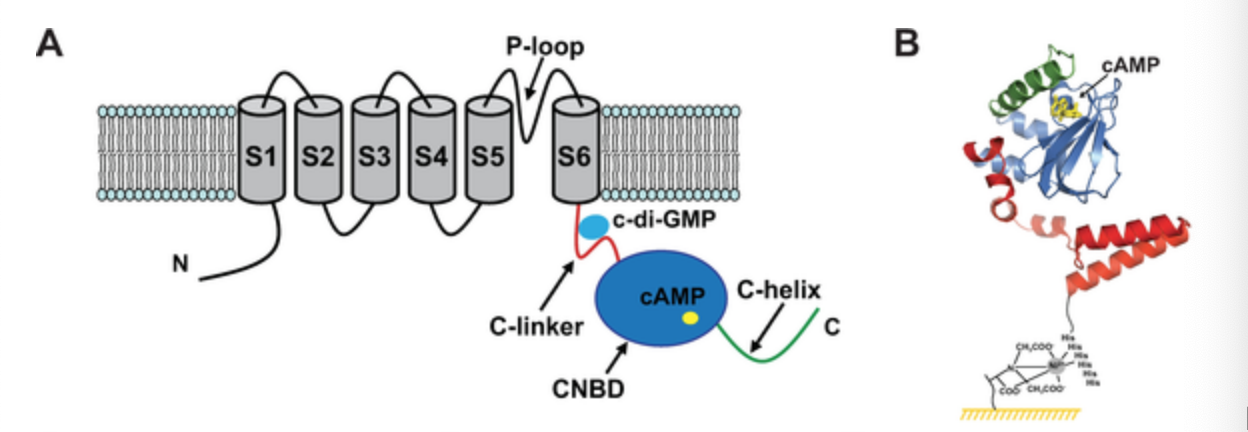
**Effects of disease-causing mutations on human HCN4 channel functionality**

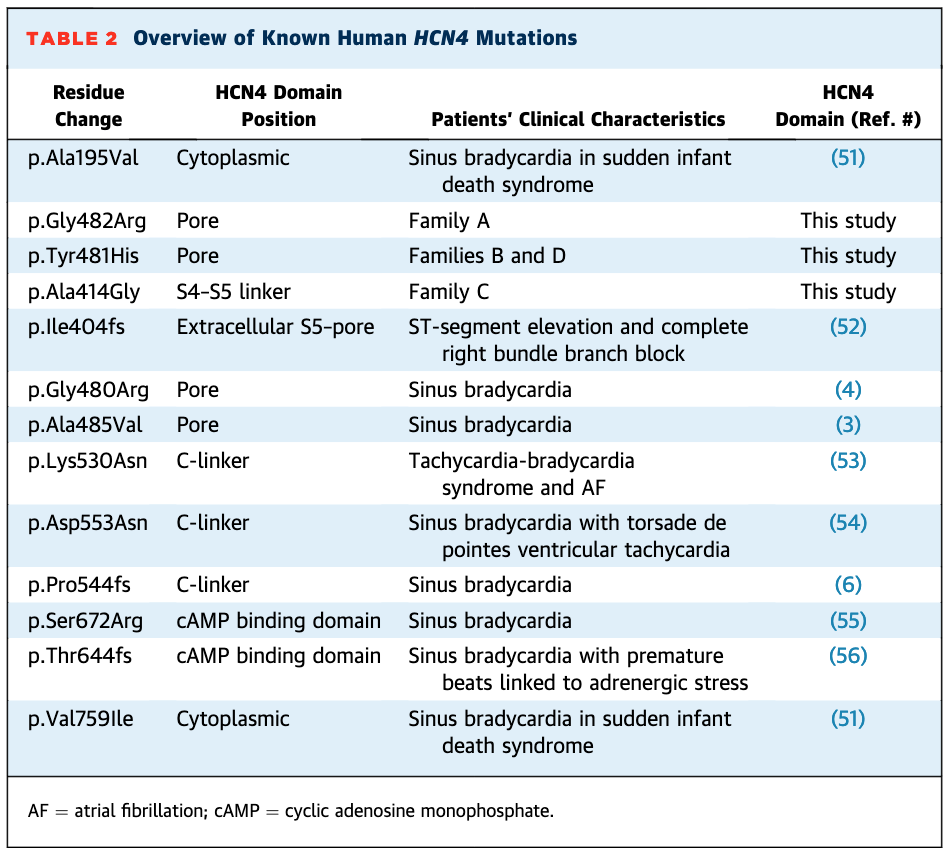
**I Introduction:**

Sinus bradycardia can be defined as a heart rate that is lower than 60 beats per minute. Asymptomatic sinus bradycardia is generally harmless and most often a signal of good physical conditioning. On the other hand, symptomatic sinus bradycardia such as sick sinus syndrome can lead to fatigue, chest pain, and shortness of breath. This condition is characterized by heart arrhythmias, which are caused by a dysfunction in the sinus node of the heart. Sinus bradycardia can be inherited, suggesting an underlying genetic basis. (Milano et al 2014)

