Michael Vuong

BNFO 300

Proposal Draft

**Introduction:**

 Alcohol is a commonly abused substance among humans that presents a variety of social and health problems. Health problems of alcohol use disorder include greater risk of certain cancers, weakened immune system, heart disorders, and damage to the central nervous system[[1]](#footnote-1). According to the National Institute on Alcohol Abuse and Alcoholism (NIAAA), an estimated 18 million people in the United States have an alcohol disorder in the form of abuse or dependence.[[2]](#footnote-2) Dependence stems from the development of alcohol tolerance, which is the acquired resistance response to the pleasurable aversive effects of the substance.

 While previous studies have shown that genetic and environmental factors can predispose humans to behavioral responses, such as alcohol abuse, sensitivity, and tolerance, the actual genes and the mechanisms underlying these behavioral responses are largely unknown[[3]](#footnote-3). There are only a handful of genes that have hold established roles in human alcohol disorders. Therefore, it is crucial to identify these genes and understand how they function in response to alcohol exposure in order to develop therapeutic treatments targeted at molecular/genetic level factors for people with alcohol disorder.

 Since genetic and molecular pathway investigations are difficult to perform in human population due to the widespread variety of the genetic and environmental factors, model organisms, specifically Drosophila fruit flies, are used instead to conduct investigations in a relatively controlled environment. This model organism is also useful for these genetic experiments due to their similar genetics to humans as well as conserved behavioral responses to alcohol. Many genes identified in fruit flies may be mapped back to human genome and could provide similar results.

 Through these experiments, the Chloride Intracellular Channel (CLIC) gene expression was identified by Bhandari et al (2012) to play a major role in development of ethanol sensitivity in Drosophila[[4]](#footnote-4). They first identified the CLIC gene from a list of possible ethanol-responsive gene candidates provided through multiple DNA microarray studies, also known as DNA chip( discussed in more detail in experiment section). In order to test the gene, this experiment overexpressed and silenced the CLIC gene, then measured organism activity in both scenarios after being exposed to ethanol. Over-expression of CLIC gene was achieved through amplification of existing CLIC DNA through PCR and injecting it as a viral substance into the organism. This injection was known as the AAV-CLIC. On the other hand, the injection to silence the CLIC gene was known as CLIC RNAi. Finally, a control group was made where nothing was injected into the organism. The animals were tested on 3 weeks after injections and were evaluated based on locomotor activity, anxiety, and loss of righting reflex(LORR) after ethanol exposure. The LORR was measured by the duration in which the animal cannot right itself back onto its paws. In flies, this was measured by climbing speed of the flies to the top of a jar after inducing ethanol. The results showed that CLIC played a huge factor in the fly sensitivity to alcohol.

 Further investigation revealed that CLIC4, a protein produced by expression of the CLIC gene, has multiple functions, such as cell trafficking between the cytoplasm and the nucleus as part of the TGF beta signaling pathway, a molecular pathway that leads to the expression of certain genes though transcription. The CLIC also interacts with and activates key proteins, Smad2/3, in the TGF beta signaling pathway.[[5]](#footnote-5) Smad 2/3 functions as transcription factors that regulated the expression of certain genes, that are unknown at this point, but may relate to the development of ethanol sensitivity in flies due to the heavy involvement of CLIC. This leads to the purpose of this investigation, which involves what genes are influenced by the TGF beta pathway, through which the CLIC protein is a major component of? The results of this experiment could lead to the opening of many branches of possible genetic experimentation to bring us closer to identifying the underlying molecular mechanisms and genetic factors, which, when mapped back to humans, can help develop therapeutic treatments for alcohol abuse.

**Experiment:**

 The aim of this experiment is to identify the genes involved in the TGF beta signaling pathway that have a high possibility with being involved in development of ethanol sensitivity in flies. Specifically, the experiment strives to assess, through a DNA microarray study, which genes are expressed under the conditions of over expression and silencing/denaturing of Smad 2/3 proteins, critical to the TGF beta pathway, in Drosophila with and without alcohol exposure. I would expect major differences in expression of certain category of genes between all four scenarios in the investigation: over-expression of Smad 2/3 proteins with alcohol exposure, over-expression of Smad 2/3 proteins without alcohol exposure, silenced Smad 2/3 proteins with alcohol exposure, and silenced Smad 2/3 proteins without alcohol exposure. The two scenarios where the Smad proteins are silenced will likely be most similar due to the fact that without Smad proteins, the TGF beta signaling pathway will not function, thus the genes which the transcription factors activate will likely not be expressed.

