to the cardiac function. It is a multifaceted system comprising to various branches which all work together cohesively to maintain normal cardiac function and contractility. At rest or under normal conditions the major control

over the heart is exerted by the parasympathetic system via the vagal innervation activating the muscarinic receptors by acetylcholine, which lead to the inhibitory control over the heart and maintain the heart rate and contractility at baseline. However during conditions which exert the heart such as stress or exercise, the parasympathetic innervations is slowly withdrawn and overtaken by the sympathetic drive which focus on increasing the heart rate and contractility to meet the increased demands of the body of blood and oxygen via the epinephrine and nor epinephrine hormones.

The nerve terminals of the sympathetic system primarily release the epinephrine whereas epinephrine majorly comes from the adrenal glands. The heart is thus innervated by both the parasympathetic as well as the sympathetic tone. However the parasympathetic innervation is mostly restricted to the atria whereas the sympathetic innervates both the atria and the ventricles. Hence the parasympathetic system controls only the heart rate whereas the sympathetic system controls both the heart rate as well as the contractility (Lymperopoulos et al., 2013; Malliani et al., 1983; Triposkiadis et al., 2009).

2.4 Adrenergic Receptors Signaling:

The Autonomic Nervous System (ANS) mediates all these function via the adrenergic receptors (ARs). The adrenergic receptors are basically comprised of

3 receptor types of G- protein coupled receptors and 9 receptor subtypes. The α_1 (α_{1A}, α_{1B}, α_{1C}), α_2 (α_{2A}, α_{2B}, α_{3C}) and the β (β_1, β_2, β_3) ARs. The β-ARs comprise the majority of the receptors in the heart whereas the α receptors comprise a very small fraction. The alpha adrenergic receptors couple to G_q leading to the activation of phopholipase C which then mediates the activation of the secondary messengers IP_3 and DAG. Activation of IP_3 and DAG further changes the calcium concentration in the cells leading to vasodilation of smooth muscles, glycogenolysis, inhibition of insulin release etc.

β-ARs are primarily responsible for maintaining the positive chronotropy, ionotropy and lusitropy of the heart. Of the three subtypes that have been identified so far, β1 are the most expressed and comprise of 70-80% of the total β receptors. β_2 receptors on the other hand comprise of 20-30% and the β_3 receptors comprise of only 2-3% of the total receptors. Of these β_1 receptors are majorly responsible for mediating the contractility of the heart. Upon stimulation by the endogenous catecholamines, epinephrine and norepinephrine the G protein coupled β receptors which are coupled to G_s get activated leading to the dissociation of the α subunit from the βγ subunit which then leads to the activation of the adenylyl cyclase (AC) and the downstream secondary messenger- cAMP (3'-5'-cyclic adenosine monophosphate). β_2 –AR on the other hand can couple to both G_s and Gi. Gi pathway functions as an inhibitory pathway, which keeps the activation of the Gs pathway in check. Coupling of β_2 –

AR to the Gi causes a reduction in the production of the cAMP and leads to the attenuation of the AC pathway(Lohse et al., 2003).

Activation of the secondary messenger cAMP by the β receptors leads to the activation of Protein Kinase A (PKA) which then phosphorylates the downstream targets like the L-type calcium channel, ryanodine receptors, cTnI and cTnC etc. leading to changes in the amplitude of the calcium present inside the myocytes which ultimately translates to the contraction and relaxation of the heart. The switching from Gs to Gi of the β_2 –AR is primarily promoted by its phosphorylation by PKA (Daaka et al., 1997). The G_i mediated signaling on the other hand propagates via the βγ subunit and leads to the activation of the ERK pathway, which mediates anti-apoptotic function via the PI3K pathway(Zhu et al., 2005).

β-ARs distinctly differ in the signaling mechanisms even though both of them signal via the Gs pathway. Recent evidences have brought under focus the distinct PKA substrate phosphorylation based on the activation by β1 –AR vs. β_2 –ARs. In canine and human hearts it was been reported that phospholamban becomes phosphorylated upon β_1 –AR activation but not β_2 –ARs. Similarly one group has reported that cTnI undergoes phosphorylation in rats upon β_1–AR activation only(Kuschel et al., 1999). On the contrary, both the proteins undergo

phosphorylation upon β_1–ARs as well as β_2–ARs activation in human cardiomyocytes (Bartel et al., 2003; Molenaar et al., 2000). These β-AR subtypes differ in their localizations in the cardiomyocytes. The β_1 ARs are uniformly present on the cardiomyocytes whereas β_2 ARs are preferentially present on the caveole. Their distinct spatial temporal associations can explain these differences in the β1–ARs vs. β2–ARs pathway (Rybin et al., 2000).

2.5 Adrenergic receptors signaling during HF

During HF there is impairment of the balance between the sympathetic and parasympathetic drive, wherein the sympathetic tone increases significantly leading to an increased amount of circulating epinephrine and norepinephrine in the system. Studies have also reported an impairment in the feedback mechanism of the α_{2A} receptors which lead to abrogation of the negative feedback mechanism to keep the levels of epinephrine and nor epinephrine under control (de Lucia et al., 2014). In the normal conditions the contractility is primarily mediated via the β1 ARs with β2 AR's contributing very little to the physiological functioning of the heart as they comprise only 20-30% of the receptors. However, during HF, this sympathetic overdrive chronically stimulates the β AR receptors due to which β1 AR's undergo downregulation and homologous and heterologous desensitization which flips the β1: β2 ratio to almost 50:50(Bristow et al., 1986). Homologous desensitization occurs as a

result of the phosphorylation of the β ARs by the GRKs, which also phosphorylate and desensitize the α_{2A} receptors that further contribute to the impairment of the feedback mechanism to regulate the levels of the circulating catecholamine. Heterologous desensitization occurs as a result of the phosphorylation of the β ARs due to PKA and PKC (Bristow et al., 1982). Also, the expression of the G-protein kinases is up regulated. GRK's are primarily responsible for the phosphorylation of the GPCR's. Two major isoforms of the GPCR is expressed in the heart – GRK2 also known as β-ARK1 majorly and GRK5 to a smaller extent. Also the constant activation of the β_1 ARs by the endogenous catecholamines leads to the activation of the CAMKIIδ pathway that further activates the pro-apoptotic pathways further worsening heart failure. Also during HF, the β_2 ARs uncouple from the G_s and mediate signaling via the G_i pathway leading to the activation of the ERK pathway, which has been shown to anti-apoptotic in nature. These changes make the β_2 AR an equally important player in HF (Brodde, 1993; Fu et al., 1991). These alterations in HF not only affect the receptors but also the downstream signaling moieties. It has been shown that the over activation of the PKA lead to the hyperphosphorylation of the L-type calcium channels and ryanodine receptors and the hypophosphorylation of the phospholamaban. Thus signaling pathways downstream of the adrenergic receptors undergo significant aberrations due to the impairment and downregulation of the adrenergic receptors. These alterations in the molecular

mechanisms and signaling pathways is one of the most important underlying pathologies of declining heart function which ultimately progresses to HF.

2.6 A-Kinase Anchoring Proteins

Tightly regulated signaling pathways following receptors activation translate into physiological responses. Thus, precise and efficient signal transduction is an extremely important phenomenon. One such family of proteins that is involved in increasing the efficiency and timing of the signal transduction pathways is A-Kinase anchoring proteins (AKAPs). They basically increase the efficiency of the signaling pathways by interacting with multiple kinases and phosphatases to co-ordinate of rapid phosphorylation and dephosphorylation of proteins, which leads to the rapid turn on, and turn off of the pathways(Langeberg and Scott, 2005).There are around 70 AKAPs that have been identified so far. All of these are structurally diverse proteins.

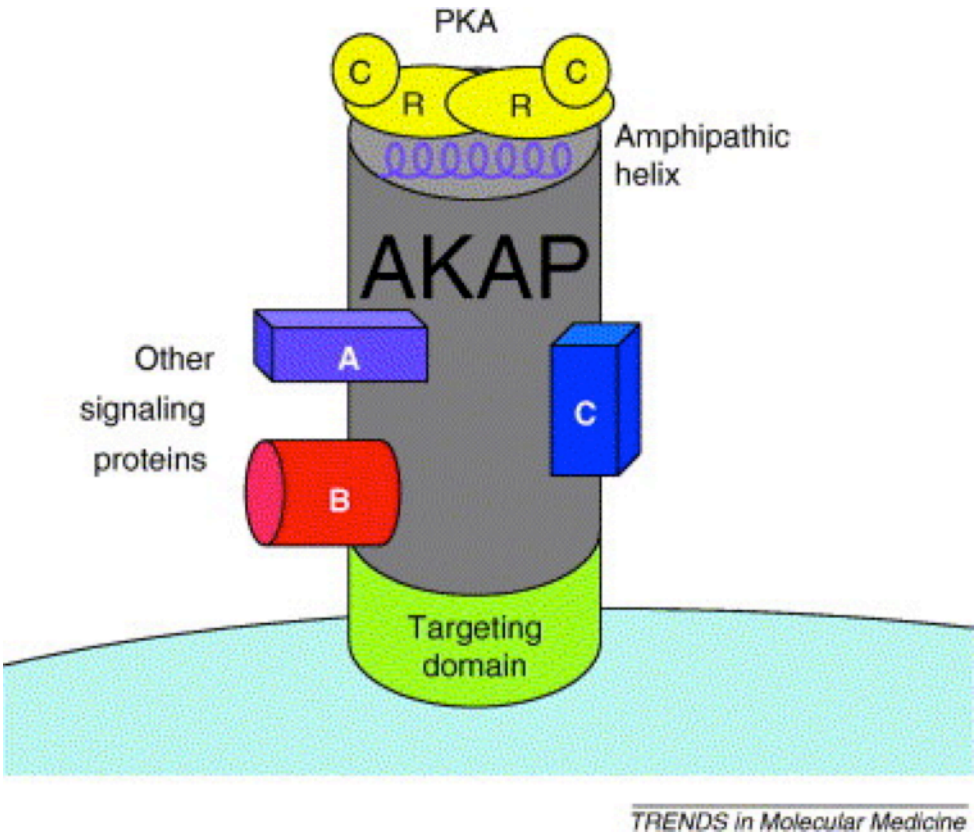


Figure 1. Schematic showing the structural organization of the AKAPs with the three distinct characteristic binding sites

All these AKAPs share 3 distinct characteristics that form the basis of their characterization as an AKAP. These features are:

1. All AKAPs have conserved amphipathic helix domain, which enables them to bind to the R subunit of PKA. However some AKAPs have been reported which bind to the RIα subunit as well and some AKAPs bind to both the

subunits(Angelo and Rubin, 1998). This targeted localization of PKA provides specificity of the PKA phosphorylation of the downstream targets.

2. A targeting domain that enables the AKAP to bind to sub cellular location such as plasma membrane, nucleus etc. which tethers and targets the signalosome complex to that subcellular location.
3. All AKAPs have multiple binding domains, which enables them to bind to proteins other than PKA, including other GPCR's, kinases, phosphatases etc.

On account of these characteristics, AKAPs are capable of forming signalosome complexes in response to activation of the receptors upstream(Colledge et al., 2000; Taskén and Aandahl, 2004).

2.7 AKAPs and Heart

Of the 70 AKAPs that have been identified so far, around 14 of them are present in the heart (rodent and human). Many of these AKAPs are involved in the localization of the PKA to various subcellular compartments in the heart. Majority of these AKAPs are involved in the regulation of the local signaling pathways in the area of the myocyte where they are localized. They are involved in the modulation of the protein-protein interaction via their scaffolding which influences important aspects of *in vivo* heart function such as excitation

contraction coupling, calcium handling, adrenergic signaling, hypertrophic pathways etc. (McConnachie et al., 2006).

Yotiao, which is an AKAP and its interaction with PKA, has been widely studied in the neurons. However Yotiao is also involved in the signalosome complex formation, which includes PKA, PDE4D, PP1 and KCNQ1 potassium channel (Marx et al., 2002). KCNQ1 generates slow outward potassium currents subsequent to beta-adrenergic stimulation. Binding of Yotiao to KCNQ1 is essential for the cAMP sensitive current generation. Moreover, studies have shown that alteration in the binding of Yotiao to KCNQ1 (due to mutation in Yotiao) leads to loss of response to beta-adrenergic signaling(Chen et al., 2007; Saucerman et al., 2004). AKAP18, is a low molecular weight AKAP present in the heart and brain. It is targeted to the plasma membrane wherein it binds and regulates the function of LTCC (Trotter et al., 1999). Studies have shown that inhibition of binding of LTCC and AKAP18 results in ablation of LTCC's to cAMP stimulation. Transgenic mouse models developed on similar premise wherein LTCC is unable to bind to AKAP18 leads to the development of cardiac hypertrophy (Fu et al., 2011).

mAKAP is one of the other AKAPs that has been well studied and characterized in the heart. It binds to PKA, PP2A, PP2B, PKA, RyR2 receptors,

nesprin 1 alpha, ERK5, PDE4D3, EPAC1, Adenyl cyclase 5 etc and tethers this entire complex to the nucleus membrane and also the sarcoplasmic reticulum of the cardiomyocytes. mAKAP has been implicated in the modulation of the hypertrophic signaling pathways(Pare et al., 2005). Studies have also shown that mAKAP is involved in the formation of the PKD1-HDAC4 complex which is essential for the activation of the hypertrophic gene expression subsequent to the β ARs chronic activation. mAKAP thus plays a very important role in the PKA complex formation associated with nuclear hypertrophic signaling mechanism(Kritzer et al., 2014). AKAPs are thus involved intricately in the modulation of several pathways that lead to important physiological responses. Other than scaffolding kinases, AKAP's also play an extremely important role in the scaffolding of phosphatases and thus modulate the interplay of kinases and phosphates to balance the responses subsequent to receptor activation(Redden and Dodge-Kafka, 2011).

AKAPs thus not only scaffold and target PKA, but also nucleate signaling complexes, which influences the physiological responses in response to stimulation. Ht31 is a membrane permeable peptide that binds to the RII subunit of PKA and inhibits the binding of AKAP to PKA thus effectively inhibiting the PKA-AKAP interaction. Studies have shown that disruption of PKA-AKAP interaction using Ht31 in cardiomyocytes has significant effect on the cardiac

contractility. Ht31 expressing cardiomyocytes show increased cell shortening in response to β AR stimulation without any change in the calcium amplitude in these cells suggesting that inhibition of PKA-AKAP interaction might result in increased myofilament sensitivity. One of the reasons for this could be the significant reduction in the phosphorylation of the major myofibril proteins such as TnI, MyBPC, PLB, RyR etc., that are involved in the cycling of calcium in the cardiomyocytes (Fink et al., 2001; McConnell et al., 2009).

AKAPs on account of their involvement in the modulation of various aspects of molecular mechanisms involved in cardiac function have significant implication in HF.

2.8 Gravin

Gravin was first identified as a cytoplasmic auto-antigen in the myasthenia gravis patients. It was later studied and characterized as an AKAP (AKAP 12 or AKAP 250) on account of it's binding to the RII subunit of PKA at the 1526-1780 residues. Since then various binding partners of gravin have been identified such as PKA, PKC, polo-like kinase 1, F-actin, FHL1 etc. Various studies report that gravin is a key modulator of the cell cycle, cytokinesis, cell migration, cell adhesion etc. Gravin via its scaffolding with various key signaling molecules mediates these functions. Gravin is highly expressed in the brain, bladder, testis,

lungs and heart. It is present both at the plasma membrane and cytosol(Nauert et al., 1997). Gravin as an AKAP has generated a lot of interest with respect to its multi-functional role and is one of the first AKAPs that has been defined as such (Carnegie et al., 2009).

One of the most well characterized roles of gravin is in cell cycle progression which is mediated via its binding with polo-like kinase 1 (plk-1). Studies have found that cyclin dependent kinase 1 (cdk1)/cyclin B phosphorylates gravin at various sites in vitro as well as in vivo and it is this phosphorylation by the cdk1 that enables gravin to interact with plk-1. All the phosphorylation sites were studied and it was found that phosphorylation of gravin at ser-766 enabled its binding to plk1. Also phosphorylation of gravin had an impact on its localization across the cell at various stages of the cell cycle. Phosphorylation of gravin enables its localization to the nucleus. These studies shed light on not only the role of gravin in cell cycle but also on the fact that gravin is able to partition between the cytoplasm and the nucleus (Canton et al., 2012; Yoon et al., 2007).

Gravin has also been implicated in the cytokineses by interacting with the cyclin D in two ways:

1. Suppressing the expression of cyclin D by the PKC-raf-mek-erk pathway.

2. Binding with cyclinD and sequestering it away form the nucleus in the cytoplasm which is evidence by the fact that phosphorylation of gravin by PKC reduces its binding of cyclinD and that leads to increase in the translocation of the cyclin D in the nucleus(Gelman, 2010).

Studies have also shown that gravin along with PKC and F-actin is co-localized on the abscission furrow of the contractile ring in the growing cells and upon stimulation by the PKC-Rho-GTPase leads to the separation of the daughter cells. This has also been confirmed by doing gravin ablation studies, which show increases in impairment of the cell division as increase in the multinucleated cells(Gelman and Gao, 2006; Lin and Gelman, 1997; Werner and Glotzer, 2008).

Studies have also shed light on some very interesting aspects of gravin with respect to oncogenic growth factors especially, tumor growth and metastasis. Increase in the expression of gravin causes significant reduction of several oncogenic factors. Further evidence came when gravin's expression was found to be upregulated with re-expression of other tumor suppressors such as p53, Smad4 and TGF- β induced cell cycle arrest. Furthermore gravin has also been established as an important role player in the suppression of angiogenesis and up regulation of the tight junction formation in the blood brain barrier (Lee et al., 2003). Thus gravin as an AKAP is very diverse with multiple binding partners and

has a significant role to play not only in cell cycle, cytokinesis, cell adhesion, angiogenesis, tumor suppressor etc. (Akakura and Gelman, 2012; Gelman, 2010; Lin et al., 2000b; Xia et al., 2001).

2.81 Role of gravin in HF

Gravin plays a very important role in the β AR pathway. It does so by not only targeting PKA but also a battery of other key components involved in the heart function such as β2 AR, PKC, PP2B etc. (Fan et al., 2001; Nauert et al., 1997; Shih and Malbon, 1994). Gravin is also involved in the PKA dependent complex formation of the β_2 AR at the membrane on account of having a docking site which involves other AKAPs such as AKAP79 (AKAP5). This is the reason that studies have shown association of AKAP79 with β_2AR in spite of no direct binding between the two proteins (Figure 2) (Cong et al., 2001; Lin et al., 2000a).

Gravin binds to β_2 ARs under basal conditions. However upon agonist stimulation there is significant increase in the gravin- β_2 ARs association. Also association of gravin-β_2 ARs complex with PKA and PKC significantly increases after agonist-induced β ARs stimulation. Now, upon chronic stimulation of the β ARs, they undergo heterologous desensitization (phosphorylation by PKA and PKC) which is assisted by gravin's targeting of PKA and PKC to β_2 ARs. Upon further chronic stimulation of the β ARs, they undergo phosphorylation by GRK

leading to a conformational change in the β ARs resulting in the recruitment of β-arrestin. The β-arrestins further act as adaptor proteins by recruiting clathrin and other assembly required for the internalization of the receptors leading to termination of the signal (Gagnon et al., 1998; Goodman et al., 1996). Studies have shown that gravin is essential for this complex formation in spite of GRK and β-arrestin not binding to gravin directly. This entire complex undergoes endocytosis along with gravin. Upon endocytosis, the β-ARs undergo dephosphorylation and are recycled back to the receptor. Studies have shown that gravin's association with the receptor is essential for the resensitization step of the receptors post chronic stimulation (Lin et al., 2000a).