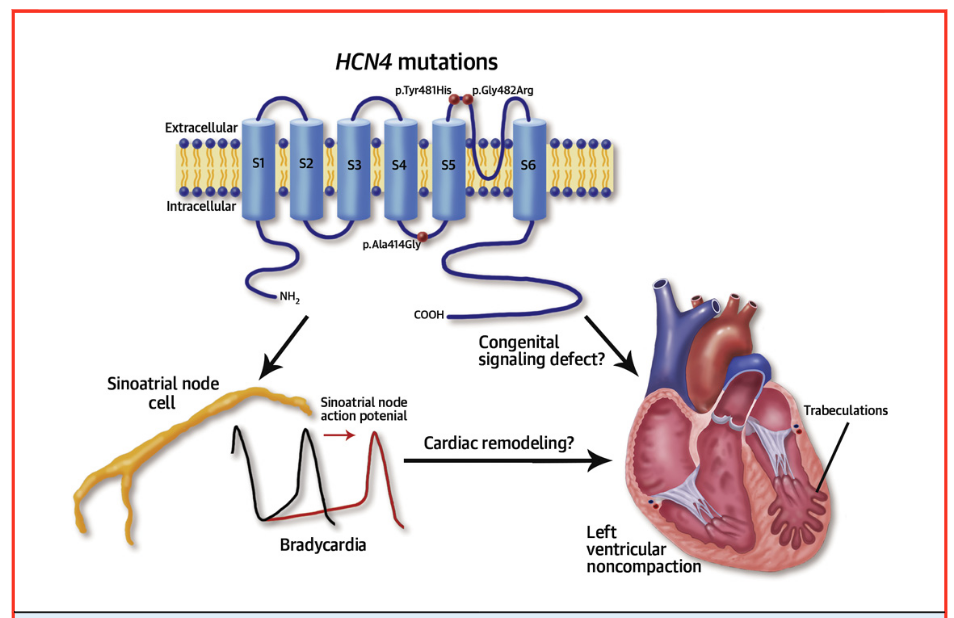
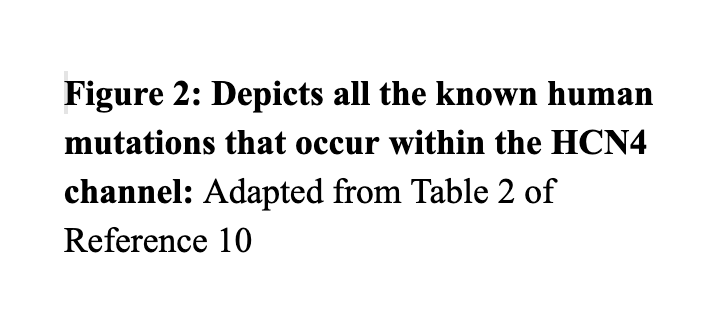
Hyperpolarization-activated cyclic nucleotide-gated (HCN) ion channels are voltage gated and ligand-activated integral membrane proteins that contribute to the pace-making activity in cardiac cells (Milano et. al 2014). They used to be referred to as pacemaker cells because they generate rhythmic activity within heart cells. A unique feature of HCN channels that distinguishes them from other voltage gated channels is that the HCN channel pore opens in response to hyperpolarizing voltages instead of depolarizing voltages (Milanesi et al, 2006). Hyperpolarization occurs when the membrane potential of the cell becomes more negative due to an efflux of potassium, whereas HCN channels help bring the membrane toward depolarization. (Flynn et al, 2018). A single unit of the HCN4 channel is composed of 6 trans-membrane domains and a pore-forming loop—the P domain—located between transmembrane domains S5 and S6, acting as the ion conducting pore and selectivity filter ( Milanesi et al. 2006). HCN channels are regulated by the binding of cyclic nucleotides such as cAMP, cGMP, or cCMP. The binding of cyclic nucleotides lowers the threshold potential of HCN channels required for channel activation.



