5-HT1B-receptor regulation by p-GSK3B due to acute ethanol exposure on mice

I. Introduction

Alcoholism is a neuropsychological condition that has plagued society for many generations. Alcohol is one of the most notorious depressants in the market and has very high rates of addiction as evidenced by Fig 1. It remains largely unknown how repeated alcohol exposures re-model the brain to eventually create an alcohol dependent state within an organism. Protein kinases play a vital regulatory role in biological pathways by turning target proteins on/off via the addition of a phosphate group (phosphorylation). Glycogen synthase kinase 3 (*GSK3*) is a serine/threonine kinase that is highly expressed in various neuronal cells within the brain and tightly regulated through phosphorylation (Zhou et al. 2012). *GSK3B* is one isoform (compound with similar function but differing peptide sequence) of this kinase and the phosphorylated form is known as *phospho-GSK3β (Zhou et al. 2012)*.

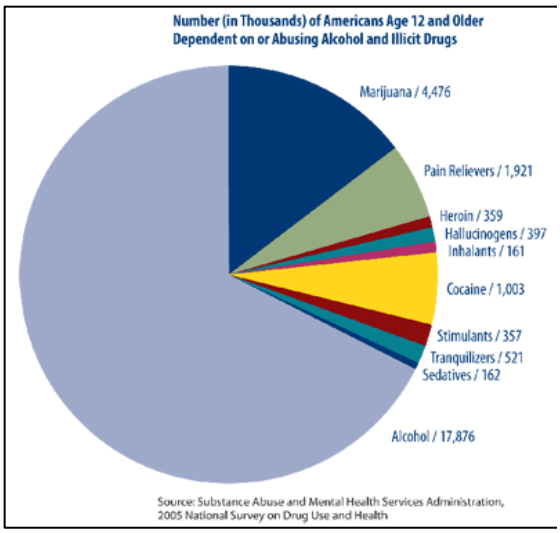

Fig 1: Number (in thousands) of Americans Age>12 that depend on alcohol or drugs; Alcohol= 17,876 while the next highest is Marijuana at 4,476 (Ref 6)

Neznanova et al (2009) investigated the mediation of pathways concerning alcohol addiction and cognitive behavior by *p-GSK3B*. One of their key results was that ethanol phosphorylates *GS3KB* in the pre-frontal cortex and the phosphorylated form of this receptor causes an increase in acute ethanol consumption in mice (Neznanova et al., 2009). Understanding how this receptor is modulated can prove to be useful in determining a prognosis for alcohol abuse. However since GSK3B is found ubiquitously throughout the brain, it is hard to pinpoint one particular pathway of regulation. Findings over the years, however, may have suggested an answer to the previous question, which lies in a neurotransmitter known as serotonin.

Serotonin is involved in the regulation of mood and behavior but also has been linked to alcoholism as well. Serotonin has actually been found to be present in large concentrations within the synapse during acute ethanol exposure to mice (Lovinger, 1997). Within serotonin neurons, serotonin release is facilitated by a group of receptors known as *5-hydroxytryptamine* receptors (*5-HT* serotonin receptors). Zhou et al (2012) showed how *GSK3B* plays an integral role in regulating Serotonin output by interaction with a subclass of receptors known as *5-HT1B*. 5-HT1B is an inhibitory receptor responsible for decrease in serotonin release into the synapse. The investigators isolated two groups of mice: one being the wild type (unaltered control mice) while the other had serotonin-neuron specific *GSK3B* gene knockouts. The investigators induced *5-HT1B* functioning by introducing an agonist into both groups (molecule that causes activation of a protein receptor) and

observed subsequent serotonin neuron firing (by looking at relative serotonin concentration in the synapse). Their key result showed that the *GSK3B* knockout mice were less sensitive to the agonist meaning that they did not have as much serotonin neuron firing as apposed to the wild type mice who had large levels of serotonin present within the synapse. The knockout results helped to show that *GSK3B* plays a major regulatory role in *5-HT1B* receptor release of serotonin (Zhou et al. 2012).