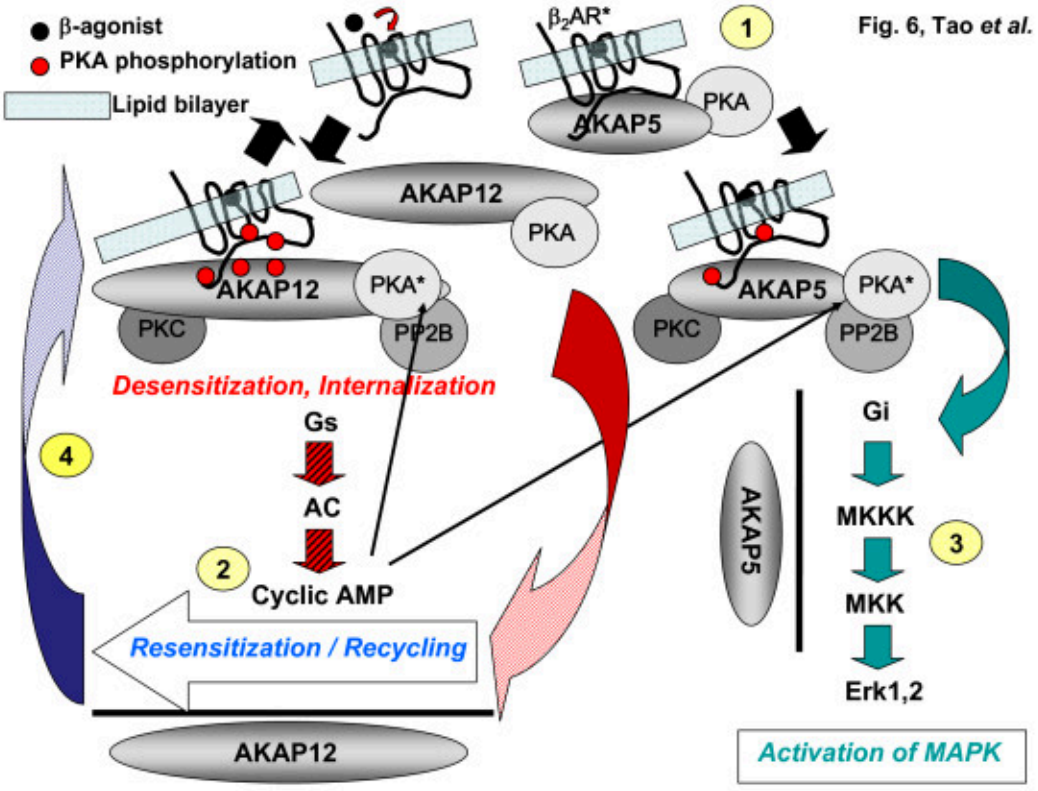


Figure 2. Schematic showing the role of gravin in docking of AKAP 5 tp β_2AR and its modulation of the desensitization and resensitization cycle of the β_2AR

2.9 Models of heart failure

There are various in vitro and in vivo tools available to study HF. However animal models of HF are the most invaluable tools to study the changes in the myocardial structure and function in response to stress or injury. One of the advantages of animal models of HF is that they provide the ability to study the progression of cardiac dysfunction to HF. Some of the frequently used models are:

a) Myocardial Infarction: This is the most widely used surgical model of heart failure developed by Pfeffer et al. In this model the left anterior descending artery is ligated to abrogate the circulation of blood, oxygen and nutrients leading to the development of necrosis and dilation. This progressively leads to left ventricular dysfunction followed by HF. One of the major advantages of this model is that is closely mimics the HF in humans.

b) Transverse Aortic Constriction (TAC): TAC model was developed by Rockman et al. and is a pressure overload induced HF model. The advantage of this model over others is that it is a more reproducible model of HF. Transverse partial constriction of the aorta causes an initial compensatory increase in heart function followed by increased pressure and gradual chamber dilation which eventually develops into HF. Also, it induces HF failure, which is far more gradient in nature compared to other

models giving more insights related to the studies that the model is being used for.

c) Isoproterenol induced heart failure: The biggest attractiveness of this model is the ease of induction and reproducibility. This model uses high doses of the non-selective β agonist to chronically stimulate the β adrenergic receptors, which triggers the HF cascade and develops HF similar to the myocardial infarction.

d) Genetic Models of Heart failure: With recent advances in the genetic approaches, it has become easier to study the implication of a protein in the development and progression of HF by employing genetic manipulations of the said proteins.

Based on these interactions our hypothesis was that absence of gravin's scaffolding would disrupt the desensitization of the β ARs as would inhibit the sequesteration and internalization of the β ARs which would rescue the failing heart by restricting the aberrations of the β AR signaling pathway. To further test this hypothesis, we induced HF mice by chronically stimulating the β ARs using Isoproterenol for a period of 14 days. The end of the 14 days various assessments of the cardiac function, alterations in the receptor density as well as molecular mechanisms were determined using a battery of in vivo and in vitro techniques.

3. Materials and Methods

3.1 Animals:

To assess the effects of absence of gravin during HF, gravin truncated (gravin t/t) mice were used. Exon 3 in these mice has been excised out using gene trap technology as exon 3 contains the binding sites for PKA, PKC, β_2 AR PP2B, Src as well as other binding proteins. (Guillory et al., 2013). The background strain of the mice was C57BL/6. Male WT and gravin t/t mice were housed in the University of Houston or Baylor College of medicine AAALAC accredited animal care facility. All methods and procedures have been approved by IACUC (Protocol #UH-ACP-14-023). Age matched (8-10 weeks) male wild type (WT) and gravin truncated (t/t) mice were used for the experiments. The mice had free access to water and chow during the entire treatment protocol.

3.2 Part I

Effect of chronic stimulation of the β-ARs in the WT and the gravin t/t mice using Isoproterenol

3.2.1 Mouse model of HF using Isoproterenol

The goal of this project was to study the role of absence of gravin in HF regulation. Since gravin is involved in the modulation of the β-AR pathway with respect to heart function, we decided to use the chronic stimulation of β-ARs induced HF. Non-selective β-AR agonist, isoproterenol was used to chronically stimulate the β-ARs to induce HF in these mice (60 mg/kg/day X 14 days). Isoproterenol (Sigma) is very labile as easily undergoes oxidation to give pink colored toxic oxidative products. Hence ISO was dissolved in 0.0002% ascorbic acid (AA) to counteract oxidation and filled in the Alzet osmotic pumps (Model 2004) as per the manufacturers guidelines (Duret Corp). The pumps were then implanted in the subcutaneous pocket through the midscapular incision(House et al., 2010). 0.0002% ascorbic acid (AA) in saline was used as control. The pumps maintained steady infusion of ISO (60mg/kg/day) for a period of 14 days at the end of which further assessments were carried out.

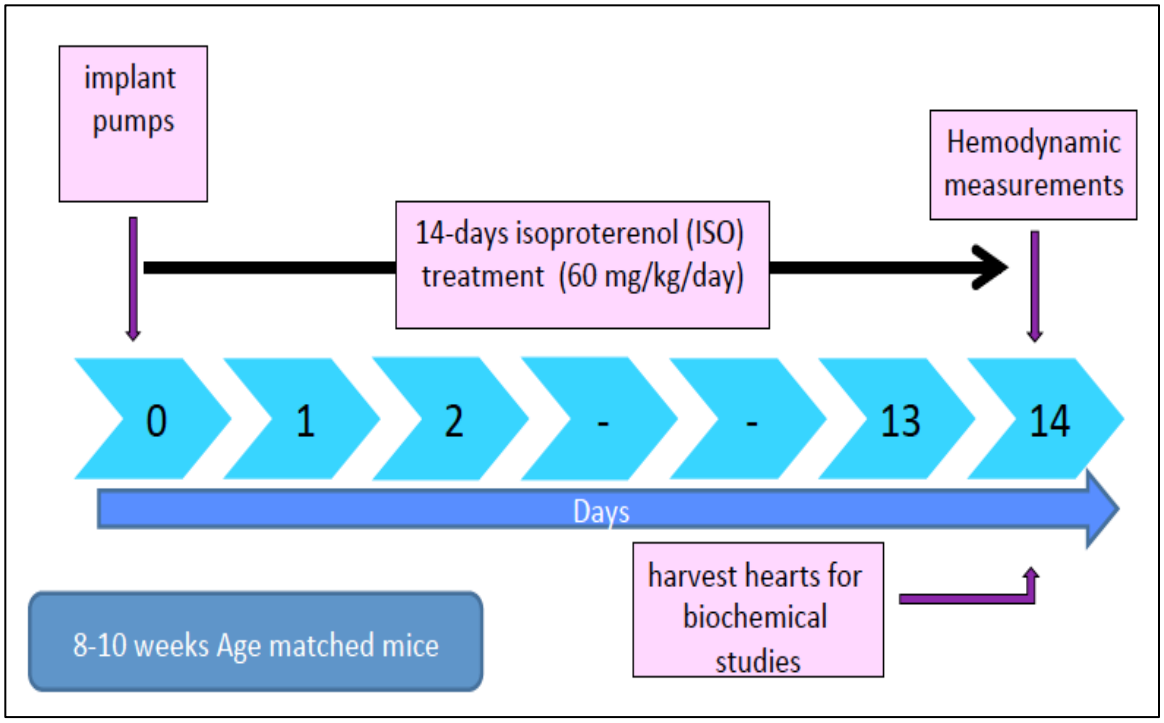


Figure 3. Schematic showing the study design involving the chronic stimulation of β-ARs induced heart failure

3.2.2 Echocardiography:

Mice were anesthetized using isoflurane (2.5% isoflurane, 97.5% O2). Echocardiography (echo) measurements were obtained at baseline following which Alzet osmotic pumps (model 2002) were implanted in the subcutaneous pocket of anesthetized mice to administer β-AR agonist isoproterenol. The measurements were made using serial M-mode echo with a VisualSonics Vevo 2100 High-Resolution In-Vivo Micro-Imaging System (VisualSonics In., Ontario,

Canada) equipped with a 30 MHz microprobe. Several M-Mode ventricular measurements were performed through the 2-week treatment protocol to determine the alteration in the cardiac function in response to chronic stimulation of β-ARs by ISO. These were baseline (before implanting the pumps), 24hours after implanting the pumps and at the end of 14 days of ISO treatment.

3.2.3 PV Loops:

To asses the in vivo hemodynamic functions in these mice, PV loops were performed as previously described (Pacher et al., 2008). Mice were anesthetized using 1.5% isoflurone and placed on the surgical table maintained at 37^0C. Post endotracheal intubation the mice were connected to a rodent respirator. The carotid artery was carefully isolated and the pressure conductance catheter (Millar Instruments - PVR 1045) was inserted through the carotid artery and advanced into the left ventricle until steady state pressure volume loops were observed. The baseline readings were taken at least 3 times followed by 3 readings during inferior vena cava (IVC) occlusion to cause a progressive decrease in the preload. Bolus injection of hypertonic saline was given via the jugular vein to do the first part of volume calibration followed by collecting the blood for cuvette calibrations. For the second part of the volume calibration, the collected blood was filled in the cuvette with known standard volumes followed by measuring the volume by inserting the catheter in the cuvette for validation.

3.2.4 Cell Shortening and Calcium Transients

Ventricular cardiomyocytes were prepared from hearts of 8-12 week WT and gravin-t/t mice using a modified Langendorff perfusion apparatus. Cardiomyocytes were isolated as described(Liao and Jain, 2007). In brief, after isoflurane sedation, hearts were removed and perfused with perfusion buffer (Ca^{2+}-free Tyrode's solution containing (in mM): NaCl 135, KCl 4.0, $MgCl_2$ 1.0, HEPES 10, NaH_2PO_4 0.33, glucose 10, butanedione monoxime 10). Collagenase mix using collagense B (0.4mg/kg) and collagenase D (0.3mg/kg) (Roche) was used to digest the hearts for 7-10 min. Left ventricles were removed and minced before being filtered. Extracellular Ca^{2+} was then added incrementally back to a concentration of 1.20 mM. The isolated myocytes were then loaded with calcium sensitive fluorescent dye – Fura 2AM for at least 20 minutes and then washed 2 times with the Fura loading buffer. Isolated cardiomyocytes were used within 8 hours of isolation. The cells were then loaded onto the IonOptix tray and perfused with fura loading buffer while maintaining them at 37^0C. For baseline measurements, the cells were electrically stimulated at 1HZ. For stimulation studies, the cells while being electrically stimulated were also superfused with 1μm Isoproterenol. Calcium contractility and intracellular calcium was measured by the IonOptix system using our previously described protocol for a period of 3-5 minutes each (Guillory et al., 2013).

3.2.5 Sample preparation:

The hearts were harvested and flash frozen in liquid nitrogen until use. The hearts were crushed and homogenized in 2ml of the homogenization buffer (50mM HEPES, 150mM KCl, 5mM EDTA (pH 7.4), and 10µl/ml protease inhibitor cocktail (Sigma)). The homogenates was then centrifuged at 800Xg for 15mins. 300ul of the supernatant was removed and labeled as the total homogenate. The pellet was discarded. The rest of the supernatant was then centrifuged at 30,000Xg for 30mins to pellet the membrane fraction. The supernatant was collected as the cytosolic fraction and the membrane fraction was re-suspended in suspension buffer (50mM HEPES (pH 7.4), 5mM MgCl2). The total homogenates, cytosolic fractions and the membrane fractions were then further used for further experiments.

3.2.6 Protein Estimation

Bicinchoninic acid (BCA) protein assay (Thermo Fisher Scientific Inc, IL) was used to quantify the amount of protein present in the total homogenates, membrane and cytosolic fractions obtained above according to the manufacturer's instructions. Absorbance was measured spectrophotometrically at 562 nm using an Eppendorf NanoDrop spectrophotometer.

3.2.7 Radioligand binding assay

Radioligand binding assay was performed as previously described to measure β-AR receptor density (Koch et al., 1995). [I^{125}]-iodocynaopindolol (Perkin Elmer) was diluted to decreasing concentrations from 10pM to 4nM and incubated with 10ug membrane samples of the heart at 37^0C. Radioligand binding buffer was used as control. Similar reaction was carried out in the presence of 20 um Alprenelol to determine non specific binding whereas 70nm ICI 118551 (specific β_2 blocker) was used to determine β_1 density and 300nm of CGP 20712A (specific β_1 blocker) was used to determine β_2 density. Each reaction was carried out in triplicate. After one hour, the reactions were harvested onto Whatman GF/B harvestor filter plates thus terminating the reaction. The plates were further washed with 3-4 times with ice-cold binding buffer and dried at 37^0C for 1-3 hours. TopCount Scintillation Counter (Perkin Elmer) was used to determine the radioactive count.

3.2.8 PKA Activity

Enzyme immune assay kit from Enzo life sciences (Cat# ADI-EKS-390A) was used to determine the PKA activity using the total heart homogenates according to the assay instructions provided by the manufacturer. The kit is an ELISA based colorimetric assay. Substrate for PKA (synethic peptide with serine-threonine site) is pre-coated onto the 96 well plates. 30ul of the cytosolic

fractions were added to the wells. ATP was added to activate the reaction followed by polyclonal antibody that recognizes the phosphorylated form of the synthetic substrate. The conjugated HRP antibody was added next following which tetramethylbenzidine was added for the color development. After 30mins of incubation for maximum color development based on the amount of the phosphorylated substrate the reaction was stopped with acidic solution. The extent of color development was measured by spectrophotometer at 405nm.

3.2.9 Histological analysis

Hearts were harvested and washed with PBS. They were then perfused with, fixed and stored in 10% formalin until they are ready for being embedded in paraffin. The embedded hearts were sectioned using a microtome at 5μm thickness. The slides were then stained using Masson's Trichome stain to study fibrosis. Masson's trichome stains the fibrosis blue. The fibrosis was further quantified using image pro plus(Challa et al., 2012; de Jong et al., 2012).

3.2.10 Western Blot

Western blotting was carried out as previously described (McConnell et al., 2009). Whole heart homogenates, membrane fraction or cytosolic fractions were resolved on SDS page gel based on their molecular weight. The resolved proteins were then transferred onto nitrocellulose membrane and blocked with

5% BSA or 5% milk for an hour. The blots were then probed with primary antibodies overnight followed by washing 3 times with TBS (with 0.1% tween 20) for 5 minutes each. After washing the blots were then probed with the appropriate HRP conjugated secondary antibodies for a period of 1hour. The blots were then developed using SuperSignal West Pico Chemiluminescent Substrate (Cat# 34078; Thermo Scientific). The blots were then stripped and probed again with appropriate antibodies. Image J (NIH) was used for the densitometric quantification of the western blots.

The list of antibodies, their concentrations and the source are as listed below.

Antibody	Concentration	Company
p-PKA	1:1000	Cell Signaling
p-β_2 -AR	1:200	Santacruz
p-PLB (Thr17p-PLB)	1:1000	Badrilla
P-TnI	1:1000	Cell Signaling
PLB	1:1000	Cell Signaling
TnI	1:1000	Cell Signaling
p-ERK1/2	1:1000	Cell Signaling
t-ERK1/2	1:1000	Cell Signaling
CAMKIIδ	1:1000	Cell Signaling
PDE4D3	1:1000	Cell Signaling
PDE4D5	1:1000	Cell Signaling
GRK2	1:1000	Cell Signaling
GAPDH	1:10000	Cell Signaling
SERCA2a	1:10000	Cell Signaling
Sodium/Calcium Exchanger NCX	1:500	Pierce
Anti-Mouse	1:10000	Cell Signaling
Anti-rabbit	1:10000	Cell Signaling

3.3 Part II

Effect of chronic stimulation of the β-ARs in the WT and the gravin t/t mice using Isoproterenol followed by treatment with β-ARs antagonists

3.3.1 Treatment with β-AR antagonists post chronic administration of isoproterenol

Our next goal was to further assess the effects of different β-ARs ligands in these mice to grasp a better understanding of the signaling mechanisms involved. β blocker therapy is the current standard used for treating chronic heart failure and is usually used alone or in combination with other therapies. For gravin inhibition to be developed as a potential therapeutic target it will probably have to undergo clinical trial in patients on some kind of blocker therapy like Carvedilol. Also, though gravin binds directly only to β_2, our studies have shown that gravin's inhibition blocks the desensitization and internalization of β_1 receptors as well. Hence, it is imperative to elucidate the role of the β adrenergic receptors in terms of specificity (β_1, β_2 or both) in the positive cardiac response in the gravin t/t mice.

To specifically determine whether this positive cardiac response in the gravin t/t animals is due to which β adrenergic subtype we selectively inhibited the β_1 and β_2 adrenergic receptors using specific antagonists (Bisoprolol and ICI-

118, 551, respectively). Also to determine whether role of absence of gravin fares in comparison to the current gold standard therapy, one cohort of mice also underwent Carvedilol treatment post chronic Isoproterenol treatment. One of the reasons for choosing these drugs was that, Carvedilol and Bisoprolol are approved drugs for use in human patients for HF. There are no approved β_2 blockers for treating HF, hence ICI-118551 was used in our studies as it is a highly specific β_2 blocker which is widely used in the β_2 receptor related studies.