**Figure 1: Structure of the HCN channel with cAMP binding site.** Adapted from Table 1 of Reference 8

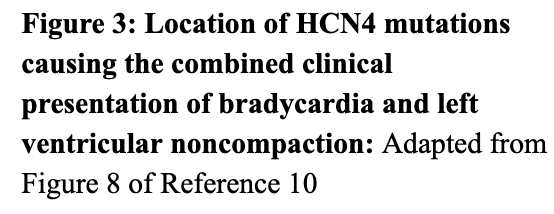
HCN channels are responsible for generating the pacemaker “funny” current that plays a crucial role in modulating the rhythm of the heart (Difransceco et al, 2007). HCN4 channels are the main isoform that are expressed within the sinoatrial node (Milanesi et al, 2006). The funny current is a mixed sodium–potassiumcurrent that activates upon hyperpolarization at voltages in the diastolic range, normally from −60/−70 mV to −40 mV (Difransceco et al, 2007) . When the end of the sinoatrial action potential is reached, the membrane repolarizes below the If threshold, -40 mV. The funny current is then activated and supplies inward current which causes diastolic depolarization. This method mechanism allows the funny current to control the activity of the sinoatrial myocytes and therefore the cardiac rate. The pacemaker current (If) that is located in the sinoatrial-node myocytes determines the slope of the diastolic depolarization of pacemaker cells and therefore has a crucial role in the generation and autonomic regulation of the sinus rhythm and rate (Difransceco et al, 2007). 

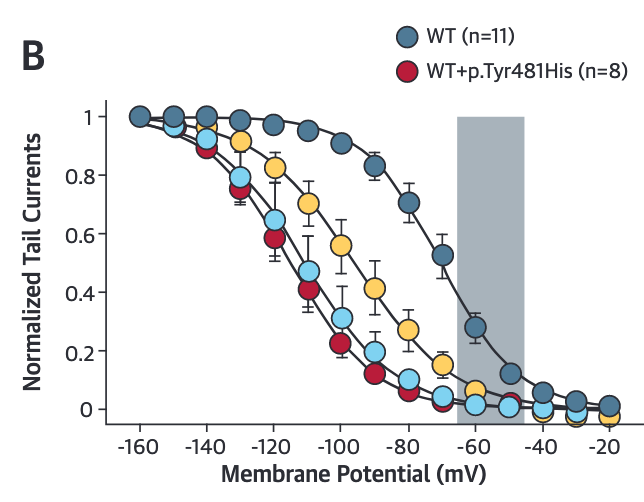
Sinus bradycardia has been linked to mutations in HCN4, SCN5A, and ANK2 genes (Milanesi et al, 2006). The focus of this proposal will be mutations located in the HCN4 channel. **Figure 2** depicts all known mutations that are found within the HCN4 channel. They all involve a change from one amino acid residue to another. Different amino acids have different hydrophobic and hydrophilic properties that can impact how the channel will respond to an influx or efflux of charged ions.

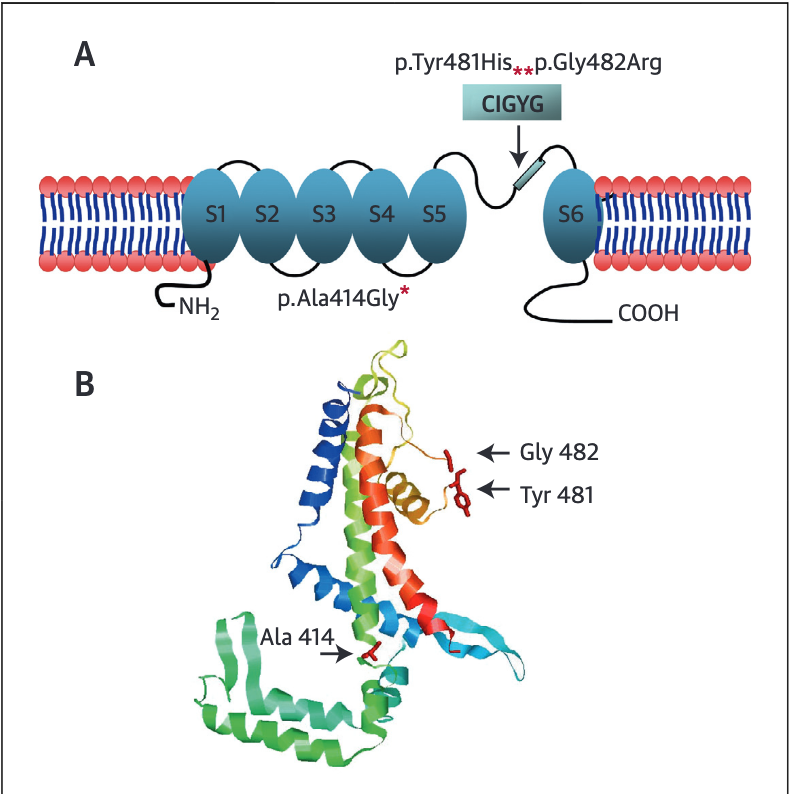
Mutations within the hHCN4 gene causes mutant channels to become activated at more negative voltages when compared with wild-type channels (Milanesi et al, 2006). This leads to less current being supplied during diastolic depolarization, which leads to a slowing of the heart rate. Diastolic depolarization occurs during the transition from the max diastolic potential (-70 mV) to the threshold potential, -40 mV (Flynn et al., 2018). The threshold potential is the critical level that the membrane potential

is depolarized to initiate the action potential.