 The main method used in this experiment is known as DNA microarray, or simply DNA chip. Originally, scientists in the 20th century studied genes by mapping them, sequencing, and analyzing the proteins they encode, one gene at a time. In the scale of 20,000 genes in the human genome, this would take many years as shown by the human genome project. Thus, the DNA microarray was invented in order to study massive amounts of genes in a single experiment. Essentially, all the genes are shown as microscopic DNA spots on a solid DNA chip. If a certain gene is being expressed or is "on", it means that molecules of messenger RNA, mRNA, are being produced. Through the microarray, it is possible to pinpoint differences in gene expression between cells that are exposed to different conditions, such as alcohol exposure. The DNA microarray chips are made through sequencing the entire genome of the organism, in this case Drosophila, identifying where each gene is located on the genome, and perform PCR to create complement primer pairs to make copies for every gene and form them into single strand DNA. These are ordered into proper rows and columns on a glass microscope slide/chip.

 The DNA chip used for Drosophila genome may be made from scratch or can be provided by a local biotechnology lab. Four groups of 200 flies each are prepared for the experiment. Two of these four groups have Smad 2/3 proteins silenced while the other two have the proteins over-expressed. The extraction and isolation of mRNA from the different groups of fruit flies are performed after two of these groups of 200 flies, one with Smad 2/3 proteins and one without, are exposed to ethanol vapor for 30 minutes. Afterwards, all the flies are frozen and samples are taken from the heads of the flies. These samples are centrifuged in order to separate the RNA from the rest of the cells and fly material. Protein, DNA, and other fly material will be in the bottom layer of the centrifuged tube and the RNA will be the upper layer. Since only mRNA reflects gene expression, the ribosomal RNA, rRNA, and transfer RNA, tRNA, are washed off to isolate only the mRNA. A complimentary copy, known as cDNA, of this isolated mRNA is made through the use of reverse transcriptase, which also includes a colored fluorescent label for easy identification on the DNA chip (figure 1).  Figure 1

This cDNA also serves the purpose of being more stable than RNA, which reduces risk of error in the experiment. In this case, a green fluorescent label will indicate the cDNA of flies that have Smad 2/3 proteins silenced while the opposite will have a red fluorescent label. All 9 different combinations of the 4 groups are prepared on different multiple array chips with two groups being on a single chip at a time. Since the microarray chip contains the complementary strand of every single gene in the Drosophila genome, the cDNA from the sample will match up, or hybridize, with its complement. Any cDNA that is not hybridized will be washed off. When the microarray is scanned and merged, there are likely multiple genes that are expressed in both groups, indicated by a yellow color. These are unimportant as they are unaffected by the change of Smad 2/3 protein presence. The genes of interest are the ones that are expressed in one condition and not the other, indicated by green or red in the merged microarray chip. These genes are cataloged and compared to identify how much Smad 2/3 proteins, and in larger terms the TGF beta signaling pathway, affects the expression of genes with and without alcohol exposure. These genes will be further investigated in terms of their role in development of ethanol sensitivity, as suggested by experiment involving the CLIC protein, which is a key part of the TGF beta signaling pathway.

**Discussion:**

 If everything goes well, there will be at multiple different microarray chips properly scanned and cataloged in terms of which genes were expressed in each unique group. All the genes that are not affected by the change in Smad 2/3 proteins will likely be removed from consideration. Welcome results would include easily discernible patterns of gene expression among the different categories. They may or may not make sense for the time, but having categories of genes would make further experimentation much simpler to approach. An unwelcome result would likely be that none of the genes are affected by the change in Smad 2/3 protein presence, which is highly unlikely but would make further investigation difficult. On the other hand, it would provoke a unique discussion as to why such a phenomenon might occur.

 In terms of errors/pitfalls during experimentation, multiple trials of each group may be necessary as contamination or unsuccessful washing of RNA may result in false positives or other types of error in the microarray. The genes that are found through experimentation may be confirmed through quantitative PCR (qPCR) as demonstrated by Kong et. al (2010).

 There are multiple limitations involved in this investigation that could be avoided, but would make the experiment much more complex and difficult. For example, this investigation only involves the genes of Drosophila, while do possess conserved behavioral responses to ethanol exposure as humans and other popular model organisms, such as C. elegan worms and mice, may not be completely accurate in genetic response. Not all genes translate into a phenotype easily measured in behavioral responses. Therefore, the information given through this experiment has a chance of being applicable to other organisms, hopefully humans, but may simply be limited to Drosophila. This can be remedied by performing the same experiment on other organisms and using those DNA microarray studies as further comparison with the results of this experiment. Therefore, even if the information is limited, the results of this experiment will regardless provide more gateways into the investigations of genetic factors that influence alcohol disorders. Additionally, the microarray method is, regardless, a most useful tool in doing studies such as these where everything is largely unknown and this is the best path to move forward with.

**References:**

Bhandari, P., J. S. Hill, S. P. Farris, B. Costin, I. Martin, C. L. Chan, J. T. Alaimo, J. C. Bettinger, A. G. Davies, M. F. Miles and M. Grotewiel (2012). "Chloride intracellular channels modulate acute ethanol behaviors in Drosophila, Caenorhabditis elegans and mice." Genes Brain Behav 11(4): 387-97

Board, P. G., M. Coggan, S. Watson, P. W. Gage and A. F. Dulhunty (2004). "CLIC-2 modulates cardiac ryanodine receptor Ca2+ release channels." Int J Biochem Cell Biol 36(8): 1599-612.

