**BK Channel Clustering in Slo-1 mutants**

By Dylan Lee

Alcohol abuse is a big problem in society. Alcoholism is thought to have a strong genetic basis at the same times we do not understand the mechanisms by which alcohol causes intoxication. It has been useful to use C Elegans as model organism to study ethanol’s effect on a molecular level. There have been several proposed targets by which ethanol acts on the brain. One of the targets is a voltage gated potassium pump called the BK(~~Burger King~~ Big Postassium) channel.

**Slo-1(lf) and Slo(T381)**

It is known through( Davies et al 2003) that the bk channel is an ethanol target. The slo-1 null mutant otherwise known as the slo-1loss of function mutant is shown to produce worms that are resistant to ethanol. In addition to being resistant to ethanol worms with the slo-1 lf mutation display a crooked neck phenotype insert a picture of the crooked neck phenotype**.** Through *(*Davis et al 2012) we know that there exists a single **cytostolic** residue at the 381stthreonine that is responsible for increased ethanol tolerance in all ethanol tolerant slo-1 mutants. When aforementioned threonine is changed to an isoleucine worms express an ethanol resistant phenotype comparable to a slo-1(lf) without the crooked neck phenotype seen in slo-1 lf. Davis et al 2012 shows The nature of this mutation. The means by which this residue confers ethanol resistance on a worm is unknown.

**The Role of Dystrophin**

Dystrophin is a protein that is responsible for connecting membrane proteins in muscles cells to the cytoskeleton and anchoring membrane proteins in position. The BK Channel is commonly found on neuron and muscle cells. It has been observed through the use of gfp that the bk channel is found in a clustered phenotype. Recently it has been shown that there exists a dystrophin mutation that effects the bk channels position on neurons and muscle cells. The dystrophin mutation causes the bk channel to lose its clustered phenotype. Worms with the aforementioned dystrophin mutation express an ethanol tolerance comparable to that of a slo-1 loss of function mutants. See figure alphafrom (Oh KH et al 2019)



Figure alpha from (OH KH et al 2019)

**Hypothesis**

**Do ethannol resistant slo-1 mutants(T381) form clusters?**

**Proposed Experimental Methods.**
To observe the clustering phenotype of an ethanol resistant T381 mutant the following steps need to be taken. Worms(slo-1(cim105[slo-1::GFP])(Oh KH, Kim H) will be mutagenized using crispr and short range homology directed repair. Microscopy will be used to observe the position of bk channels on neurons and sacrolemma of the worms.

**Crispr Methods**

Crispr works by taking a generated marker(sgRNA) and using the marker as a guide to make a double stranded break using the cas9 enzyme. For crispr to work Cas9 attaches to guide sgRNA. SgRNA is made up of two components crRNA and tracRNA. crRNA is an rna strand that acts as a target for where to dircect the location of the double stranded cut. tracRNA binds the crna to the cas 9 proteins that makes the double stranded cut. To select the location of the crRNA sequence we need to find a pam or a short sequence NGG or NCC see figure 2 that will be directly preceded in the 5’ direction by the template that will be used to make our crRNA. One codon upstream of our pam region will be where the double stranded break will occur fig2. Once we have assembled our sgrna sequence we need to insert said sequence into a plasmid with the sequence for cas 9 already in it. Oligo nucleotides will be ordered their sequence being (ecorv+crRNA+tracRNA+kasl) the oligo nucleotides will be annealed together **make citation to create the final sgrna complex**. The plasmid mentioned in (Dickinson et al 2014)will be ordered off of add gene and the restriction enzyme ecoRV and kasl will be used to create two cuts that will create two strands of dna of different sizes both strands will be ligated together with the sgrna that had been exposed to the ecorv and kasl restriction enzymes**.** After ligation two products will exists one long and one very short. In order to separate the two products a restriction enzyme digest using gel electrophoresis will be performed to select the longer product.



figure 1

The design of the plasmid used to insert cas 9 into c elegans genome.