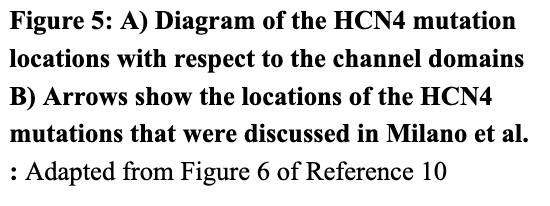
This transition is called the pacemaker phase and its duration governs how fast the heart beats (Flynn et al., 2018).

Mutant channels respond normally to cAMP binding but are activated at negative voltage when compared with wild type channels (Milanesi et al, 2006). For example, mutant channels open at -60 mV while wild-type channels open at -20 mV (Milano et al. 2014). This can be seen in **Figure 4.** The wild-type channels are more easily opened and are open longer than the mutated channel.

**Figure 4 : Comparison of membrane potential in mV of wild-type and mutated channels:** Adapted from Figure 7 of Reference 10

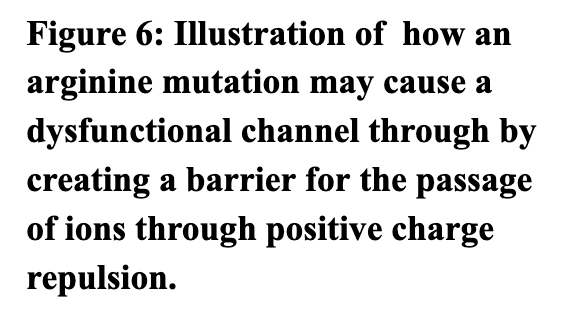


Mutations within the S5-S6 pore domain region have been linked to bradycardia (Milano et al. 2014). The S5-S6 linkage region acts as the pore forming loop of the HCN4 channel. The S5-S6 region acts as the ion conducting pore and a selectivity filter (Milano et al. 2014). Mutations in this region will directly impact the selectivity of the channel since the pore itself is being affected. There have been two mutations that have been identified in this region, Tyr481His and Gly482Arg (Milano et al. 2014). **Figure 5** shows the mutations and the CIGYG region where these mutations occur. This region

is highly conserved, which means that it has remained relatively unchanged far up the phylogenetic tree. These two mutations involve the mutation of an uncharged amino acid to charged amino acid. This changes the properties 

of the amino acids from hydrophobic to hydrophilic.

In both of the identified mutations, Tyr481His and Gly482Arg, uncharged, hydrophobic amino acids are mutated to a charged amino acid. Armstrong et al (2016) speculated that arginine side chains provide a potent barrier to the passage of ions within voltage gated channels. They tested this by causing mutations in a human skeletal muscle sodium channel cDNA clone through site-directed mutagenesis within human embryonic kidney cells. They engineered different mutations within the channel converting hydrophobic amino acids to charged arginine or lysine amino acids. They then measured peak levels of Na+ depolarization through patch clamp recordings. They found that in voltage-mediated transitions the positive side chains provide a barrier to the passage of ions. Bendahhou at el (2007) goes in depth in the opposite direction by focusing on how mutations from charged to uncharged amino acids can affect how voltage-gated sodium channels. They found that mutations from hydrophobic to charged amino acids can cause comparable or even larger shifts in activation and inactivation curves when compared to charged substitutions. A balance between hydrophobic and charged amino acid interactions are crucial for the overall function of the gate. **Figure 6** diagrams how a charged amino acid mutation present in the CIGYG region may interfere with the movement of charged ions attempting to pass through the channel by charge repulsion. The original wild-type channel does not contain a charged amino acid and this may be the reason why ions are able to pass through easier.

 Mutations in this pore region have been limited to mutations that have gone from uncharged to positively charged and the results have shown that the function of the channel has decreased causing bradycardia. There have been no cysteine mutations discovered within the region. Cysteine is a polar amino acid. The purpose of this proposal is to explore whether mutating the cysteine, present within the S5-S6 pore forming region, to an arginine will also cause a dysfunctional HCN4 channel.