Carvalho E, Solferini VN, Matioli SR. " Alcohol dehydrogenase activities and ethanol tolerance in Anastrepha (Diptera, Tephritidae) fruit-fly species and their hybrids." Genet Mol Biol. 2009 Jan;32(1):177-85.

Devineni, AV. Eddison, M. Heberlein, U. "The novel gene tank, a tumor suppressor homolog, regulates ehtanol sensitivity in Drosophila." J Neurosci 2013 May 8: 33(19): 8134-43

Dzitoyeva S, Dimitrijevic N, Manev H. " Gamma-aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in Drosophila: adult RNA interference and pharmacological evidence." Proc Natl Acad Sci U S A. 2003 Apr 29;100(9):5485-90.

Dulhunty, A. F., P. Pouliquin, M. Coggan, P. W. Gage and P. G. Board (2005). "A recently identified member of the glutathione transferase structural family modifies cardiac RyR2 substate activity, coupled gating and activation by Ca2+ and ATP." Biochem J 390(Pt 1): 333-43.

Ghezzi, A., Y. M. Al-Hasan, L. E. Larios, R. A. Bohm and N. S. Atkinson (2004). "slo K(+) channel gene regulation mediates rapid drug tolerance." Proc Natl Acad Sci U S A 101(49): 17276-81.

Guarnieri, D. J. and U. Heberlein (2003). "Drosophila melanogaster, a genetic model system for alcohol research." Int Rev Neurobiol 54: 199-228.

Kapfhamer D, King I, Zou ME, Lim JP, Heberlein U, Wolf FW. " JNK pathway activation is controlled by Tao/TAOK3 to modulate ethanol sensitivity." PLoS One. 2012;7(12):e50594

Kong EC, Allouche L, Chapot PA, Vranizan K, Moore MS, Heberlein U, Wolf FW. " Ethanol- regulated genes that contribute to ethanol sensitivity and rapid tolerance in Drosophila." Alcohol Clin Exp Res. 2010 Feb;34(2):302-16

Lasek AW, Giorgetti F, Berger KH, Tayor S, Heberlein U. " Lmo genes regulate behavioral responses to ethanol in Drosophila melanogaster and the mouse." Alcohol Clin Exp Res. 2011 Sep;35(9):1600-6.

McClure KD, Heberlein U. " A small group of neurosecretory cells expressing the transcriptional regulator apontic and the neuropeptide corazonin mediate ethanol sedation in Drosophila." J Neurosci. 2013 Feb 27;33(9):4044-54.

Morozova TV, Ayroles JF, Jordan KW, Duncan LH, Carbone MA, Lyman RF, Stone EA, Govindaraju. " Alcohol sensitivity in Drosophila: translational potential of systems genetics." Genetics. 2009 Oct;183(2):733-45, 1SI-12SI.

Ronnov-Jessen, L., R. Villadsen, J. C. Edwards and O. W. Petersen (2002). "Differential expression of a chloride intracellular channel gene, CLIC4, in transforming growth factor-beta1-mediated conversion of fibroblasts to myofibroblasts." Am J Pathol 161(2): 471-80.

Shanks, R. A., M. C. Larocca, M. Berryman, J. C. Edwards, T. Urushidani, J. Navarre and J. R. Goldenring (2002). "AKAP350 at the Golgi apparatus. II. Association of AKAP350 with a novel chloride intracellular channel (CLIC) family member." J Biol Chem 277(43): 40973-80.

Shukla, A., M. Malik, C. Cataisson, Y. Ho, T. Friesen, K. S. Suh and S. H. Yuspa (2009). "TGF-beta signalling is regulated by Schnurri-2-dependent nuclear translocation of CLIC4 and consequent stabilization of phospho-Smad2 and 3." Nat Cell Biol 11(6): 777-84.Berryman, M. A. and J. R. Goldenring (2003). "CLIC4 is enriched at cell-cell junctions and colocalizes with AKAP350 at the centrosome and midbody of cultured mammalian cells." Cell Motil Cytoskeleton 56(3): 159-72.

Urizar, N. L., Z. Yang, H. J. Edenberg and R. L. Davis (2007). "Drosophila homer is required in a small set of neurons including the ellipsoid body for normal ethanol sensitivity and tolerance." J Neurosci 27(17): 4541-51.

1. Morozova et al. (2006) [↑](#footnote-ref-1)
2. National Institute on Alcohol Abuse and Alcoholism of the National Institutes of Health [↑](#footnote-ref-2)
3. Kong et al. (2010) [↑](#footnote-ref-3)
4. Bhandari, P. et al (2012) [↑](#footnote-ref-4)
5. Shukla et al (2009) [↑](#footnote-ref-5)