(Dickinson et al 2014) see magnified figure 1 at the end

Electric blue = t381 mutation T→ I

crna template = GAAGCACATAGTGGTCTGTG

Pink= pam sequence

Black = cut site

AATTGCCGATTTGATTGGAAACCGGCAAAAATACGGTGGGGAGTACAAAGGAGAGCACGGGAAGAAGCACATAGTGGTCTGTGGCCATATCACCTACGATTCGGTGTCCCATTTTCTTCAAGATTTCCTACACGAGGACCGTGATGACGTGGATGTCGAAGTGGTGTTTTTGCATCGTGTCGTGCCGGATTTGGAGCTGGAAGGCTTGTTT

figure 2

N(ECORV)CACATAGTGGTCTGTGGCCATATCATCTACGATTCGGTGTCCCATTTTN(ECOIV)

ssDNA Oligonucleotide that will be used as a homology template

green = homology arms

blue = sites to be changed

figure 3

**HDR(Homology Directed Repair)**

To create the edits to the bk channel. We are creating a cut 5’ upstream of the codon that codes for the 381st threonine (see figure 2 for the cut location). Once the cut is made a single strand oligo sequence will be introduced as the repair template. The repair template has one edit. For short range HDR to occur a double stranded break needs to be made near(within 10 bp) the site that is going to be repaired and a repair template needs to be created for homologous recombination to occur. Once the break has been created and the repair template introduced t5 exonculease chews back the five prime end of the broken strands creating a 3' overhang. the repair template anneals to the 3’ end then dna polymerase fills the gap the process is repeated again for the other side of the repair template as seen in figure 4.

Homology directed repair will occur using the ssDNA oligo sequence as a template.

The single stranded black region below is the change to be made. The single stranded blue and green regions are homology arms that direct homology directed repair.





Figure 4

the glowing portion in the diagram is t5 exo nuclease activity.

https://www.youtube.com/watch?v=4wv4ETxL5PE

**MICRO INJECTION**

The crispr plasmid along with the ssDNA repair template mentioned above will be injected using microinjection into the gonad (eggs) of the hermaphrodite worm slo-1(cim105[slo-1::GFP]).

**PCR Screen**

Once the worm has been injected its progeny will be grown up and separated. Care needs to be taken to separate each generation on plates of their own. Once the F2 generation has been reached worms will be separated and allowed to lay eggs. The f2 generation’s worm will be lysed and the dna will be extracted.

To see if transgenesis was successful a screen of the animals using pcr will be carried out. To do this primers were created. A start primer was made based on the region that was being modified an end primer was made for a random region about 1000 bp down stream of start.

Figure 5

start primer : TGTGGCCATATCACC

end primer :taagaagaaaatcttaaaa

blue = mutation point

These primers create one product 1109 base pairs long verified with biobike in a wild type worm

If this product cannot be created from the progeny of the worm then we can say that transgenesis has taken place because our modification will have broken the region the start primer would have bound to. If this product can be created than we can be confident that transgenesis has not taken place.

**Microscopy**

Once the animals are screened using pcr. The progeny of the animals that carry the T381 mutation will be grown into adults and microscopy will take place.

For Microscopy an animal will be moved under the microscope illuminated with blue light and then the the dorsal cord(nerve) the sacrolemma(muscle cell) and the egg laying nerve will be observed under magnification. The puncta that appear or do not appear should then be counted on each of the different areas over a box 150 pixels long. Compare the number of puncta to the numbers in see figure6 (Oh KH, Kim H 2019).



figure 6 (Oh KH et al 2019)

The wild type neuron on top shows clustering the dystrophin mutant on bottom shows a diffuse phenotype.

**Strains and plasmids used**

A strain of worm with gfp attached to the end of its slo-1 gene was used.

slo-1(cim105[slo-1::GFP])(Oh KH et al 2019)

# Peft-3::Cas9 + ttTi5605 sgRNA Dickinson et al 2014



Works cited

Davies AG, Pierce-Shimomura JT, Kim H, et al. A Central Role of the BK Potassium Channel in Behavioral Responses to Ethanol in C. elegans. *Cell*. 2003;115(6):655-666. doi:10.1016/s0092-8674(03)00979-6.

Davis SJ, Scott LL, Hu K, Pierce-Shimomura JT. Conserved single residue in the BK potassium channel required for activation by alcohol and intoxication in C. elegans. *J Neurosci*. 2014;34(29):9562–9573. doi:10.1523/JNEUROSCI.0838-14.2014

Dickinson DJ, Ward JD, Reiner DJ, Goldstein B. Engineering the Caenorhabditis elegans genome using Cas9-triggered homologous recombination. *Nat Methods*. 2013;10(10):1028–1034. doi:10.1038/nmeth.2641

Oh KH, Kim H. BK channel clustering is required for normal behavioral alcohol sensitivity in C. elegans. *Sci Rep*. 2019;9(1):10224. Published 2019 Jul 15. doi:10.1038/s41598-019-46615-9

5 https://benchling.com/protocols/wTQojorb/option-1-clone-grnas-into-plasmids