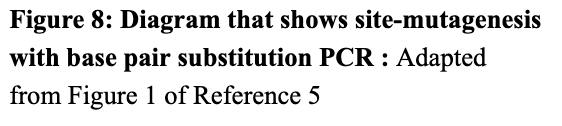
**II** **Experiment:**

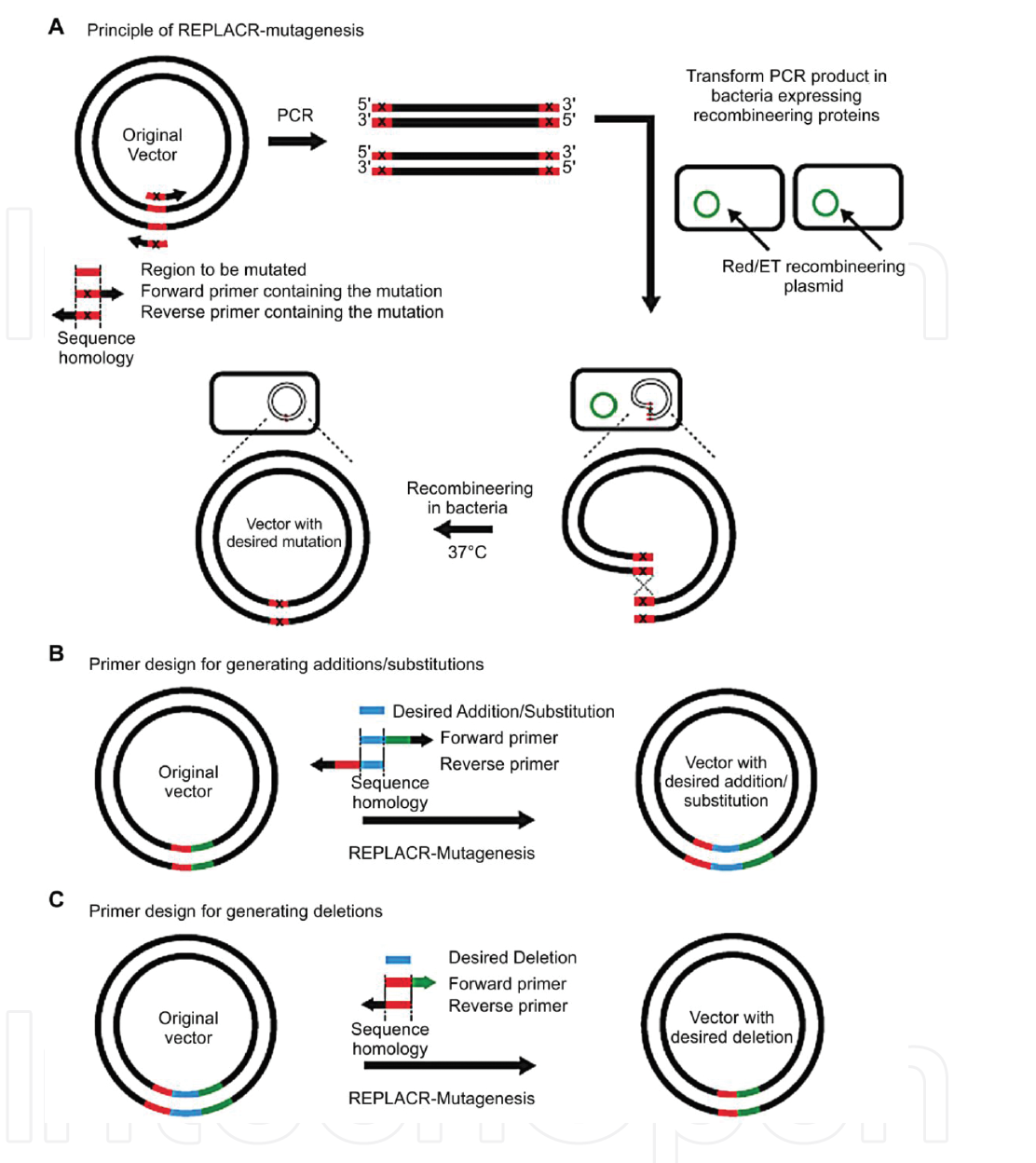
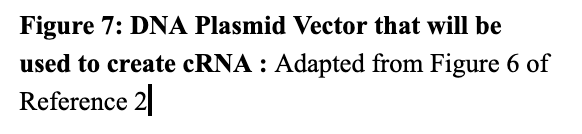
The overall goal of the experiment will be to compare mutated human HCN4 channels with wild-type human HCN4 channels. cDNA, one with the mutation and one without, will be created within E. Coli and injected within the nuclei of the frog oocytes. The oocyte that is expressing wild-type HCN4 channels will be the control group. The frog oocytes will then be allowed to go through the processes of translation to create functioning HCN4 channels and be measured through two-electrode voltage-clamp (TEVC).