Mice were randomly divided in to different cohorts for the drug treatment. The treatment, dose and mode of drug administration was as follows:

The isoproterenol induced HF model validated in part one was used in second part as well followed by treatment with antagonists. The timeline for the treatment and subsequent echo measurements is as shown in figure 2. The mice were chronically treated with isoproterenol (60mg/kg/day) for a period of 2 weeks via Alzet osmotic pumps similar to 3.2.1. At the end of 14 days the pumps were removed and a 36-48 hour washout period was given to eliminate the Isoproterenol from the system following which the mice were started on the drug treatment. The washout period was important before starting the blocker therapy to ensure that there is no trace isoproterenol in the system so that there is no event of competitive binding between Isoproterenol (agonist) and blockers (antagonists). Carvedilol was administered to the mice via powdered food at the

dose of 24mg/kg (2400ppm). Bisoprolol was also administered via powdered food at the dose of 100mg/kg. ICI 118551 is very unstable and has a very low half-life and hence was dissolved in saline and administered via alzet osmotic pumps (model 2004). These doses were determined based on previous reports, which shows that these are the effective doses , which occupy more than 90% of the receptors (Callaerts-Vegh et al., 2004; Callaerts-Vegh et al., 2003; Stati et al., 2014).

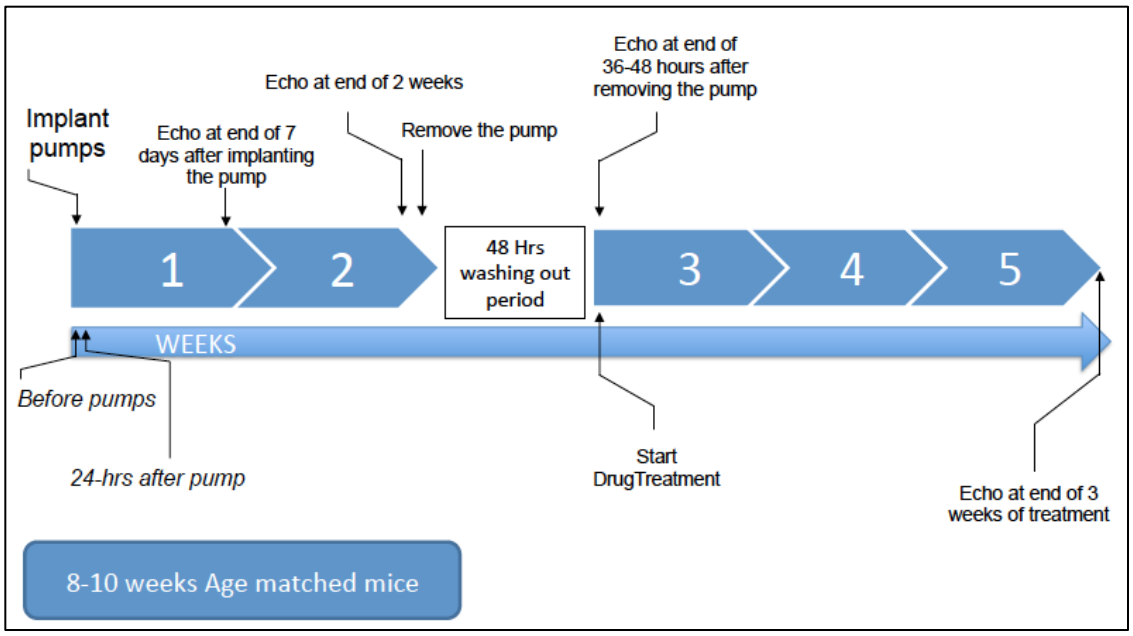


Figure 4. Schematic showing the study design involving the chronic stimulation of β-ARs induced heart failure followed by 3 weeks of drug treatment. The drug treatment comprised of Carvedilol, Bisoprolol and ICI 118551.

3.3.2 Echocardiography

The experiment was performed similar to section 3.3.2

3.3.3 β AR Receptor Density

The experiment was performed similar to section 3.3.7

3.4 Statistical Analysis

Data were processed using GraphPad Prism 5.0. All values are expressed as the mean ± S.E.M. Analysis was performed using one-way ANOVA, two-way ANOVA test as appropriate. For comparison of multiple groups with significant differences Tukey's post hoc multiple comparison tests was performed. P values of less than 0.05 were considered significant.

4. Results

4.1 Morphometric changes in response to chronic treatment with isoproterenol

Isoproterenol induced cardiac hypertrophy is one of the widely used pharmacological mouse model of HF. Administration of low dose of Isoproterenol is known to induce hypertrophy. However high dose of isoproterenol induces hypertrophy followed by decline in cardiac function and morphometric alterations, gene expression changes etc., which mimics the HF symptoms in humans (Brooks and Conrad, 2009; Galindo et al., 2009). To examine the role of absence of gravin in the HF induced by chronic stimulation of β-ARs, osmotic pumps delivering isoproterenol (dissolved in ascorbic acid) were implanted in WT and t/t mice. Ascorbic acid delivery pumps were used as control. The mice were randomly divided into four groups: WT and t/t mice treated with 2 weeks of isoproterenol (WT ISO and t/t ISO) and WT and t/t mice treated with ascorbic acid for 2 weeks (WT AA and t/t AA).

Two weeks of isoproterenol treatment causes an increase in the dimension of the heart along with significant amount of remodeling. To assess the hypertrophy induced by 2-week treatment of isoproterenol Heart weight to body weight (HW/BW) ratio was analyzed. The HW/BW ratio of the WT ISO mice was significantly higher than the ascorbic treated counterpart. On the contrary

the t/t mice after two weeks of Isoproterenol treatment did not show any significant changes in the HW/BW ratio (Figure 5). Increase in HW/BW ratio is accompanied by significant amount of remodeling and fibrotic lesions. The WT mice treated with isoproterenol also showed significant amount of fibrosis as indicated by masson's trichome staining compared to the vehicle treated mice. t/t mice did not show any fibrosis (Figure 6).

4.2 Changes in cardiac function in response to chronic isoproterenol treatment

HF has significant implication on the cardiac function. We assessed the cardiac function in these mice using two methods.

4.2.1 Echocardiography

One of the ways that changes in cardiac dimensions and function were evaluated was using echo in the WT and t/t mice. Based on the cardiac dimensions, left ventricular mass was calculated. There was no difference in the LV mass of the WT AA and the t/t AA mice. However post 2 weeks of treatment with Isoproterenol we found that there was significant increase in the LV mass of the WT ISO mice compared to the control whereas there was no difference between the t/t ISO mice compared to the control. At baseline, i.e. in the absence of the any chronic agonist stimulation, there were no changes in the cardiac dimensions in the WT or t/t mice. However post 2 weeks treatment with

Isoproterenol, the cardiac dimensions showed significant alterations as well. There was significant increase in the left ventricular inner diameter (LVIDd) at systole as well as diastole in the WT ISO mice indicating presence of significant amount of dilation in the left ventricle chamber in response to 2 weeks of Isoproterenol treatment. No such increase was seen in the t/t ISO mice. There was significant increase in the inter ventricular septum at diastole as well in the WT ISO mice along with significant increase in the Left ventricular Posterior Wall Thickness at systole as well as diastole. The gravin t/t mice on the other hand did not show any such change in interventricular septum at diastole (IVSd), left ventricular posterior wall diameter at diastole (LVPWDd) or LVPWDs (table 1).

To further study whether these alterations in the cardiac dimensions affected the cardiac function we looked in to parameters that define the physiological functioning of the heart-using echo. There was no change in the cardiac function in the WT AA and the t/t AA mice. However after 2 weeks of Isoproterenol treatment there was significant increase in the heart rate of the WT ISO mice compared to the control mice coupled with significantly reduced fractional shortening as well as decrease in the stroke volume. None of these parameters showed any changes in the gravin t/t mice even after 2 weeks of Isoproterenol treatment, which was consistent with no changes in the cardiac dimensions. Consistent with decreased stroke volume, we found that there was a

trend of decreased ejection fraction in the WT ISO mice, though this trend did not reach statistical significance (p value = 0.68). However the ejection fraction of the t/t ISO mice was similar as that of the control mice (Figure 7).

4.2.2 Pressure Volume Relationship

We also determined the in vivo cardiac function in these mice using pressure volume loops since this technique has an advantage over the echo in terms of variables in data due to the changes induced by anesthesia. Hence PV loops is means to determine the in vivo hemodynamics in mice and it generates data that is more robust than the data generated by Echo. Similar to the echo data at baseline we found no change in the cardiac function between the WT AA or t/t AA groups. However post 2 weeks of Isoproterenol treatment we saw significant changes. There was significant reduction in the cardiac output, ejection fraction and stroke volume in the WT ISO mice compared to the control whereas there was no change in the t/t ISO mice (Figure 8). The PV loops obtained using this technique define the cardiac function and various other parameters. Representative loops are shown in Figure 6. During IVC occlusion there is a progressive increase in the afterload and the changes obtained in the slope of the loop define the End Systolic Pressure Volume Relationship (ESPVR). Decrease in the slope (rightward shift) of the ESPVR is indicative of declining ionotropy. In the representative loops, the loops as well as the slopes of the

ESPVR of the WT AA and the t/t AA mice are similar (Figure 10). However there are obvious changes in the loops of the WT ISO mice with a right ward shift in the slope of the ESPVR. The gravin t/t ISO mice on the other hand show no such alteration.

4.3 β AR Expression changes in response to chronic isoproterenol treatment:

Previous studies have shown that gravin in involved in the modulation of the β-ARs desensitization. Specifically, post agonist stimulation the association of PKA, PKC, GRK2, β-arrestin and clathrin is increased with β_2-ARs leading to the sequestration of the receptor (Lin et al., 2000a; Siegel et al., 2012).

After 14 days of isoproterenol treatment the effects of absence of gravin's scaffolding on the receptor density was evaluated. Chronic stimulation of β-ARs with agonist leads to their down regulation. The total β-ARs receptor density was assessed after subtracting the non-specific binding evaluated with alprenolol. We found that there was significant reduction in the total β-ARs receptor density in the WT ISO mice compared to the control WT AA mice. On the contrary the gravin t/t mice (t/t ISO) did not show any significant alteration in the total β-ARs density in spite of 2 weeks of isoproterenol treatment (Figure 11).

To further understand the alteration involved in the β-ARs down regulation we assessed the total β-ARs subtype density using ICI to determine total β_1-ARs density and CGP to determine total β_2-ARs. We found that similar to the total β-ARs, there was significant reduction in the total β_1-ARs density in the WT ISO mice compared to the WT AA, whereas there was no significant difference between the t/t ISO and t/t AA mice (Figure 12). Interestingly in the case of the β_2-ARs, we found that the t/t AA mice had significantly higher β_2-ARs compared to the WT AA mice. On the contrary was no significant difference in the β_2-ARs density in WT ISO or t/t ISO mice (Figure 13).

4.4 PKA activity and substrate phosphorylation

Subsequent to β-ARs activation, PKA gets activated and phosphorylates the downstream substrates, which then leads to cardiac events like contraction and relaxation. Studies have shown that during HF, one of the major reasons for progressive deterioration of contractility is the significant perturbation in the substrate (ryanodine receptors, cTnI, phospholamban etc) phosphorylation by PKA. However some studies show differences in the PKA activity and expression in the failing vs. non-failing hearts whereas other do not (Manni et al., 2008; Wang et al., 1999).

To study the effect of HF induced by chronic stimulation of β-ARs, we determined the total PKA activity in the total homogenates of the heart using ELISA assay. This assay analyses the efficiency of the PKA present in the homogenates to phosphorylate the serine threonine substrate plated in the bottom of the ELIZA plate. We found that there was no significant difference between the PKA activities in any of the four treatment groups (WT AA, WT ISO, t/t AA and t/t ISO). This proved that the kinase activity of PKA in itself had not undergone any kind of alteration and the activity was the same across the groups (Figure 14).

Our next step was to evaluate whether the upstream alterations in the β-ARs density expression were effecting the activation of PKA and subsequent substrate phosphorylation of the substrates. Some studies have reported a change in the expression of RII alpha during HF whereas some report no change in expression (Enns et al., 2010; Kompa et al., 1999). In our studies we did not find any significant difference in the PKA – RII alpha expression between the WT and t/t mice in the vehicle treated or Isoproterenol treated group (Figure 16). Further the total expression of the PKA catalytic subunit did not show any significant difference between the four treatment groups as well. PKA upon activation undergoes phosphorylation at threonine-197, and this phosphorylation is required for optimum activity of the PKA (Cauthron et al., 1998). However

during HF, this auto-phosphorylation undergoes impairment as some studies report hyper-phosphorylation at Thr-197 whereas some report hypo-phosphorylation in the failing hearts(Han et al., 2013). We found that there was significant increase in the p-PKA at Thr-197 in the WT ISO group compared to the WT AA, whereas there was no significant difference between the t/t ISO group and t/t AA group (Figure 15).

PKA is the major effector of β-ARs activation. To understand the downstream effects of the changes in the PKA activation we looked in to the substrate phosphorylation of some of the most important targets of PKA. Upon chronic stimulation, one of the first proteins to undergo phosphorylation is the β-ARs itself. In our studies we found that there was no difference in the phosphorylation of the β_2-ARs in the t/t AA mice compared to the WT AA mice. However after 2 weeks of Isoproterenol treatment, there was significant up regulation in the phosphorylation of the β_2-ARs in the membrane fraction in the WT ISO group whereas no change was seen in the t/t ISO group (Figure 17). Another major substrate of PKA, cTnI, is phosphorylation by PKA, which reduces the calcium sensitivity of the cTnC that augments the relaxation of the heart during the EC coupling. During HF, the phosphorylation levels of TnI are altered which further impairs the calcium cycling during the EC coupling. We found that after two weeks of Isoproterenol treatment there was significant decrease in the

levels of p-TnI in the WT mice compared to the WT AA whereas there was no difference in the p-TnI levels in the gravin AA vs. t/t ISO group (Figure 19).

The next target that we looked into was phospholamban. Phosphorylation of phospholamban results in the removal of its inhibition from SERCA2a. This allows the reuptake of calcium back into the SERCA2a after contraction resulting in relaxation and prepares the calcium stores for the next contraction cycle. We found that the gravin t/t mice did not show any changes in the p-PLB (at Serine 16) after 2 weeks of Isoproterenol treatment whereas WT ISO hearts had significant reduction in the p-PLB (Figure 18).

To further assess the calcium handling in these mice we also looked into the changes in the PKA phosphorylation of the cMyBPC. There are three known sites that cMyBPC – Ser-273, Ser-282 and Ser-302 that under undergo phosphorylation by PKA, PKC, CAMKII, and PKD. The exact mechanism and effect of phosphorylation of each of these sites is not known. However it is well established that PKA phosphorylates at site ser-273. Also, phosphorylation of cMyBPC contributes to the desensitization of the myofilaments to calcium leading to relaxation. We determined the phosphorylation levels of cMyBPC post treatment for 2 weeks with ISO. We found that there was significant reduction in the phosphorylation of ser-273 in the WT ISO group compared to the WT AA

group. For the other sites (p-282 and p-302) that undergo phosphorylation, we did not find any significant difference in the phosphorylation levels between the WT ISO and WT AA group. However there was no change in the phosphorylation levels between the t/t ISO group and the t/t AA group at any of the phosphorylation sites (Figure 20).

4.5 GRK2

Chronic stimulation of the β-ARs leads to the desensitization and internalization. Phosphorylation of the β-ARs by GRK leads to these events. GRK2 is the major subtype of GRK present in the heart and plays a very important role. During HF, there is significant up regulation in the GRK2 expression in the heart, which further impairs the β-ARs on the cell surface. In our studies we found that there was no difference in the expression in the WT and t/t control mice. However, WT mice treated with ISO for 2 weeks had significant increase in the GRK2 expression compared to the t/t mice treated with ISO for 2 weeks (Figure 21).

4.6 PDE4D Expression

PDE basically keep the PKA signaling in check by decreasing the concentrations of cAMP by degrading it. There are two major isoforms present in the heart- PDE4D3 and PDE4D4 which gravin scaffolds. In our studies we found

that there was no difference in the expression of PDE4D3 and PDE4D5 between the WT AA and gravin t/t AA. However after 2 weeks of ISO treatment we found that there was significant reduction in the PDE4D3 expression in the WT ISO as well as t/t ISO mice compared to their respective controls. However in the case of PDE4D5 we found that there was no change in the expression in the WT ISO or t/t ISO mice compared to the control (Figure 23).

4.7 Calcium Transients and Sarcomere Shortening

To study if the effect of chronic stimulation of β-ARs leading to impaired contractility of the WT ISO group is accompanied by alterations in the calcium homeostasis in the cardiomyocytes, we studied the cell shortening and intracellular calcium concentration in the isolated cardiomyocytes from the hearts of the four treatment groups. The measurements where made in cardiomyocytes loaded with Fura-2AM, at baseline while the cells were paced at 1Hz using electrical stimulation and also after stimulating them with 1μm ISO.

We found that in the vehicle (AA) treated mice, the amplitude of the calcium transients of the t/t mice were significantly smaller compared to the WT AA mice. However the corresponding sarcomere shortening of the t/t AA mice were significantly higher than that of the WT AA mice. Moreover cardiomyocytes from both the groups responded to acute β-AR stimulation achieved using 1μm

ISO. There was significant increase in the transients of both the groups post stimulation, however the corresponding difference in the calcium transients between the WT AA and t/t AA was preserved.

Further more, when the cardiomyocytes from the 2-week ISO treated WT and t/t mice were studied, we found that there were significant alterations in the calcium transients of the ISO treated WT mice. There was significant increase in the baseline of the calcium transient of the WT ISO mice compared to the control with no changes in the baseline of the t/t ISO mice. Also there was significant reduction in the amplitude of the calcium transient and sarcomere shortening of the WT ISO mice compared to the WT AA mice. Further, when the cardiomyocytes isolated from the 2 week chronically treated WT mice were further acutely stimulated with 1μm ISO, they did exhibit increase in their calcium and sarcomere shortening however their response was severely blunted compared to the acutely stimulated WT AA cardiomyocytes. The calcium transient and sarcomere shortening of the cardiomyocytes of the t/t ISO mice at baseline were similar to that of the t/t AA mice. Also upon acute stimulation the cardiomyocytes of the t/t ISO group and t/t AA group responded similarly (Figure 25 and Figure 26).

4.8 Expression of proteins involved in calcium cycling

Our next step was to further elucidate whether the alterations in the calcium handling were due to changes in the protein involved in calcium handling. To confirm this we looked into the alterations in the expression of major proteins involved in the cycling of calcium form the cytosol of the cardiomyocytes. Sodium Calcium exchanger (NCX) is involved in the efflux of calcium out of cytosol of the cardiomyocytes whereas SERCA2a is a major calcium pump that regulates the calcium concentration in the cytosol during contraction and relaxation. Both NCX and SERCA2a did not show any change in expression between the WT AA and t/t AA that indicated, absence of gravin did not affect the protein expression of these proteins. Even after 2 weeks of ISO treatment there was no change in the expression of NCX SERCA2a in both WT ISO and t/t ISO (Figure 27 and Figure 28).

4.9 CAMKII expression

During HF, studies have shown that there is an up regulation in the CAMKII dependent pathway, which leads to activation various, adverse pathways such as apoptosis, alteration in calcium handling etc. In our studies we found that there was no change in the expression of the CAMKII in any of the four groups indicating that absence of gravin or 2 weeks treatment with ISO does not alter the expression of CAMKIIδ (Figure 24).

