IRB

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Title of Project: Determination of the functional role of cleavable residues in the C-terminus of L-type calcium channel ($Ca_V1.2$) in cardiac myocytes.

A. Study Purpose and Rationale: Excitation- contraction coupling in cardiac myocytes is dependent on calcium flow through voltage dependent L-type calcium channels ($Ca_V1.2$) (1). Changes in the activity or expression of these channels on the cellular membrane have been shown to trigger atrial fibrillation or structural remodeling of the heart leading to pathologic hypertrophy (2, 3). These channels are localized at t-tubules, which positions them in close proximity to the ryanodine receptor of the sarcoplasmic reticulum. It is this close relationship that underlies cardiac excitation-contraction coupling (4, 5). Two important differences exist between neonatal and adult excitation-contraction coupling. Specifically, t-tubules are nearly absent during fetal development, and fetal and neonatal cardiomyotes are dependent on extracellular calcium influx through calcium channels (including L-type calcium channels) in order to initiate excitation-contraction coupling due to immaturity of the sarcoplasmic reticulum (6).

The trafficking of the L-type calcium channel has been suggested to result from residues within the C-terminus of the α_{1C} subunit; specifically Ala_{1800} has been proposed to undergo proteolytic processing via an unidentified protease, in addition, Ser_{1700} and Thr_{1704} are proposed to be phosphorylated by PKA. However, this cleavage has never been reproducible in heterologous expression studies (7). Additionally, there are very unclear and contradictory results involving the roles of residues in the C-terminus depending on the expression system by which the studies are performed. In heterologous expression, truncation at residue 1905 of the C-terminus reduced surface expression of the $Ca_V1.2$ channel and enhanced single channel open probability. However, in knock out mice, the channel expression was so dramatically reduced that the mice underwent cardiac failure and died perinatally (8). Therefore, the laboratory has undertaken the development of transgenic mice with inducible, tissue

specific expression of multiple C-terminal residue mutations of the α_{1C} subunit of $Ca_V1.2$ channels. The overall goal of the project is to study the role of specific residues in the region on receptor trafficking, regulation, and functional targeting of the channels in neonatal cardiac myocytes, using a system which is inducible, developmentally complex, and more similar to human cardiac physiology than heterologously expressed channels.

Aim:

1.Characterize $Ca_V1.2$ channels role in fetal/neonatal cardiac development, and investigate physiological changes that occur to channel function as sarcomere anatomy develops.

2.To study $Ca_V1.2$ trafficking and functional modulation directly in fetal/neonatal cardiac myocytes, using a system that is more physiologic in nature.

3.To characterize the role of C-terminal residues of $Ca_V1.2$ in pathological conditions, including cardiac arrhythmias and sudden death.

B. Study Design and Statistical Analysis: Transgenic doxycycline inducible mice expressing key mutations in the C-terminus of Cav1.2 channels (S1800A and T1704A) were generated, with the approval of the Institution for Animal Care and Use Committee at Columbia University. Pregnant female mice will have induced expression of mutant Cav1.2 channels in fetal pups at gestational day 17-21 using 0.2mg/kg doxycycline-impregnated feeding pellets. Determination of a phenotype for the transgenic Cav1.2 channels will be determined by comparing post-natal pups at DOL 3, 7, 14 with wild type controls using cellular electrophysiology comparing peak Ca^{2+} currents, the Cav1.2 current-voltage relationship, and the fractional shortening of freshly isolated myocytes. The possibility exists that the dosing of the pregnant female and days of doxycycline exposure may need to be adjusted if the mutations result in fetal demise as has been previously describe with the non-inducible transgenic mouse (8).

In brief, each procedure is provided below:
Cellular Physiology: A whole cell patch-clamp method using a Multiclamp 700B amplifier. Ca^{2+} currents will be measured by holding the membrane potential at -70mV with to +10mV every 5-10 seconds to stimulate the voltage-activated Cav1.2 channels. Current-voltage relationships will be determined using a similar protocol starting at -50mV to +50mV with interval increases of 10mV.
Fractional shortening: Fractional shortening of sarcomere length will be measured using the Sarclength module of Ionoptix. Myocyctes will be stimulated at 1Hz. Nisoldipine and other calcium channel blocking drugs will be used as antagonist to determine effect of channel mutations on fractional shortening.

Hypotheses to be tested:
Hypothesis A: The phenotype of the c-terminal mutated Cav1.2 channels as determined by peak Ca^{2+} current, the current-voltage relationship, and fractional shortening of the sarcomere length, will differ significantly from wild type Cav1.2 channels.
Null hypothesis A: The phenotype of the C-terminal mutated Cav1.2 channels will not differ significantly from the phenotype of the wild type Cav1.2 channels.
Hypothesis B: T-tubule formation and maturation of the SR will result in differences in peak Ca^{2+} current of the Cav1.2 channel and shortening of the sacromere observed over time points (DOL 3, 7, 14) in early neonatal life of WT mice cardiac myocytes.
Null hypothesis B: No differences will exist in peak Ca2+ current of the Cav1.2 channel and shortening of the sacromere observed over time points (DOL 3, 7, 14) in early neonatal life of WT mice cardiac myocytes.

Statistical analysis: Data will be presented with means and standard errors. A minimum of 3 mice from at least 2 litters will be used for each time interval (DOL 3, 7, 14) for each group (transgenic and wildtype). Graphpad Prism will be used for statistical analysis and data presentation. For comparison between two groups (transgenic mutations and wildtype mice), unpaired t-test will be used,

with significant determined to be p>0.05. For multiple group analysis, one way ANOVA will be used.

Using a power calculation for unpaired t-test, 6 cardiomyocytes should be sufficient to detect an effect between the two groups to the standard deviation observed in wild-type mice. Each mouse will contribute more than the required number of cardiomyocytes to detect a difference, however using 3 mice from 2 different litters will provide intra-animal and intra-litter validity to the results.

		DOL 3	**DOL 7**	**DOL 14**
WT	Peak Ca *i*	Mean (#) SEM (+/-)	Mean (#) SEM (+/-)	Mean (#) SEM (+/-)
	i-v relationship	# +/-	# +/-	# +/-
	Δ in length (sarcomere)	# +/-	# +/-	# +/-
Mutations	Peak Ca *i*	# +/-	# +/-	# +/-
	i-v relationship	# +/-	# +/-	# +/-
	Δ in length (sarcomere)	# +/-	# +/-	# +/-

Section C- R: not applicable

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