A. Why *Xenopus* oocytes:

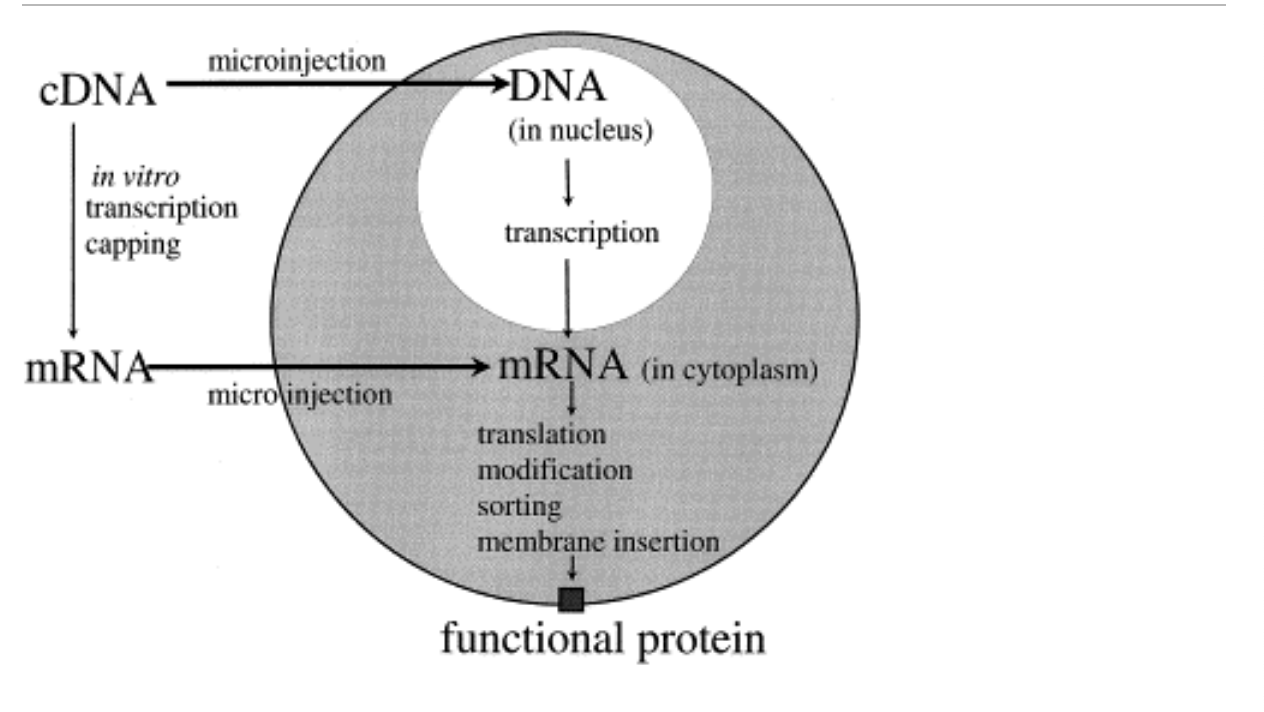
*Xenopus* oocytes have been used extensively as a heterologous expression system to study ion channels *in vivo*. A primary advantage of *Xenopus* oocytes is their cells do not express large numbers of ion channels and receptors, so exogenous proteins can be studied with minimum endogenous interference (Goldin and Alan, 2006). There are also advantages in the use of oocytes with respect to electrophysiology. The oocyte system is very well suited for the study of different mutations since injection and two-electrode voltage-clamping can be performed rapidly (Goldin and Alan, 2006).

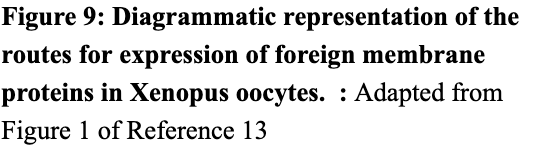
B Vectors and Site-directed mutagenesis:

A close up of a map

Description automatically generated The first step in this experiment in this to use a viable vector that can successfully create complementary DNA that can be injected into the oocyte nucleus (Miller & Zhou, 2000). The vector that will be used for this experiment is commercially available and will be provided by The ORFeome Collaboration. The vector (pENTR223.1) contains the appropriate HCN4 DNA. **Figure 7** depicts the structure of the vector that is available through ORFeome Colloboration. Human HCN4 channel cDNA clone will be used as a template to engineer the mutation of cysteine to arginine by a point mutation through site directed mutagenesis PCR that will change one thymine to a cysteine. The overall point mutation will cause a shift from TGC to CGC effectively creating the desired cDNA. **Figure 8** depicts how base substitution functions in detail. The DNA plasmid is made up of two sets up complementary DNA. During the first step the plasmid is heated in vitro to 95 º C which destroys the hydrogen bonds between the base pairs and allows the two strands to separate. PCR primers are ordered which contain the sequence with the point base mutation present. The PCR primer sequence that will be used will be TCGGTGTACGACCCGTAGCCGATG. The region itself has a sequence of AGCCACATGCTGTGCATCGGCTAC .The sequence of the region was found using the National Center for Biotechnology Information’s website. The point mutation is depicted in red and does not hydrogen bond to its complement region because they are not complementary. In the annealing step, the solution is allowed to cool which allows the primers to attach to their complementary regions. DNA polymerase and free nucleotides are added in vitro as well. The solution is then heated once more which allows the DNA polymerase to begin to synthesize new DNA in the 5’ to 3’ direction that contains the new codon sequence. One of the DNA strands is the parent strand while the other is the mutated strand. The parent strand is then removed using methylation-dependent endonuclease (Laursen, 2016). Another round of separation and replication occurs which yields the new modified double stranded DNA which is created by DNA polymerase and free nucleotides. Thus, the new product double stranded complementary DNA is created which contains the desired mutation.

II In vivo injection into *Xenopus* oocyte:

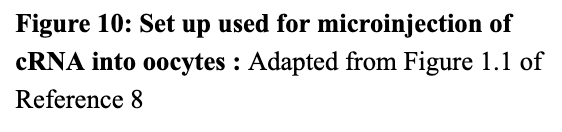


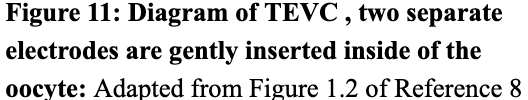
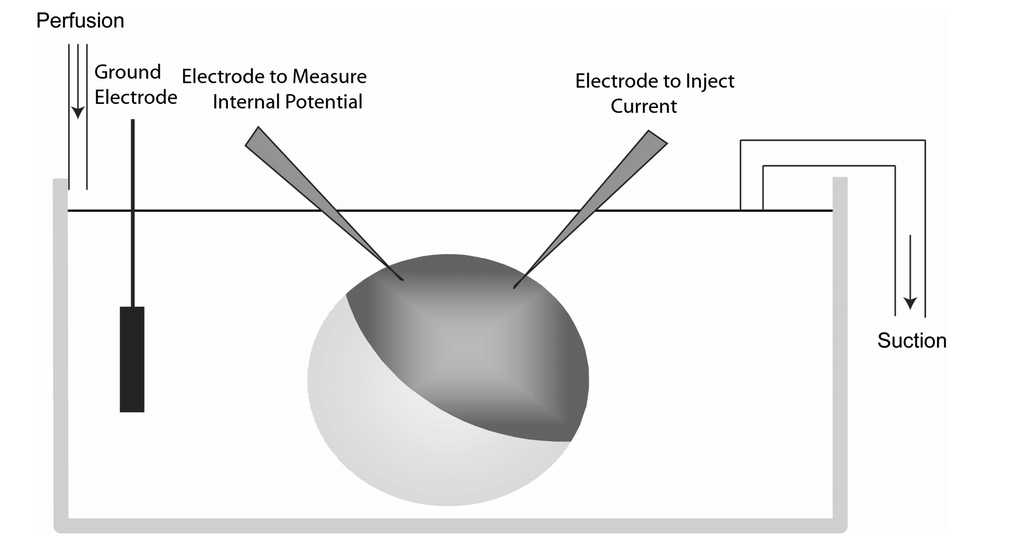
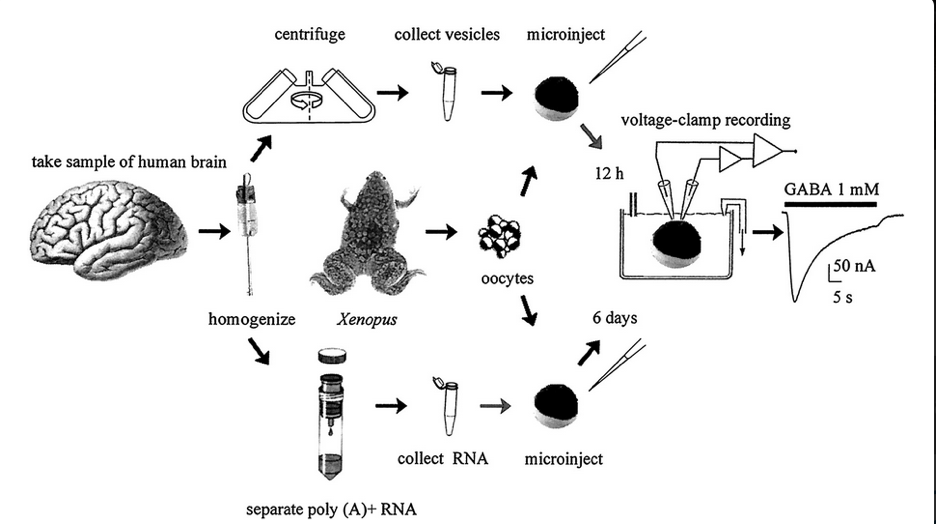