4.10 ERK Phosphorylation

Aberrant ERK activation is one of the molecular dysfunctions during HF. ERK activation is protective in nature, as it leads to activation of the pro-hypertrophic factors that are essential for adaptive hypertrophy. However prolonged activation is detrimental and leads to activation of transcription of hypertrophic genes(Dong et al., 2006; Rose et al., 2010; Zhang et al., 2003). In our studies we found that WT ISO had significantly increased levels of ERK activation (phosphorylated ERK) compared to the control mice. The gravin t/t ISO mice on the other hand had similar activation of ERK levels as compared to the control mice (Figure 22).

PART II

4.11 Effects of Treatment with β-Antagonists following Chronic Rreatment with Isoproterenol

We looked into the effects of the treatment of the antagonists on the cardiac function.

We looked into the heart to body weight ratio to determine the morphometric changes in response to treatment with antagonists and found that the t/t ISO

mice did not show any increase in the HW/BW ratio as t/t mice resist hypertrophy. This did not change in spite of treatment with Carvedilol, Bisoprolol or ICI. The WT ISO mice on the other hand showed significant increase in the HW/BW ratio after treatment with ISO. Treatment with antagonists did not change this hypertrophy. Hypertrophy is always accompanied by fibrosis. WT ISO Control mice had significantly increased induction of fibrosis compared to the WT AA control mice. Treatment with Carvedilol, Bisoprolol or ICI did not affect fibrosis. On the other hand the t/t ISO control mice did not exhibit any fibrosis and treatment with any of the antagonists did not change this.

To assess the changes in the functional aspects in response treatment with antagonists, we studied the cardiac function of these mice using echo. We found that WT ISO control mice had significant increased LVIDs, LVPWDd as well as LVPWDs indicating that there was significant dilation in the WT hearts coupled with increase in the wall thickness as well. These morphological changes also translated into the physiological alterations. There was significant decrease in the fractional shortening in the WT ISO control mice compared to the control mice. The gravin t/t mice on the other hand did not show any aberrations in the morphology of the heart in response to ISO treatment due to which we did not see any alterations in the functional aspects as well. There was some increase in the cardiac function post treatment with Carvedilol and Bisoprolol as

seen by a significant decrease in the LVIDd in both the treatment groups (Table 2 and Table 3). ICI 118551 on the other hand did not show any significant change on the cardiac parameters post treatment (Table 4).

Our next step was to look into the changes in the β-ARs expression in response to the treatment with the antagonist (Figure 29, Figure 30 and Figure 31). In our studies we found that as expected the WT ISO control mice show significant reduction in the total β-ARs receptor density. When we looked into the β-ARs subtype we found that there was significant reduction in the β_1-ARs density with no change in the β_2-ARs subtype. The t/t ISO Control mice on the other hand showed no change compared to the vehicle control mice in the total β-ARs. Further elucidation of the subtypes showed no change in the β_1-ARs between the control mice or the ISO treated group. The β_2-ARs subtype on the other hand similar to the previous studies had significant increase in the β_2-ARs subtype in the control as well as the ISO treated group. However in the group of mice undergoing treatment with Carvedilol, even though we saw an increase in the cardiac function, there is no change in the total β-ARs, β_1-ARs or the β_2-ARs density in the WT cohort. However interestingly in the t/t cohort treated with Carvedilol, we found that thought the total β_1-ARs density did not change compared to the control t/t mice, there was a significant increase in the β_2-ARs density. When we further looked into the changes in the receptor density after

ICI118551 treatment we found that in the case of the WT mice there was a modest increase in the total β-ARs receptor however this increase was not statistically significant. On further looking into the subtype receptor expressions we found that ICI 118551 treated WT mice had no change in the β_1-ARs receptor density but showed a significant increase in the β_2-ARs subtype compared to the 2 weeks ISO treated time point in the same cohort. This increase in the β_2-ARs subtype was significantly higher than the 2-week ISO treated mice but significantly lower than the baseline. Incase of gravin t/t mice, we found that there was again an increase in the total β-ARs density which however di not reach significance and no change in the β_1-ARs subtype compared to the post 2 weeks treatment with ISO time point. However incase of the β_2-ARs we found that post ICI 118551 treatment there was a significant increase in the β_2-ARs expression compared not only to the 2 week ISO time point but also significantly higher than the baseline β_2 ARs expression. This increase was however lower than the β_2-ARs increase induced by the Carvedilol treatment. Incase of Bisoprolol treatment we did not see any differences between the WT and gravin t/t mice. Bisoprolol treatment increased the β_1-ARs expression in both the WT and t/t mice compared to both the 2 week ISO treated time point as well as the baseline of their respective cohorts.

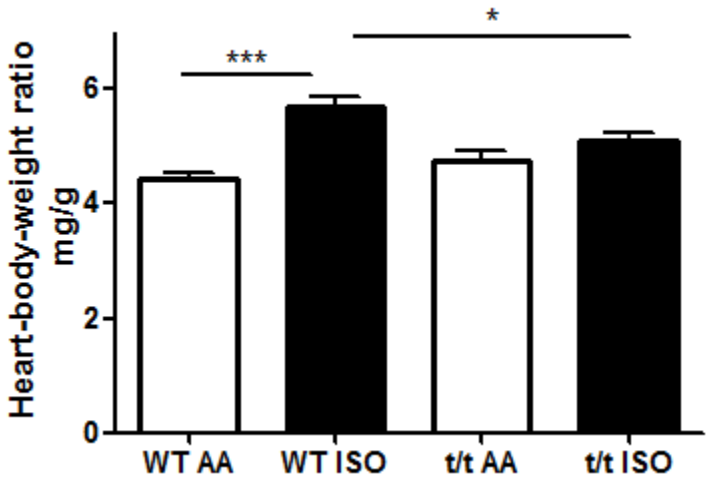
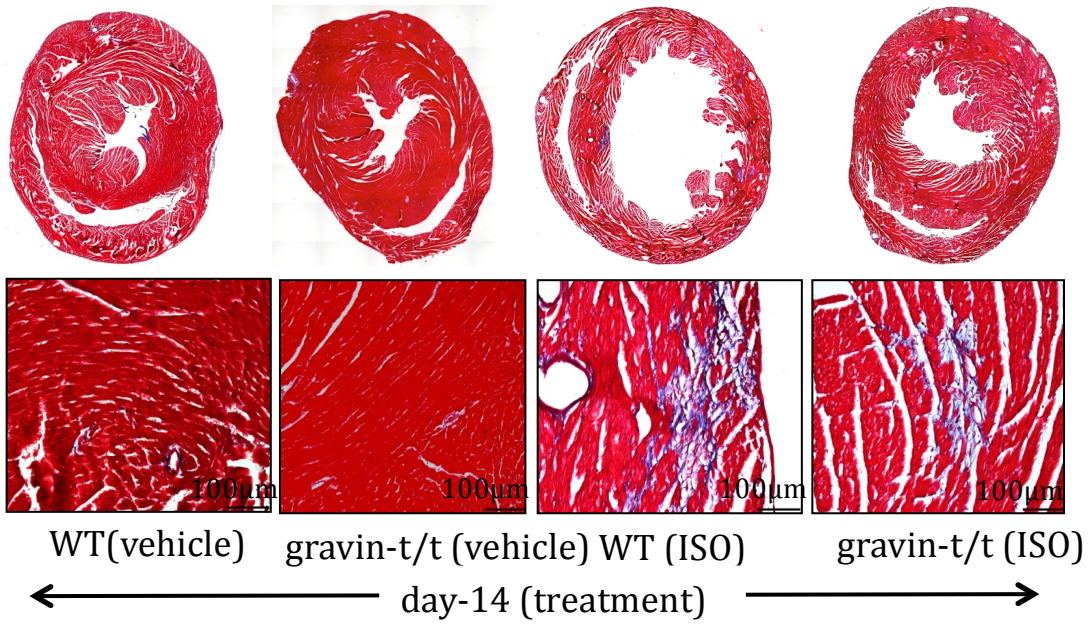


Figure 5. Disruption of gravin's scaffolding blocks ISO-induced increases in heart-to-body-weight ratio. Heart-to-body-weight ratio of WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Data are expressed as mean ± S.E.M; n=10; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

A



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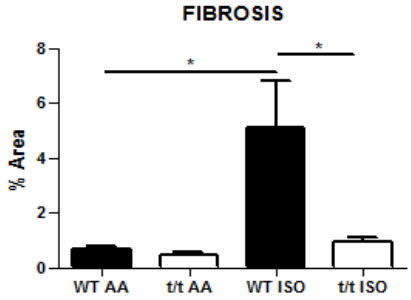

Figure 6. Fibrosis induced in response to chronic ISO stimulation in WT and t/t mice. Masson's Trichrome (MT) stained paraffin sections of excised WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

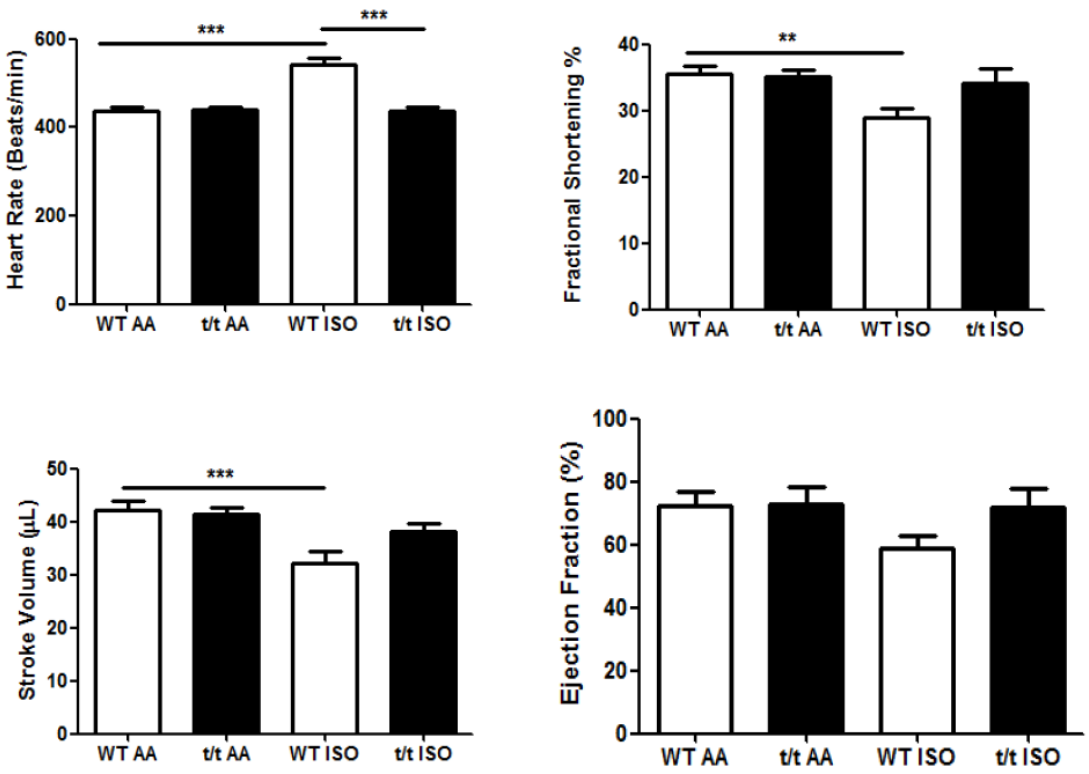

Figure 7. Physiological parameters assessed by Echo. (A) Heart Rate and (B) Fractional Shortening (C) Stroke Volume and (D) Ejection Fraction in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Results are presented as the mean ± SEM; n = 7-8; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

	WT AA	t/t AA	WT ISO	t/t ISO
IVSDd	0.6878 ± 0.02001	0.6753 ± 0.01952	0.7983 ± 0.03760*	0.7031 ± 0.01952
IVSDs	0.9647± 0.03939	0.9435± 0.03438	0.7649± 0.02442*	0.9249± 0.05369
LVIDd	0.6878± 0.02001	0.6753± 0.01952	0.8131± 0.03919*	0.7275± 0.02000#
LVIDs	2.398± 0.09380	2.564± 0.07862	2.800± 0.09305*	2.557± 0.1257#
LVPWDd	0.7771 ± 0.03719	0.7850 ± 0.02335	1.060 ± 0.05473*	0.8825 ± 0.04490#
LVPWDs	1.109 ± 0.05937	1.087 ± 0.03611	1.559 ± 0.1055*	1.304 ± 0.08628

Table 1. All values are expressed as mean ± SEM; ISO, isoproterenol; IVS, intraventricular septum; LVID, left ventricular internal dimensions; LVPW, left ventricular posterior wall; s, systole; d, diastole; *p<0.05 vs. baseline of same genotype; # p<0.05 WT ISO vs. t/t ISO.

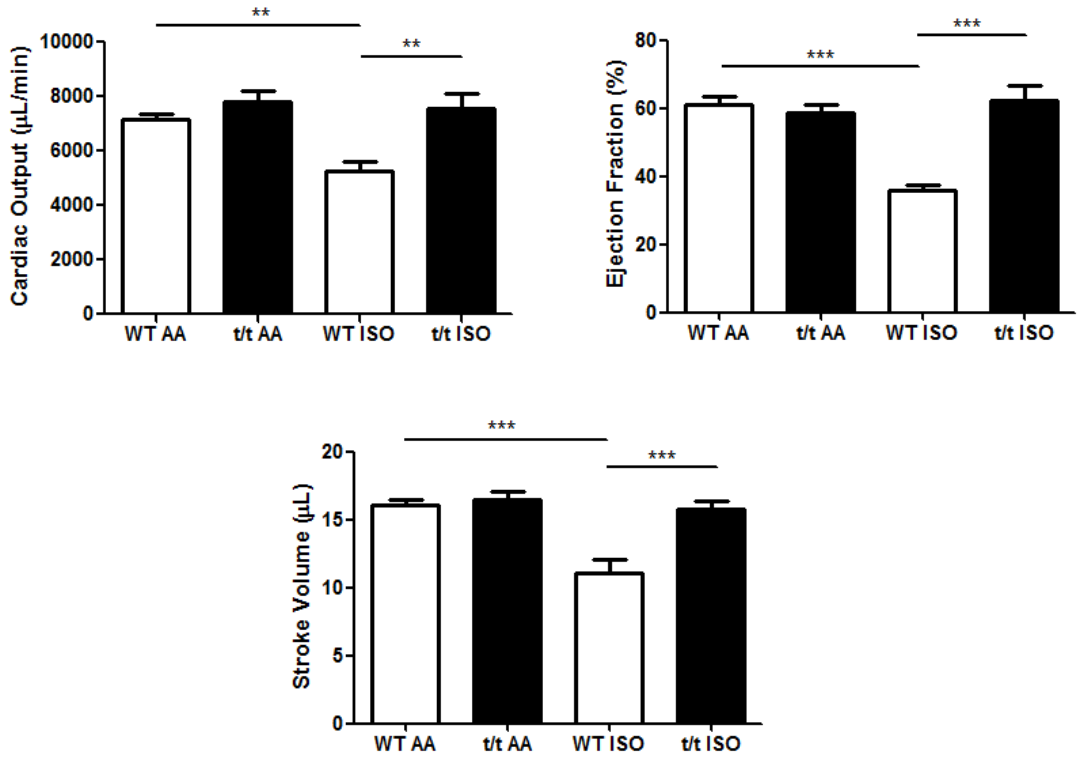

Figure 8. In vivo hemodynamic assessment using Pressure Volume Loops. (A) Cardiac Output (B) Ejection Fraction and (C) Stroke Volume in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Results are presented as the mean ± SEM; n = 7-8; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

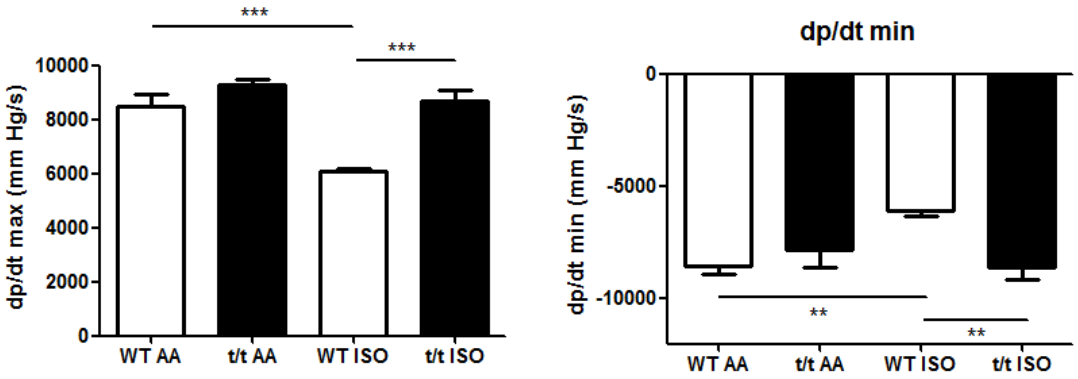

Figure 9. Physiological parameters assessed by PV Loops. (A) dp/dt max and (B) dp/dt min in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Results are presented as the mean ± SEM; n = 7-8; *p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.

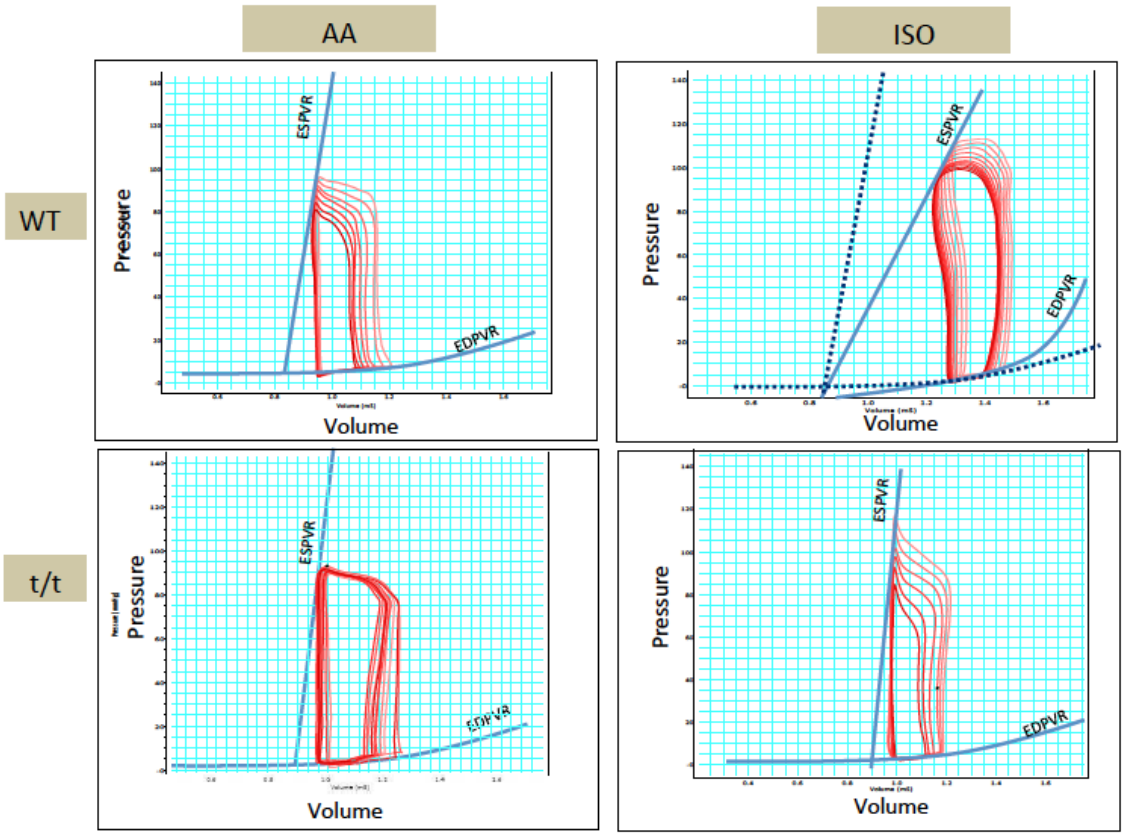

Figure 10. In vivo assessment of contractility with LV P-V relationships. Representative P-V loops under inferior vena cava occlusions (IVC) in WT and t/t vehicle (AA) and ISO treated mice. P-V loops were recorded under baseline condition and during inferior vena cava occlusions (IVC) to decrease the prel

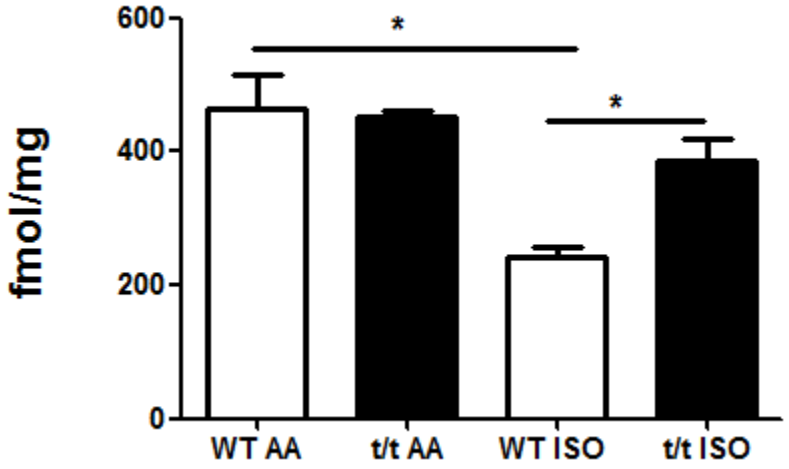

Figure 11. Total β-AR density following chronic ISO stimulation determined in left ventricular membrane fractions isolated from in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of20μM alprenolol. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6.

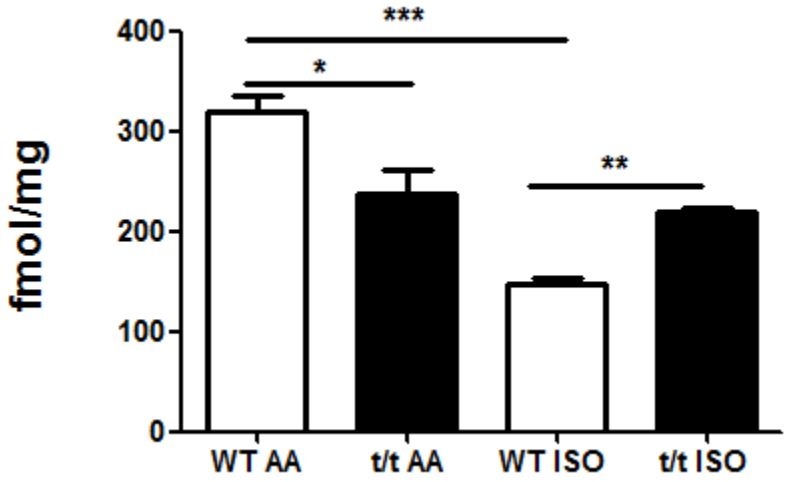

Figure 12. Total β_1-AR density following chronic ISO stimulation determined in left ventricular membrane fractions isolated from in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of 20μM alprenolol. β_1-AR density was determined in the presence of 70nM ICI 118,551 to block β_2-AR. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6

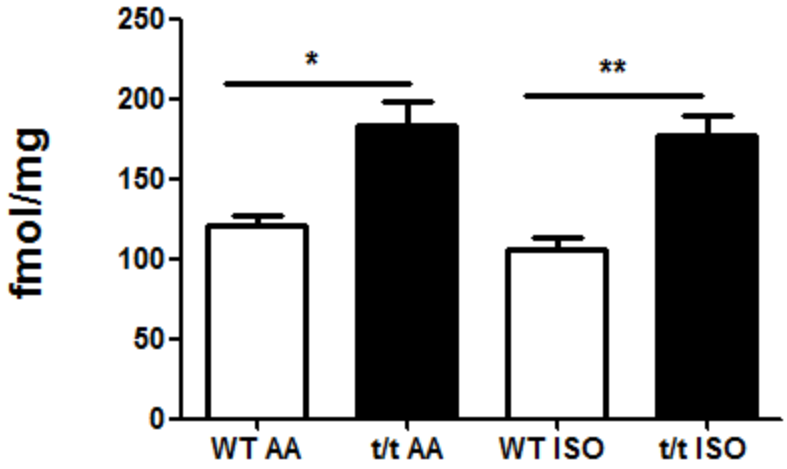

Figure 13. Total β_2-AR density following chronic ISO stimulation determined in left ventricular membrane fractions isolated from in WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of 20μM alprenolol. β_2-AR density was determined in the presence of 300μM CGP 20712A to block β_1-AR. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6.

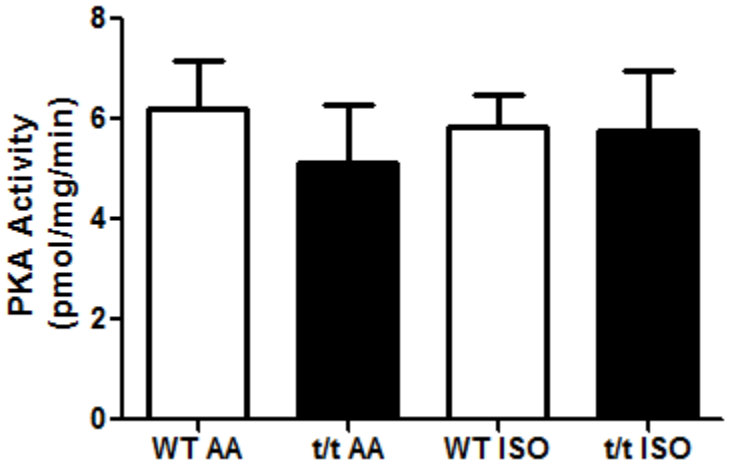

Figure 14. PKA activity was determined in WT and t/t mice following chronic ISO stimulation. PKA activity in left ventricular cytosolic fractions isolated from WT and t/t mice following chronic vehicle (AA) or ISO treatment. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples

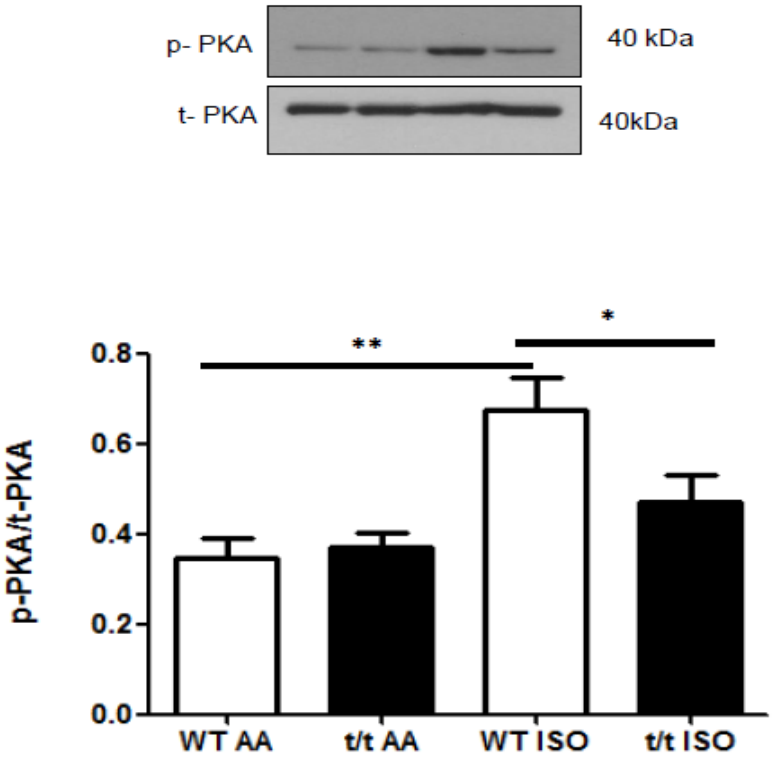

Figure 15. Western blot analysis of PKA phosphorylation at Thr-197 in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with anti-phospho-protein antibody and the lower panel shows a Western blot with an antibody to total-protein (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of phosphorylated to total protein. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

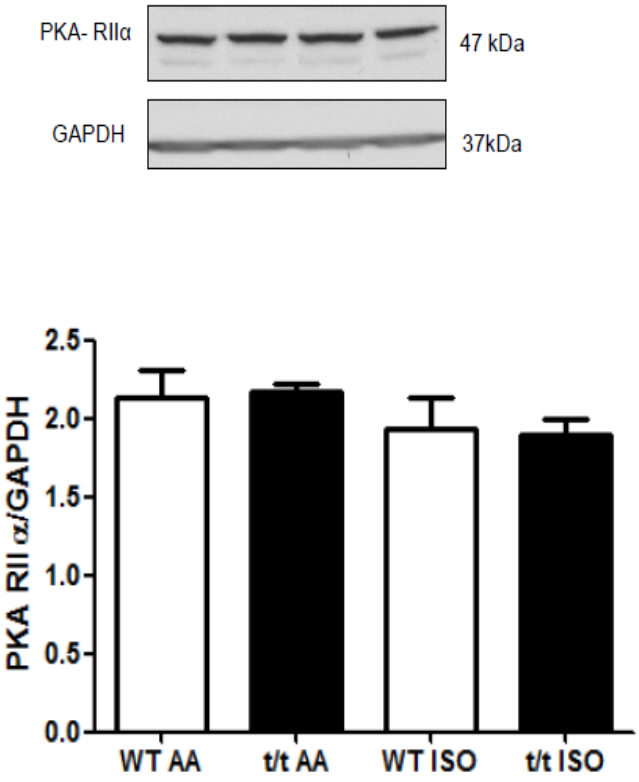

Figure 16. Western blot analysis of PKA RII α expression in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with PKA RII α antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of PKA RII α to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001

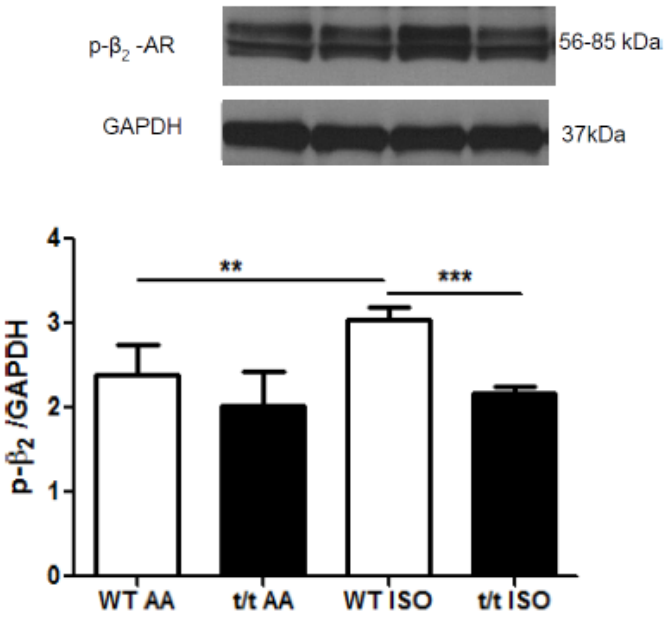

Figure 17. Western blot analysis of phosphorylated β_2 expression in membrane fractions of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with phosphorylated β_2 antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of phosphorylated β_2 to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

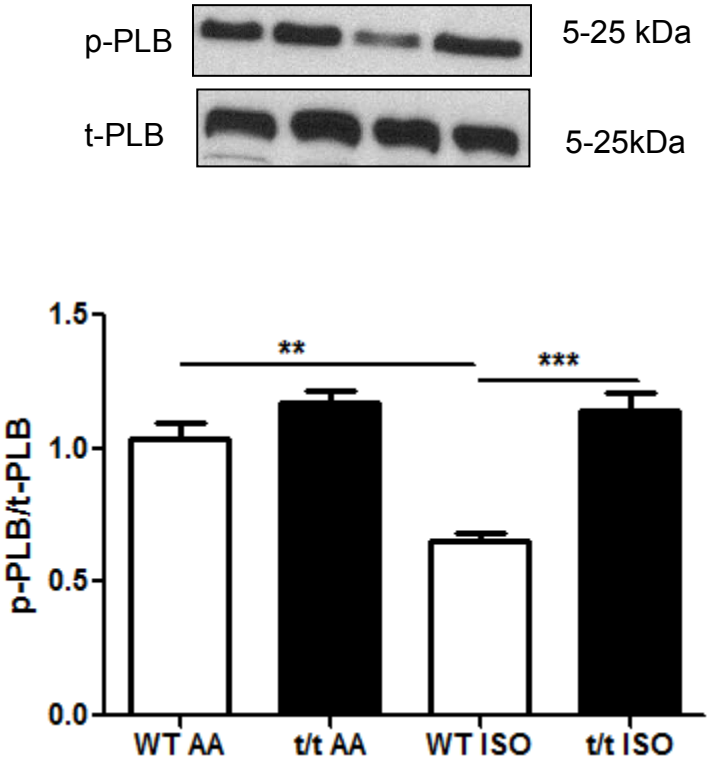

Figure 18. Western blot analysis of PKA substrate phosphorylation of PLB (p-PLB) in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with anti-phospho-protein antibody and the lower panel shows a Western blot with an antibody to total-protein (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of phosphorylated to total protein. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

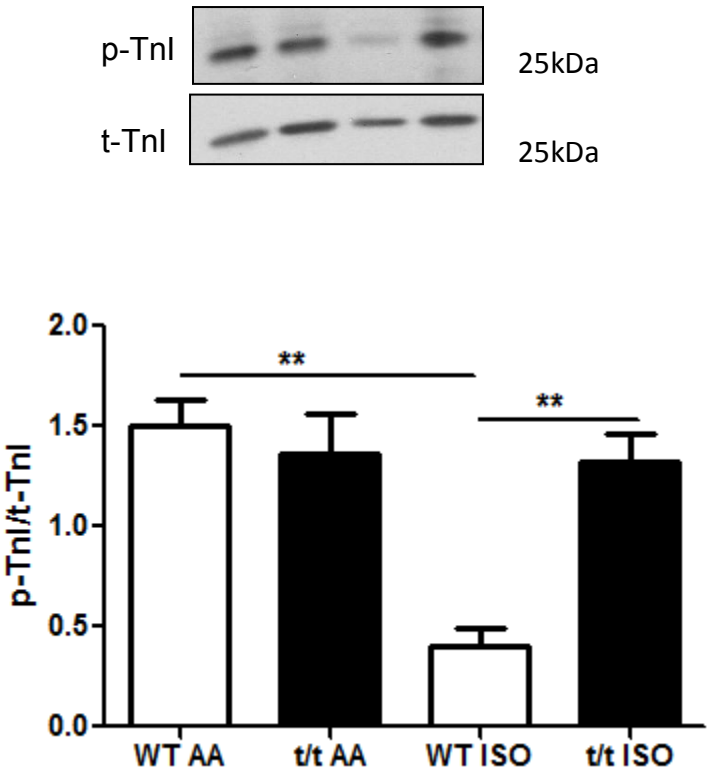

Figure 19. Western blot analysis of PKA substrate phosphorylation of cTnI (p-TnI) in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with anti-phospho-protein antibody and the lower panel shows a Western blot with an antibody to total-protein (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of phosphorylated to total protein. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05 vs.vehicle of same phenotype; *P<0.05, **P<0.01, ***P<0.001.

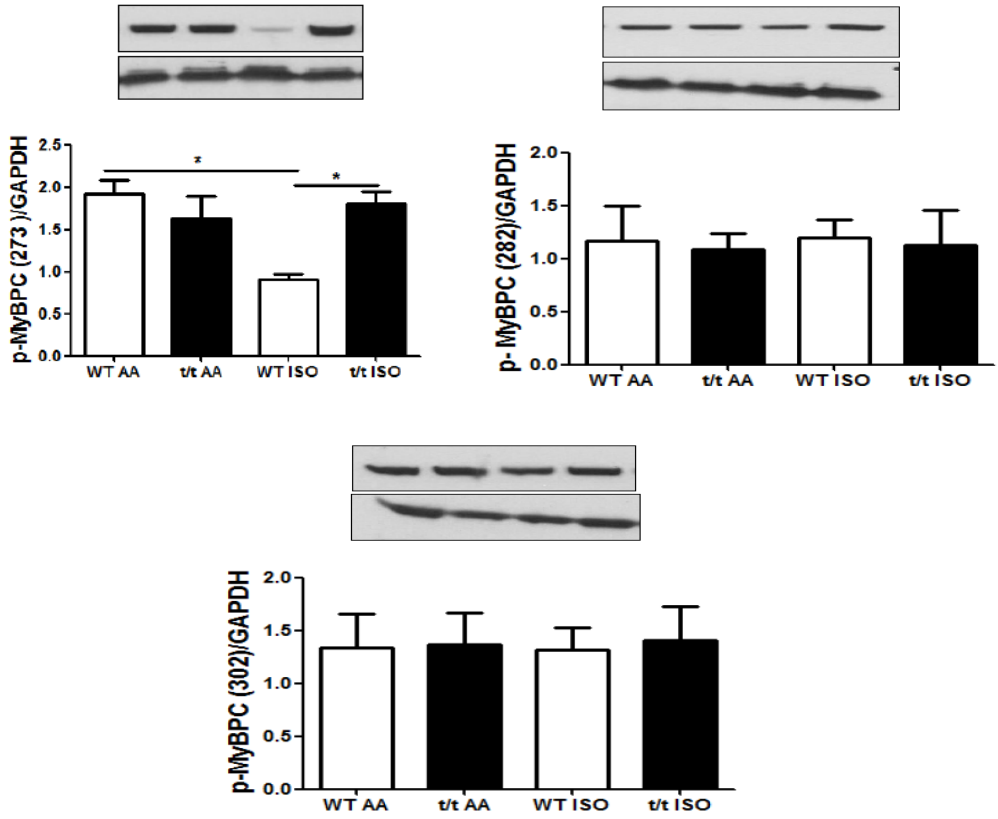

Figure 20. Western blot analysis of p-MyBPC (p-273, p-282 and p-302) present in the total homogenate of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with antibody to total-protein and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of total protein to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

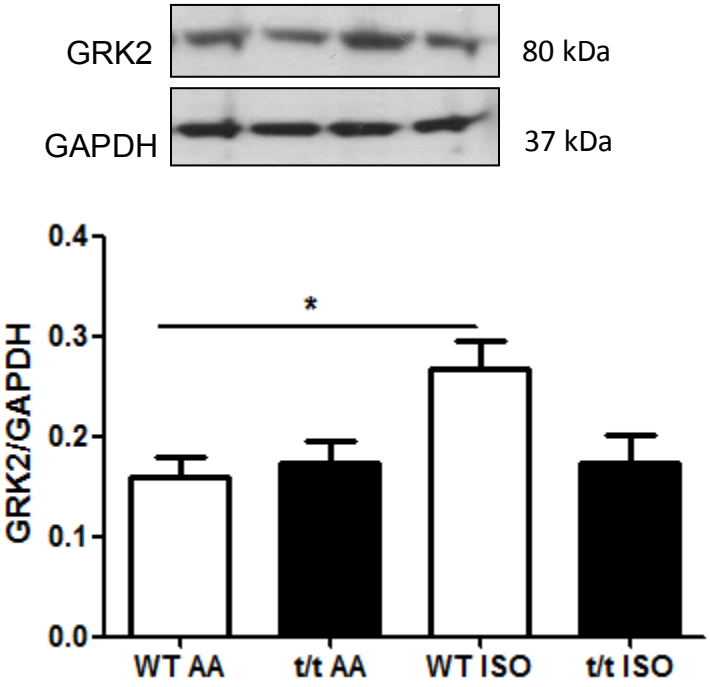

Figure 21. Western blot analysis of GRK 2 present in the membrane fraction of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with antibody to total-protein and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of total protein to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