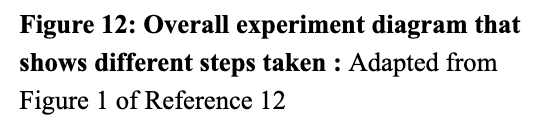
Once cDNA has been created the next step is to microinject cDNA into the nucleus of the oocytes using a microinjector. This step should be done for both the wild-type and mutant oocyte. The advantage of injecting DNA is that there is no need to perform in vitro transcription reactions, which saves both time and money (Goldin and Alan, 2006). Cytoplasmic injection is rapid and simple procedure. All that is needed is a dissecting microscope, micromanipulator, and an injector (Goldin and Alan, 2006). **Figure 9** depicts how once the cDNA is injected translation is allowed to occur and the oocyte will begin to express the mutant or wild-type human HCN4 channel. The cDNA goes through the process of translation which creates mRNA that translates into a functional protein that expresses the mutation. **Figure 10** depicts the overall microinjection setup that contains the dissecting microscope, micromanipulator, and the injector.



II Measuring electrophysiology with TEVC (two electrode voltage clamp):

To compare both oocytes and the impact of the mutation on the HCN4 channel the electrophysiology of each set of oocytes will be measured. The method that will be used will be two-electrode whole cell voltage-clamp (TEVC). Whole cell voltage clamping of oocytes involves using two electrodes inserted into the oocyte with one measuring the internal potential of the oocyte and the other electrode being used to inject current (Goldin and Alan, 2006). The large size of the oocyte makes this system possible since it contains enough room for both electrodes. The electrodes 

are made of glass and are filled with 3 M KCl solutions and have an electric resistance between .2 and 3 M (Bianchi, 2004). Ca2+ buffers are used to inhibit endogenous calcium currents ( Bianchi, 2004). The electrodes are then mounted on electrode-holders that contain a silver wire with a layer of AgCl2 that allows the transmission of the signal from the KCl solution into the oocyte. To be able to visualize the oocytes, a stereomicroscope is equipped with a cold light source and is placed under the vibration isolation table beneath a Faraday cage ( Bianchi, 2004). The Faraday cage is used to reduce electronic noise. Oocyte impalement is done in clamp (controlled) mode and successful impalement is determined by reading the resting membrane on the amplifier display. A healthy non-injected oocyte should have a resting membrane potential of -40 mV ( Bianchi, 2004). Once both electrodes have been inserted, the amplifier is set in voltage clamp mode and the voltage-clamp feedback gain, which is used to compensate for membrane capacity, is increased to speed up the time response of the voltage clamp. Currents are then filtered and recorded using a digital converter and a computer. The activation curves for the HCN4 currents should be recorded under whole-cell conditions that are obtained by standard activation and deactivation protocols and analyzed with the Boltzmann equation, y =1/{1 +exp (V-V1/2 /s) (Milanesi et al, 2006). Where y is the fractional activation, V is the voltage in millivolts, V1/2 is the half-activation voltage in millivolts, and s is the inverse slope factor in millivolts (Milanesi et al, 2006). Mean activation curves are obtained by fitting individual curves from each cell to the Boltzmann equation and averaging half-activation voltages and inverse slope factors (Milanesi et al, 2006). **Figure 11** shows a diagram of TEVC in action with both electrodes present inserted within the oocyte. **Figure 12** shows the entire experimental process from start to finish.



**III Discussion:**

The results of the experiment may show whether or not the point mutation would have a detrimental impact on the HCN4 channel, or it may show that there was no impact at all on the mutation. The experiment may show that a mutation from a cysteine to an arginine makes the channels behave differently when compared to the wild-type channel. If the experimental results show that the mutant HCN4 does not responded correctly it may show that any mutations within the P-form region that contains the CIGYG region will lead to a heart rate disfunction. A limitation of this experiment is that it since there have been no experiments looking into the effects of a polar amino acid to a positive charged amino acid mutation within the HCN4 it is difficult to say how the channel will respond. Furthermore, it is not known whether the channel may even be able to function with a point mutation that causes a cysteine to convert to an arginine.

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