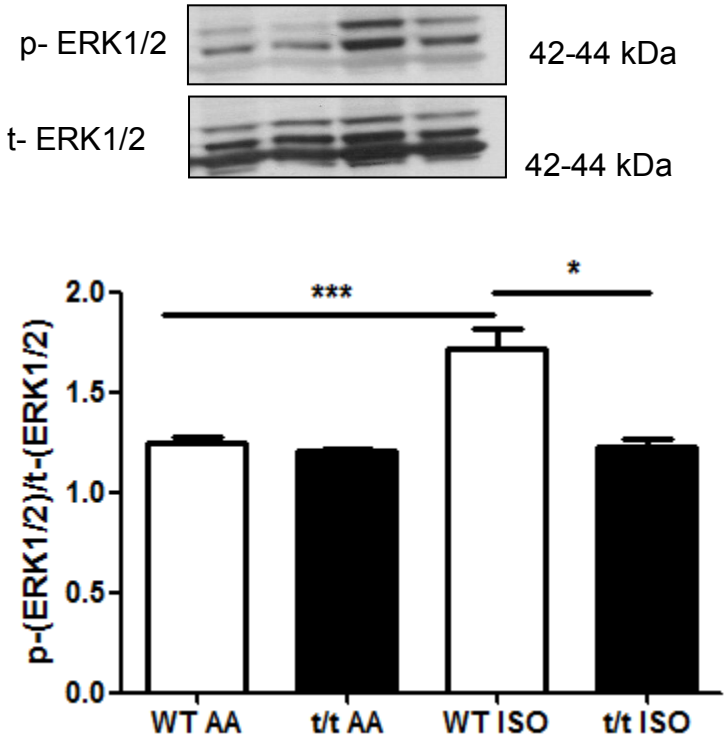

Figure 22. Western blot analysis of the expression of phosphorylated Erk-1/2 in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with anti-phospho-protein antibody and the lower panel shows a Western blot with an antibody to total-protein (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of phosphorylated to total protein. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001

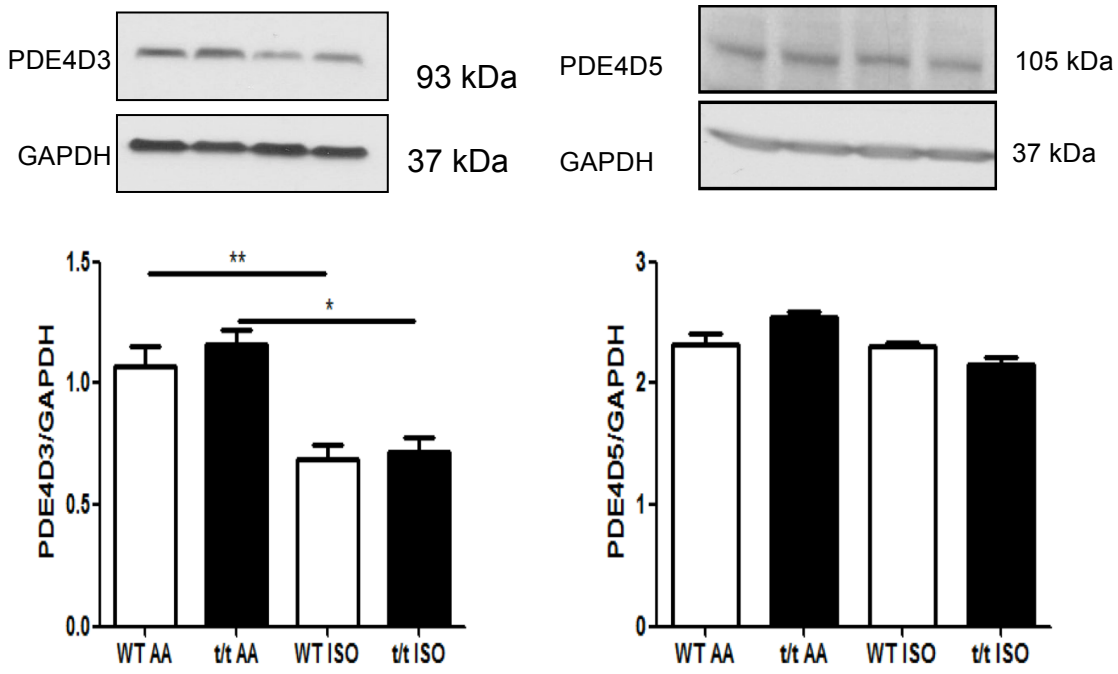

Figure 23. Western blot analysis of PDE4D3 and PDE4D5 in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with respective protein antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of PDE4D to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001.

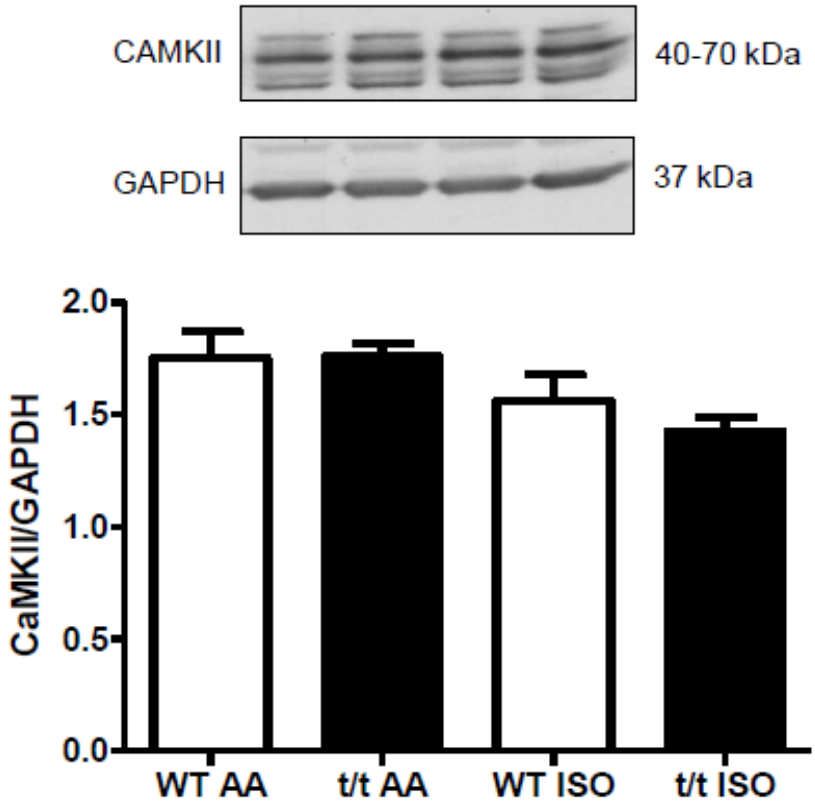

Figure 24. Western blot analysis of CaMKIIδ in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with CaMKIIδ antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of CaMKIIδ to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001

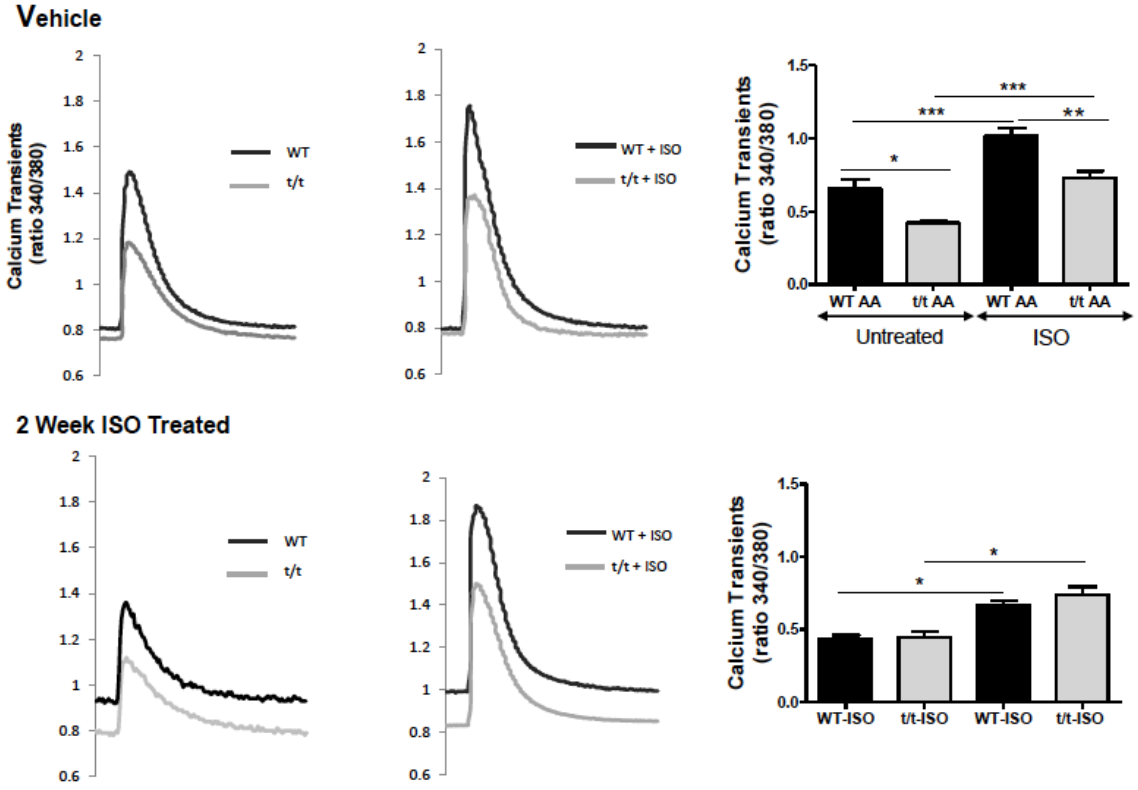

Figure 25. Calcium transients, in response to ISO stimulation in WT and gravin-t/t cardiomyocytes. (A) Representative cardiomyocyte Ca2+ transients and (B) corresponding bar graph of the 340/380 ratio in WT (n=18) and gravin-t/t (n=30) cardiomyocytes perfused with control buffer and WT (n=14) and gravin-t/t (n=29) cardiomyocytes perfused with ISO. The bar graphs show the Ca2+ transient 340/380 ratio from cardiomyocytes collected from 4 to 6 mice per group. Data are expressed as the mean ± S.E.M.; *P<0.05, **P<0.01, ***P<0.001

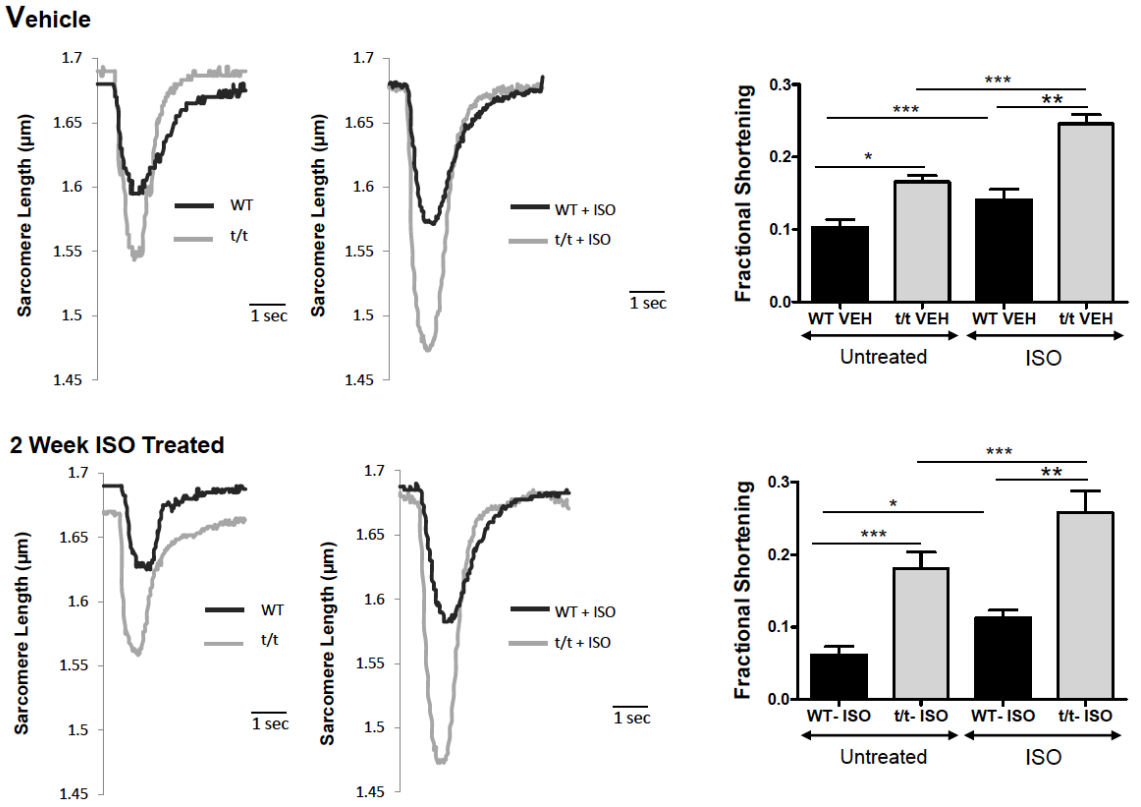

Figure 26. Sarcomere length shortening in response to ISO stimulation in WT and gravin-t/t cardiomyocytes. (A) Representative cardiomyocyte sarcomere length fractional shortening transients of WT (n=9) and gravin-t/t (n=13) cardiomyocytes perfused with control buffer and WT (n=8) and gravin-t/t (n=8) cardiomyocytes perfused with ISO. (B) Shows the corresponding bar graph of the percent shortening in sarcomere length. The bar graphs show the percent shortening from cardiomyocytes collected from 4 to 6 mice per group. Data are expressed as the mean ± S.E.M.; *P<0.05, **P<0.01, ***P<0.001

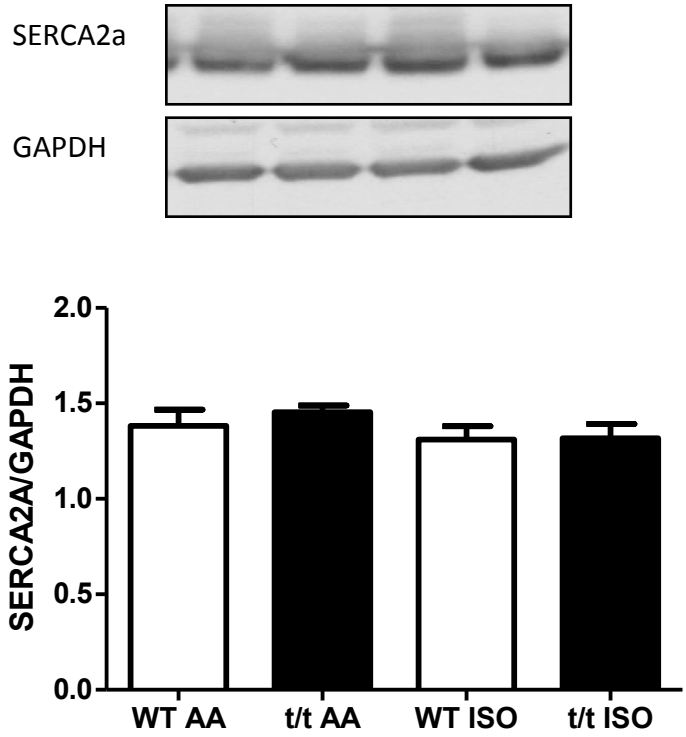

Figure 27. Western blot analysis of SERCA2A in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with SERCA2A antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of SERCA2A to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001

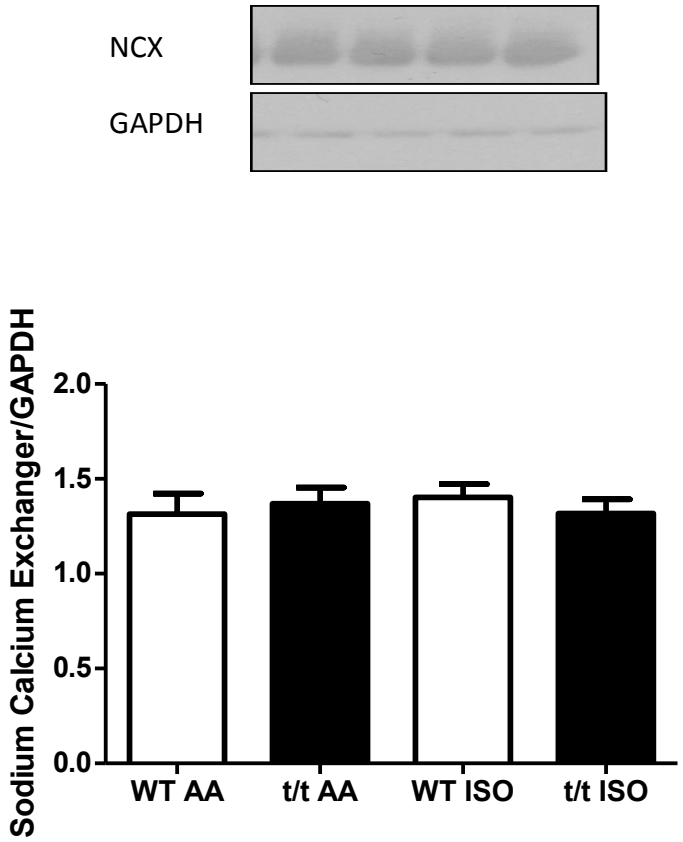

Figure 28. Western blot analysis of Sodium calcium exchanger (NCX) in total homogenates of the left ventricles isolated from WT and t/t hearts vehicle (AA) and ISO treated after 14 days of ISO treatment (60mg/kg/day). The upper panel shows a Western blot with Sodium calcium exchanger (NCX) antibody and the lower panel shows a Western blot with an antibody to GAPDH (Lane 1: WT AA; Lane 2: t/t AA; Lane 3: WT ISO; Lane 4: t/t ISO). The bar graphs show the ratio of SERCA2A to GAPDH. Data are expressed as the mean ± S.E.M.; n= 4 to 6 samples; *P<0.05, **P<0.01, ***P<0.001

	WT			t/t		
	Baseline	**2 weeks ISO treated**	**3 weeks carvedilol treated**	**Baseline**	**2 weeks ISO treated**	**3 weeks carvedilol treated**
LVIDd	3.706250 ± 0.09793413	4.574417± 0.1497907*	4.152667± 0.08601759	3.455833 ± 0.2015115	3.661667 ± 0.135049†	3.473667 ± 0.202853@
LVPWDd	0.817750 ± 0.05046909	1.031000 ± 0.05550915	0.9756666 ± 0.05116358	0.7603333 ± 0.03693568	0.9540833 ± 0.06316704	0.8494167 ± 0.06660786
IVSd	0.6631666 ± 0.01931738	0.8596667 ± 0.030122*	0.606000 ± 0.0250772#	0.6168333 ± 0.02359578	0.7443333 ± 0.019736*†	0.576250 ± 0.0284182#

All values are expressed as mean **±** SEM. LVIDd, left ventricular inner diameter; LVPWDd: left ventricular posterior wall diameter; IVSd: intraventricular septum. All these measurements were made at diastole.

*P <0.05 either baseline in WT vs 2 weeks WT ISO or baseline in t/t vs 2 weeks t/t ISO;

#P <0.05 either 3 weeks carvedilol treated WT vs 2 weeks WT ISO or 3 weeks carvedilol treated t/t vs 2 weeks t/t ISO;

†P <0.05 2 weeks WT ISO vs 2 weeks t/t ISO; @P <0.05 3 weeks carvedilol treated WT vs 3 weeks carvedilol treated t/t

	WT			t/t		
	Baseline	**2 weeks ISO treated**	**3 weeks bisoprolol treated**	**Baseline**	**2 weeks ISO treated**	**3 weeks bisoprolol treated**
LVIDd	3.675000 ± 0.072410	4.462000 ± 0.128600*	4.097000 ± 0.105900	3.303000 ± 0.141100	3.407000 ± 0.205300†	3.567000 ± 0.200500@
LVPWDd	0.764800 ± 0.022510	0.992000 ± 0.057400*	0.906000 ± 0.045540	0.872300 ± 0.016760	0.779700 ± 0.030470†	0.965300 ± 0.047920
IVSd	0.670200 ± 0.017030	0.872500 ± 0.037490*	0.693500 ± 0.041560#	0.635200 ± 0.024950	0.716500 ± 0.017720†	0.739800 ± 0.017250

All values are expressed as mean **±** SEM. LVIDd, left ventricular inner diameter; LVPWDd: left ventricular posterior wall diameter; IVSd: intraventricular septum. All these measurements were done at diastole.

*P <0.05 either baseline in WT vs 2 weeks WT ISO or baseline in t/t vs 2 weeks t/t ISO;

#P <0.05 either 3 weeks bisoprolol treated WT vs 2 weeks WT ISO or 3 weeks bisoprolol treated t/t vs 2 weeks t/t ISO;

†P <0.05 2 weeks WT ISO vs 2 weeks t/t ISO; @P <0.05 3 weeks bisoprolol treated WT vs 3 weeks bisoprolol treated t/t

	WT			t/t		
	Baseline	**2 weeks ISO treated**	**3 weeks ICI 118 551 treated**	**Baseline**	**2 weeks ISO treated**	**3 weeks ICI 118 551 treated**
LVIDd	3.709000 ± 0.129400	4.226000 ± 0.170200	4.418000 ± 0.147300	3.688000 ± 0.043720	3.640000 ± 0.109300†	3.721000 ± 0.152500@
LVPWDd	0.784300 ± 0.044640	1.072000 ± 0.034610*	1.063000 ± 0.060250	0.785200 ± 0.030910	0.921700 ± 0.073070	0.871400 ± 0.069270@
IVSd	0.655600 ± 0.022750	0.828300 ± 0.031340*	0.826400 ± 0.022700	0.668200 ± 0.022240	0.699700 ± 0.014760†	0.709000 ± 0.014200@

All values are expressed as mean **±** SEM. LVIDd, left ventricular inner diameter; LVPWDd: left ventricular posterior wall diameter; IVSd: intraventricular septum. All these measurements were done at diastole.

*P <0.05 either baseline in WT vs 2 weeks WT ISO or baseline in t/t vs 2 weeks t/t ISO;

#P <0.05 either 3 weeks ICI 118 551 treated WT vs 2 weeks WT ISO or 3 weeks ICI 118 551 treated t/t vs 2 weeks t/t ISO;

†P <0.05 2 weeks WT ISO vs 2 weeks t/t ISO; @P <0.05 3 weeks ICI 118 551 treated WT vs 3 weeks ICI 118 551 treated t/t

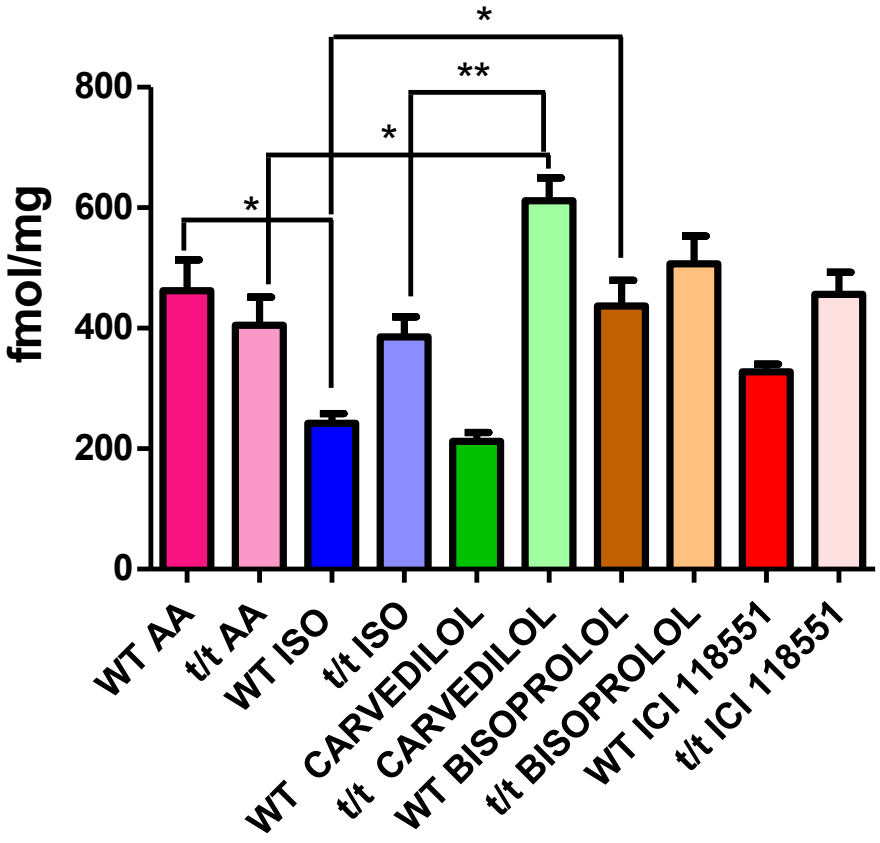

Figure 29. Total β-AR density determined in left ventricular membrane fractions isolated from in WT and t/t hearts after chronic ISO stimulation for two weeks followed by 3 weeks of chronic treatment with Carvedilol, Bisoprolol and ICI118551. Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of20μM alprenolol. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6

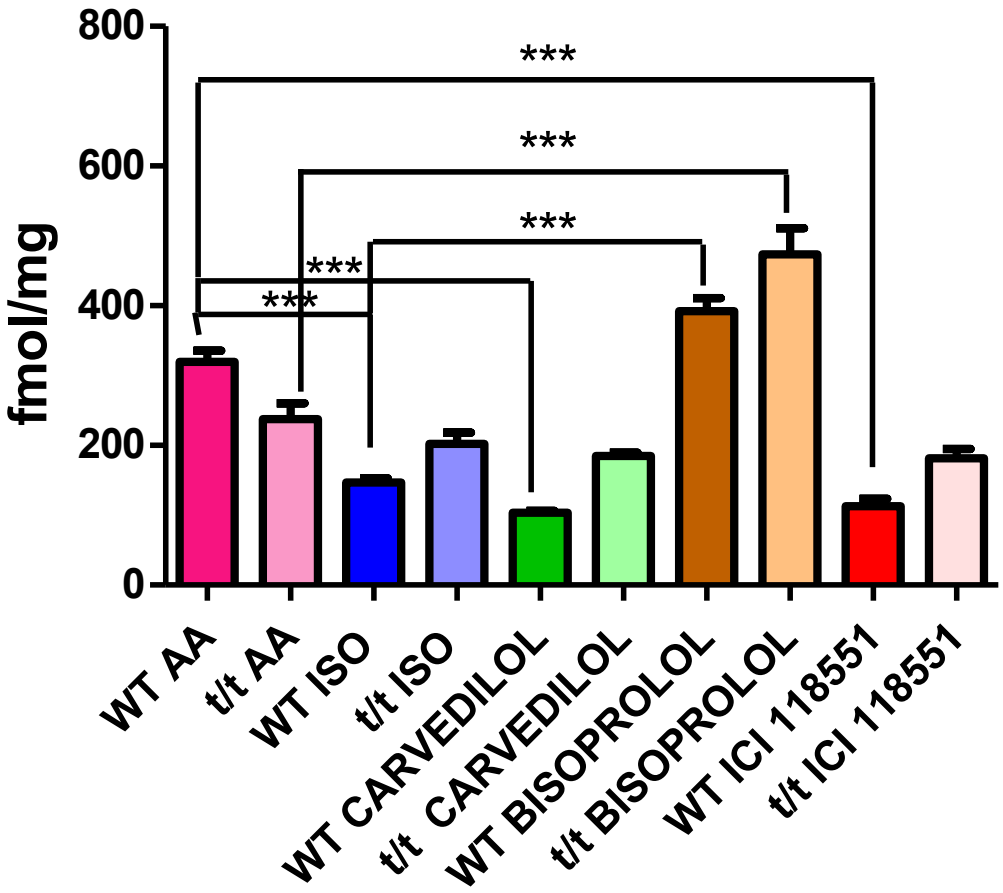

Figure 30. Total β_1-AR density determined in left ventricular membrane fractions isolated from in WT and t/t hearts after chronic ISO stimulation for two weeks followed by 3 weeks of chronic treatment with Carvedilol, Bisoprolol and ICI118551. Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of20μM alprenolol. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6.

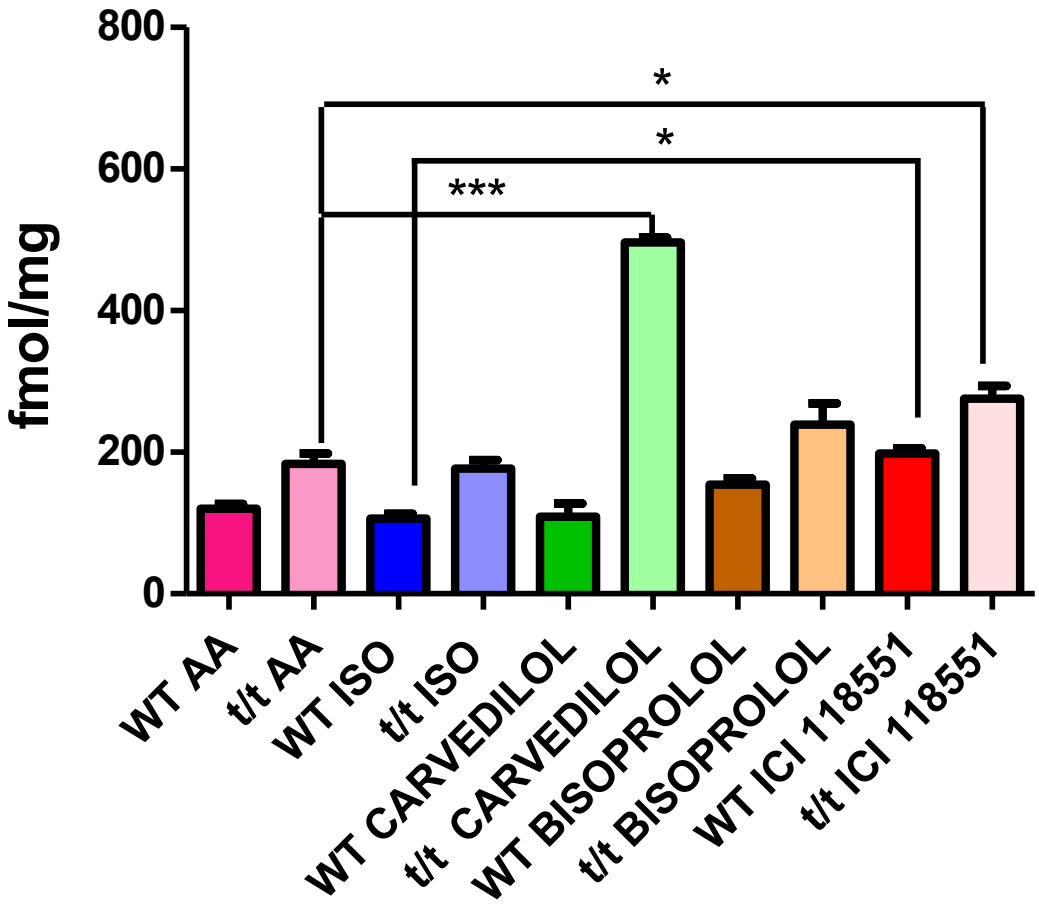

Figure 31. Total β_2-AR density determined in left ventricular membrane fractions isolated from in WT and t/t hearts after chronic ISO stimulation for two weeks followed by 3 weeks of chronic treatment with Carvedilol, Bisoprolol and ICI118551. Saturation curves were made using [125I]-cyanopindolol (10pM-4nM). Nonspecific binding was determined in the presence of20μM alprenolol. Bmax (receptor density) was normalized to fmol/mg. Data expressed are means ± S.E.M.; n=5-6.

5. Discussion

Cardiac contractility is primarily mediated through the β-ARs, which mainly signal via their coupling to the Gs pathway leading to the activation of PKA resulting in a positive ionotropic effect. The β-AR subtypes however i.e. β_1 and β_2 have their distinct signaling mechanisms as well as distinct roles to play during pathological conditions. The β_1-ARs: β_2-ARs ratio in the healthy heart is 70:30%. However during HF, the elevated circulating levels of catecholamines leads to severe desensitization of the β-ARs. The β-ARs undergo phosphorylation by PKA and GRK leading to their desensitization as well as internalization. This decreases the number of receptors available on the membrane of the cardiomyocytes. This changes the ratio of the β-ARs subtype to 50:50. The β_2-ARs are also dysfunctional in nature as during HF they uncouple from Gs and signal via Gi. These significant alterations in the β-ARs have severe implications on the downstream pathway especially with the respect to the PKA dependent phosphorylation. Major substrates downstream of β-ARs activation undergo aberrant phosphorylation that worsens the contractility, relaxation and calcium homeostasis and triggers adverse events such as apoptosis in cardiomyocytes.

Gravin scaffolds PKA and PKC and targets them to β_2-ARs. Upon agonist stimulation, the association of PKA with β_2-ARs increases only in the presence of gravin. Gravin thus increases the phosphorylation of the β_2-ARs by PKA and

PKC. Moreover, gravin is also essential for the recruitment of GRK and β-arrestin to the β_2-ARs for the desensitization and internalization following chronic stimulation of the receptors. This leads to the abrogation of the β-ARs signaling. Hence, disruption of gravin's scaffolding will block the desensitization and internalization of the β-ARs which should resist the induction and progression of HF.

In our studies, we found that the truncation of the gravin protein wherein the exon 3 has been excised from the genome resulting in the truncation of the protein. This truncation renders the protein non-functional as it looses all the binding sites for important partners such as PKA, PKC and β_2-ARs. We found that truncation of gravin resulting in abrogation of gravin's scaffolding action leads to increased cardiac function in spite of 2 weeks of isoproterenol treatment. Other studies have found that 2 weeks of isoproterenol mimics the chronic stimulation of the β-ARs, as is the case during HF. In our studies we found that the enhanced hemodynamic function in spite of the chronic stimulation of the β-ARs in the t/t mice could be the result of the increased β_2-ARs pathway. We found that absence of gravin caused a significant increase in the β_2-ARs density compared to the WT mice. This increased β_2-AR in the gravin t/t mice may be contributing to the resistance of induction of HF and enhanced hemodynamic in these mice. In our studies we found that, 2 weeks of ISO administration

significantly reduced the total β-ARs density as well total β_1-ARs density in the WT cohort. However no significant change was seen in the β_2-ARs density in these mice. Gravin t/t mice on the other hand showed no significant alteration in their total β-ARs density, β_1-ARs density or β_2-ARs density.

Studies have shown that administration of isoproterenol at high doses causes severe reduction in the β-ARs expression due to desensitization as well as sequestration of the receptors. Chronic stimulation of the β_2-ARs causes the receptor to uncouple and signal via the Gi pathway. Also the receptors, which are present, are impaired and dysfunctional due to phosphorylation by the PKA and GRK. Even though gravin does not bind to the β_1-ARs directly, it is involved in the signalosome complex formation which includes GRK, β-arrestin, clathrin coated pits etc. which are essential for the endocytosis and sequestration of the β_1-ARs receptors post chronic stimulation. Absence of gravin inhibits this entire complex formation leading to the abrogation of the internalization and sequestration of the β-ARs.

We found that there was no change in the GRK2 expression in the membrane in the gravin t/t and WT mice in the control group indicating that absence of gravin does not effect the GRK translocation under normal conditions. However post chronic stimulation of the β-ARs there was significant increase in

the GRK2 translocation to the membrane, which was also associated with increased β_2-ARs phosphorylation in the WT mice. However in the absence of gravin, this increased recruitment of the GRK2 to the β_2-ARs post chronic stimulation was abolished. This also translated into no change in the phosphorylation of the β_2-ARs post treatment with ISO.

In case of GRK, studies have shown that inhibition of GRK can prove beneficial in HF. A peptide inhibitor known as β-ARKct was developed which has the Gβγ-binding domain of the GRK. It competitively inhibits the translocation of the GRK to the membrane thus inhibiting the GRK mediated decline of the β-ARs pathway (Jaber et al., 1996; Koch et al., 1995). Studies have also shown that over expression of the β-ARKct not only rescues the failing heart but also enhances the baseline contractility of the healthy heart in vivo. The role of β-ARKct has been well characterized in surgical models of HF as well crossing with transgenic models of HF (Esposito et al., 2000; Freeman et al., 2001; Rockman et al., 1998). It has been found that the protective effects of β-ARKct against HF are primarily through the prevention of the impairment in the β-ARs signaling pathway. Similar results have been reported using the adenoviral transgene of β-ARKct in larger animal models of HF. Correction of the impaired β-ARs signaling mechanisms seems to be the mechanism by which the β-ARKct (inhibition of

GRK) delay the onset of HF and enhance the hemodynamic function (Akhter et al., 1997; Watari et al., 2014; White et al., 2000).

These findings draw a parallel with the β-blocker therapy as well. One of the ways that the β-blockers improves the declining function in HF is by blocking the chronic stimulation of the β-ARs which inhibits their further desensitization and give the desensitized receptors a chance to recover and become functional again. Similarly β-ARKct abrogates the phosphorylation of the β-ARs and subsequent recruitment of β-arrestin. This prevents the impairment and desensitization of the β-ARs and more receptors are available to respond to catecholamine stimulation. Absence of gravin functions in a similar way and inhibits the desensitization carried out by not only the GRKs but also PKA and prevents the cascade of HF. Thus blockade of the desensitization and internalization of the β-ARs may be one of the ways that disruption of gravin's scaffolding imparts protection against isoproterenol induced HF in mice.

One of the hallmark pathologies of HF is the downstream effect of dysfunctional β-ARs due to desensitization and sequestration. β-blockers, the gold standard therapy for HF significantly improves mortality by rescuing the impaired β-ARs (Hjalmarson et al., 2000). However β-blockers do not restrict the progression of HF indicating that the impairment in the downstream effectors of

the β-AR pathway continues to exist long after the β-blockers rescue the β-ARs. Alteration in the PKA pathway is one of the severely impaired pathways as a result of impairment of the β-ARs. In our studies we evaluated the activity profiles of PKA in all the treatment groups. We found no change in the PKA activity in any of the four groups, which indicated that absence of gravin did not affect the PKA activity in the heart. Also treatment with 2 weeks of ISO treatment did not affect the PKA activity in heart in the WT or t/t mice. The assay is based on the intrinsic ability of the kinase to phosphorylate the substrates at the serine threonine site. This indicated that the PKA as a kinase was not undergoing any alteration and retained the ability to phosphorylate the substrates.

One of the reasons being that PKA pathway is impaired is the significant posttranslational modification as a result of altered β-AR signaling. Normally, PKA gets activated in a two-step manner. Subsequent to β-AR stimulation, PKA undergoes phosphorylation first at Thr-197 and followed by phosphorylation at Ser-338 (Keshwani et al., 2012; Shoji et al., 1981; Shoji et al., 1979). Phosphorylation of Thr-197 is required for the optimum activity of the kinase. Studies have shown that the phosphorylation levels of p-PKA (Thr-197) are elevated during HF possibly due to post translational modification especially in the catalytic subunit (Han et al., 2013). We found that there was no change in the expression of the catalytic subunit in before or after ISO treatment in either WT or

t/t mice. So gravin's absence did not affect the expression levels of the catalytic subunit of PKA. Moreover we found that the phosphorylation levels of p-PKA at Thr-197 were the same between the WT and t/t mice in the control groups. However, post 2 weeks of treatment with ISO, there were significant changes. The WT ISO had significantly higher levels of phosphorylation at Thr-197 compared to the control whereas in the gravin t/t ISO mice, the levels were same as control mice. Since there was no change in the activity of the kinase between the WT ISO and t/t ISO, the possible reasons for changes in the PKA phosphorylation levels (PKA activation) could be due to alterations in the post translational modification due to chronic stimulation of the β-ARs. These findings indicate that absence of gravin mediated restriction of the β-ARs desensitization limited the impairment in the downstream activation of PKA.

Our next goal was to further elucidate the implication of the PKA activation and how absence of gravin's disruption affected the downstream β-AR pathway. Sarcomeric proteins are the major contributors of maintaining the cardiac function. They are also the major targets of PKA and hence any changes in the PKA activity, localization, activation or posttranslational modification affects the heart function. Studies have shown that in HF, there are significant aberrations in the phosphorylation of the sarcomeric proteins, which further worsens the HF and triggers many adverse pathways.

Cardiac TnI has three isoforms: slow skeletal, fast skeletal and cardiac specific isoform. The cardiac specific isoform is present in the heart. cTnI is a myofibril protein and is involved in the modulation of the actin myosin interaction by manipulating the calcium sensitivity that determines the sarcomere shortening in the heart. TnI upon phosphorylation basically inhibits the actin myosin interaction by decreasing the sensitivity of cTnC to calcium, which increases the rate of dissociation of calcium from cTnC (Layland et al., 2005; Westfall et al., 2002). One of the other key targets of PKA is cMyBPC. Cardiac MyBPC is a thick filament protein and undergoes phosphorylation at three sites via PKA, CAMKII, and PKC etc. Of the 3 known sites, p-273 andp-302 is phosphorylated by PKA whereas other sites are phosphorylated by CAMKII, PKC etc(Gautel et al., 1995; McClellan et al., 2001). Phosphorylation of cMyBPC by PKA inhibits the myosin binding which decreases the distance between the actin and myosin resulting in the alteration of the cross bridge(Weisberg and Winegrad, 1996). The alteration can significantly affect the force of contraction of the cardiac muscle. One of the other proteins that is phosphorylated along with cTnI and PKA is PLB. PLB is an important regulator of calcium as after undergoing phosphorylation by PKA it disinhibits the inhibition of SERCA2a pump leading to uptake and release of calcium resulting in contraction and relaxation of the heart(MacLennan and

Kranias, 2003). Thus phosphorylation of both TnI, PLB and MyBPC play a very important role in the myofilament function (Tong et al., 2004).

In our studies we found that there was no change in the phosphorylation levels of any of the sarcomeric proteins namely, TnI, PLB and MyBPC, between the WT and the gravin t/t control mice. This indicated that absence of gravin does not effect the localization of PKA involved in the phosphorylation of these sarcomeric proteins. Since PKA is compartmentalized in the cardiomyocytes to different subcellular locations by different AKAPs, it proves that gravin is not the AKAP that is involved in the targeting and localization of the PKA pool responsible for the activation of the sarcomeric proteins. However as mentioned before the phosphorylation and dephosphorylation of these proteins plays a very important role subsequent to β-AR activation in the modulation of the cardiac contractility and relaxation. In our studies we found that after chronic stimulation of the β-ARs, there was significant reduction in the phosphorylation of the p-TnI in the WT group, which would affect the desensitization of cTnC further affecting the actin myosin cross bridging impairing the contractility. However the phosphorylation levels were similar between the gravin t/t AA and the gravin t/t ISO group. Similarly, PLB undergoes phosphorylation at Ser 16 by PKA. After 2 weeks of ISO stimulation we found that WT ISO group had significantly hypophosphorylated PLB whereas there was no significant difference in the p-

PLB between the gravin t/t AA and gravin t/t ISO mice. The hyperphosphorylation of the PLB can result in significantly altered calcium loading and unloading during each cycle of contraction and relaxation which further impairs the calcium cycling in subsequent cycle of contraction and relaxation thus progressively impairing the contractile function of the heart. One of other important proteins, which we looked into, was the MyBPC. We found that there was no significant difference between the phosphorylation levels at site 273 in the gravin t/t AA and gravin ISO mice. However 2 weeks ISO treatment caused significant reduction in the phosphorylation at site 273 in the WT ISO mice compared to the WT AA group. Interestingly, site 273 was the only site that we saw a difference in the phosphorylation levels in. Site 282 and 302 did not show any alteration in any of the four groups. One of the reasons could be that site 273 is phosphorylated by PKA, whereas the other sites 282 and 302 are also phosphorylated by PKA, PKC and also CAMKII. Since the model used for inducing HF was the chronic stimulation of β-ARs, the major pathway that undergoes significant alterations is the β-AR-PKA dependent pathway. This could be the reason that we see significant alterations only at site 273. However, this is one of the most important sites as only after phosphorylation at 273 by PKA, the MyBPC undergoes conformational changes exposing the other two sites for phosphorylation. MyBPC is a thick filament protein, which reduces the actomyosin ATPase activity after phosphorylation. The aberrant phosphorylation levels of MyBPC induced by

2 weeks of ISO treatment thus further worsen the contractile machinery. This shows that there was significant impairment in the β-AR dependent PKA pathway in the WT ISO mice. However absence of gravin's scaffolding restricted the impairment in the β-AR dependent pathway, which reflected in the normalized phosphorylation of the PKA targets. This reflected in the normalized cardiac function of the gravin t/t mice in spite of 2 weeks of isoproterenol treatment.

All these proteins are involved in regulating the homeostasis of calcium- one of the most important ionophores required for normal cardiac function. Subsequent to β-AR activation, the downstream pathway in involved in the homeostasis of this ionophore, which further modulates the cardiac contractility. Studies have shown that during HF calcium cycling in the cardiomyocytes is severely impaired. This not only affects the pumping capacity of the heart but also significantly reduces the responsiveness of the heart (β-ARs) to acute stimulation (e.g. during exercise etc.).

We studied the calcium cycling and contractility in the cardiomyocytes isolated from the four treatment groups we found that the baseline calcium transients were similar between the WT AA and the t/t AA mice indicating that the concentration of calcium in the resting cardiomyocytes was similar between the two groups. The amplitude (peak height) of the calcium transient of the

cardiomyocytes from t/t AA mice was significantly smaller than that of WT AA mice. However the corresponding sarcomere shortening of the cardiomyocytes of the t/t AA group was significantly larger than that of the WT group indicating that decreased transients had no effect on the contractility in the t/t group. On the contrary, absence of gravin significantly increased the sarcomere shortening of the cardiomyocytes at the baseline. Upon acute stimulation of the β-ARs with isoproterenol (1um) there was significant rise in the amplitude of the calcium transients of both the groups. However the transient of the gravin t/t AA mice were still smaller than that of the WT AA mice post stimulation with corresponding higher sarcomere shortening. Studies by the Meredith Bond group have also shown that inhibition of the AKAP/PKA binding using Ht31 (a peptide that binds to PKA and inhibits the binding of AKAP) yields similar results wherein there is increased contractility in response to β-AR stimulation. Further when the cardiomyocytes isolated from WT ISO and t/t ISO group were studied we found that the impairment in the β-AR pathway in the WT ISO group had translated in to aberrant calcium homeostasis as well. There was not only a significant increase in the baseline concentration of calcium in the resting cardiomyocytes in the WT ISO mice but there was significant reduction in the responsiveness of the cardiomyocytes to β-AR stimulation as indicated by the bluntness in the amplitude of the calcium transients after β-AR stimulation. Also the corresponding sarcomere shortening was significantly reduced for the WT ISO

group both at baseline and post acute β-AR stimulation. This indicated that the aberrant phosphorylation of the sarcomeric proteins was taking a toll on the calcium cycling in these cardiomyocytes leading to a higher baseline calcium concentration. Also, significant reduction and impairment in the β-ARs could be the reason for reduced responsiveness of the cardiomyocytes in response to β-AR stimulation. This mishandling of calcium also affected the contractility of the cardiomyocytes as indicated by the reduced sarcomere shortening. The cardiomyocytes isolated from gravin t/t ISO mice on the other hand showed no significant alteration in the baseline calcium concentration or the responsiveness to β-AR stimulation. The corresponding increase in sarcomere shortening was preserved in the t/t ISO mice in spite of the chronic stimulation of the β-ARs. Further the protein expression levels were the same for SERCA2a and NCX before and after treatment with ISO in both the WT and the gravin t/t mice indicating that absence of gravin does not affect the homeostasis of calcium cycling in and out of cytosol. Also treatment with Isoproterenol does not affect the expression of these proteins. Hence the significantly reduced calcium transients that we see might be due to increased calcium sensitivity due to which lower concentration of calcium is required for the activation of myofilaments to attain contractility similar to that of the WT.

Chronic stimulation of the β-ARs also leads to aberrant activation of other pathways such as CAMKIIδ etc, which prove detrimental in HF. CAMKIIδ is the major isoform present in the cardiac muscles and chronic stimulation of the β_1 receptors leads to its activation leading to aberrant calcium cycling eventually resulting in apoptosis. In our studies we did not find any change in the expression of CAMKII delta in any of the treatment groups indicating that 2 weeks of ISO induced HF does not lead to the activation of CAMKII. One of the reasons could be that, 2 weeks of Isoproterenol infusion does not induce severe enough heart failure to warrant activation of CAMKIIδ and more severe HF models such as myocardial infarction or pressure overload induced HF models need to be studied. This could also be the reason as to why we did not see differential phosphorylation levels in MyBPC at all the site as CAMKII is also involved in the phosphorylation of MyBPC at site 282 and 302. Further more the levels of PDE4D3 and PDE4D5, the two major isoforms of PDE4D present in the heart showed different expression levels. PDE4D5 levels were unaffected by the ISO treatment in both the WT and t/t ISO treated mice. However incase of PDE4D3 we found that there was a significant decrease in both the WT ISO and t/t ISO mice. Studies have shown that PDE's have differential activation levels in various HF conditions. Molecular dysfunction in the WT ISO mice could be the reason for decreased PDE4D3 expression. PDE4D3 forms a complex with gravin and β_2-ARs and is involved in the attenuation of the hypertrophic signaling (Berthouze-

Duquesnes et al., 2013). Also PDE4D3 is involved in the modulation of cardiac contractility by regulating the sarcoplasmic reticulum calcium release(Beca et al., 2011). Decrease in the PDE4D3 levels in the t/t ISO mice may be a compensatory mechanism in response to ISO stimulation to accommodate for the increased calcium sensitivity and increased β_2-ARs expression in these mice. Further investigations such as evaluating the cAMP levels and PDE activity assays are required to elucidate the exact mechanisms involved.

We also found that there was a significant increase in the ERK activation levels in the WT ISO group compared to the control. However there was no change in the ERK activation levels in the t/t mice. ERK activation occurs as a result of the β-ARs stimulation to initiate the prohypertrophic signaling mechanisms that is initially protective in nature. However chronic activation of ERK as happens during HF is detrimental and further worsens the HF. This leads to increase in the transcription of the hypertrophic genes leading to induction of fibrosis. We too found in our studies that there was significant increase in the amount of fibrosis in the WT ISO mice compared to the t/t ISO mice.

Disruption of gravin's scaffolding thus proved protective against isoproterenol-induced HF. Our next step was to validate whether this approach would prove more beneficial in rescuing a failing heart compared to the current

gold standard therapies as well as to elucidate the effects of β_1-ARs vs. β_2-ARs pathway. To do so we compared its effectiveness to Carvedilol (Non specific β-AR specific blocker), Bisoprolol (β_1-AR specific blocker) and ICI 118551 (β_2-AR specific blocker). The WT and gravin t/t mice were treated with these drugs for a period of 3 weeks after inducing HF with ISO for two weeks. We found that after 3 weeks of treatment with these drugs Carvedilol and Bisoprolol were successful increasing the cardiac function in the ISO treated WT group as indicated by decrease in the LVIDd as well is IVSd.

Beta-blocker therapies basically rescue the dysfunctional β-AR that forms the basis for their positive effects during HF. They also cause an increase in the receptor density that further contributes to their positive effects (Barry and Gilbert, 2003; Haeusler, 1990). Carvedilol is one of the most superior blocker drugs that not only alleviates the symptoms of the HF but also improves mortality even though the exact mechanisms involved in the superficial effects is not known. Most of the positive effects of Carvedilol seen in the clinical trials have been attributed to the biased signaling of Carvedilol at β_2-ARs coupled with some agonistic activity at the alpha receptors. Carvedilol however does not cause a change in the receptor density even after chronic treatment and also is known to cause further internalization of the β_2-ARs(Wisler et al., 2007). In our studies we found that Carvedilol did not change the total β-AR, β_1-AR or β_2-AR receptor

density after 3 weeks of treatment in the WT mice in spite of exhibiting improvement of the cardiac function. However in gravin t/t mice we found some interesting results. There was no change in the total β-AR or β_1-AR receptor density in the t/t mice. However there was a 2.5 fold increase in the β_2-AR density. Bisoprolol and ICI 115881 on the other hand showed similar changes in the receptor density. With β_1-AR blockade there was an increase in the β_1-AR density in the WT as well as t/t mice with no effect on the β_2-AR density and vice versa with β_2-AR blockade. Thus it shows that there are additional mechanisms involved in the absence of gravin that cause a 2.5 times increase in the β_2-AR density with Carvedilol treatment. The increase in the β_2-ARs density as a result of Carvedilol treatment in absence of gravin;s scaffolding can be harnessed to develop combination therapy of Carvedilol and inhibition of gravin's scaffolding for treatment of HF. However further studies are required to evaluate the action of Carvedilol at β_2-AR in the absence of gravin's scaffolding.

In conclusion the gravin t/t mice restrict the induction and progression of HF. Majorly it restricts the internalization and desensitization of the β-ARs, which inhibits the downstream molecular dysfunction that is usually accompanied by chronic stimulation of the β-ARs. This leads to normalized β-AR signaling pathway, which is one of the most severely, compromised pathways that lead to worsening of the HF. Also, absence of gravin leads to enhanced calcium

sensitivity, which can be seen by enhanced contractility coupled with smaller calcium transients. Thus disruption of scaffolding of gravin imparts protection against isoproterenol-induced HF. Thus absence of gravin can be further explored as a potential therapeutic target that not only rescues the dysfunctional β-ARs but can also circumvent the side effects of current therapies like cardio toxicity on account disruption of gravin's subsequent beneficial action of enhanced calcium sensitivity.

6. Summary and Conclusions

1. Gravin t/t mice did not show hypertrophy and fibrosis after 2-week of isoproterenol treatment

2. There were no alterations in cardiac morphology and dimensions of gravin t/t/ mice after 2 weeks of ISO treatment which also reflected into normal heart rate, fractional shortening, stroke volume and ejection fraction. Validation also came along with PV loops data which showed that gravin t/t mice in spite of 2 weeks of ISO treatment exhibited normal cardiac output, ejection fraction and stroke volume. The contractility of the gravin t/t mice was also preserved even after ISO treatment for 2 weeks. The systolic function of the left ventricle was also comparable to the control.

3. Absence of gravin did not affect the total β-AR density or the β_1-AR density. However these mice exhibited an increase in the β_2-AR density at baseline. Upon 2 weeks ISO treatment there was no loss in the total β-AR, β_1-AR or β_2-AR receptor density indicating that absence of gravin blocked the sequestration and internalization of the β-ARs. The desensitization of the β_2-ARs was also restricted post 2-week treatment with ISO as indicated by no change in the phosphorylation levels of β_2-ARs compared to the control.

4. Absence of gravin's scaffolding did not alter PKA activity and expression of PKA RII alpha or total catalytic PKA expression. Further phosphorylation of PKA at Thr-197, which is required for optimal enzymatic activity of PKA, was significantly increased in WT ISO mice as

compared to control whereas no change was seen in the t/t ISO mice. This aberrant activation translated into altered substrate phosphorylation of the substrates in the WT ISO mice but was normalized in the gravin t/t mice.

5. To our surprise, disruption of gravin's scaffolding did not affect the PDE4D3 or PDE4D5 expression at baseline. However post 2-week treatment with ISO the expression of PDE4D3 was significantly decreased in WT as well as t/t mice whereas PDE4D5 did not show any change.

6. Gravin t/t mice showed enhanced myofilament sensitivity as indicated by significantly smaller calcium transients coupled with significantly increased sarcomere shortening as compared to the WT mice in the vehicle treated cohort. Also post 2 weeks of ISO treatment there was significant reduction in both the calcium transients as well as the sarcomere shortening of the WT ISO group indicative of altered calcium handling. The gravin t/t ISO mice on the other hand showed no changes in the calcium homeostasis compared to the control. SERCA2A and NCX showed no change in WT and gravin t/t mice before and after ISO treatment which indicates that the alteration of the calcium handling is probably due to molecular dysfunction in the β AR pathway rather than changes in the calcium cycling in the cardiomyocytes.

7. Significant increase in the ERK phosphorylation along with increased apoptosis was seen in WT ISO mice whereas gravin t/t mice showed no such change. These alterations were also reflected in the amount of fibrosis that was induced in these hearts.

8. Treatment with Carvedilol causes a 2.5 fold increase in the β_2-AR receptors whereas treatment with ICI 115881 causes only a modest increase in the receptor increase.

7. Limitations of the Study

One of the biggest limitations of this study is the mouse model of HF. Gravin t/t mice resist the induction of HF by chronic administration of ISO. Hence with the treatment of β-AR blockers it is difficult to discern their effect as the gravin t/t mice do show any alterations post 2-week treatment with ISO. Hence surgical models of HF like, myocardial infarction by ligation of the left anterior descending artery or pressure overload model by transverse aortic constriction that would induce injury to the same extent in both WT and t/t(deAlmeida et al., 2010; Wang et al., 2006). Using these HF models would better help understand the role of antagonisms of the β-ARs using the beta-blockers.

Also, gravin is involved in the scaffolding of the β-arrestin to the β_2-AR complex. Also β-arrestin signaling has been shown to be beneficial in HF. It is possible that the beneficial effects of the β-arrestin are shadowed by the disruption of scaffolding of the gravin. Hence further studies elucidating this pathway are also required.

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