The means by which this occurs is speculated in a different study, this time done by Polter and Li in 2011. They also showed the regulatory effects *GSK3B* has on *5-HT1B* but furthers this conclusion by proposing a model (Fig 2) that suggests that its actually direct binding of *GSK3B* to *5-HT1B* in serotonin neurons that induces regulatory effect of the neurotransmitter. The model suggests that as serotonin concentration increases within the synapse a signal is sent to phosphorylate GSK3B, which binds with 5-HT1B to reduce levels of Serotonin as a means of regulation. This phosphorylation occurs on the Serine site of GSk3B, which is known to cause inhibitory effects such as the decrease in serotonin levels, mentioned previously.

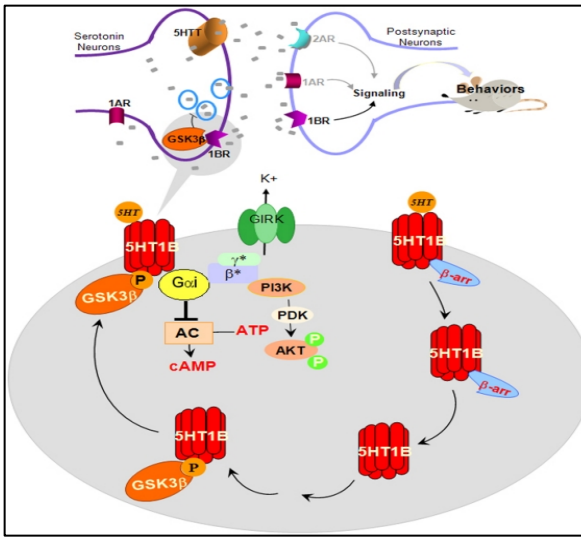

Fig 2: Proposed GSK3B interaction with 5HT1B in Serotonergic (Serotonin) neurons. P-GSK3B binds to 5-HT1B to illicit serotonin release in synapse. (Polter et Li. 2011)

GSK3B also has another region in which phosphorylation can occur known as the Tyrosine 216 site. While ethanol does phosphorylate GSK3B it has not been investigated if it could target Tyrosine Phosphorylation as well. This begs the question that does ethanol acts to phosphorylate the tyrosine unit of GSK3B causing an excitatory response and prevents binding with 5-HT1B, which would further develop the understanding of GSK3B regulation of 5-HT1B.

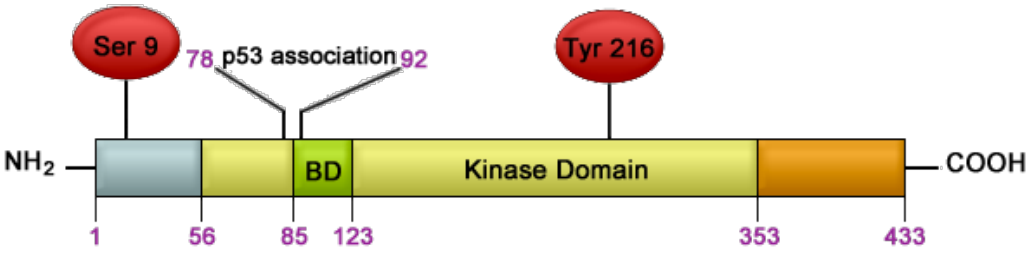

Fig 3:DNA structure of GSK3B; shows Ser9 and Tyr216 sites (Bax et al, 2001)

II. Experiment

IIA: General Overview

For this particular experiment three groups of mice will be tested: one being a control that contains a fully functional *GSK3B* gene and another that will have a mutation in the serine 9 site of *GSK3B* (the serine will be replaced with alanine). The

serine-9 site is where phosphorylation of the receptor occurs so by preforming this mutation the second group will have an active *GSK3B* but will not be able to become phosphorylated. The second group will contain a mutation in the Tyrosine 216 site in the same manner. These mutations will be done by a technique known as site-specific mutagenesis. Data collection will be done using a technique known as micro dialysis probing as the mice in mutated groups are given acute amounts of ethanol (2ug/kg). The data will be quantified using a HPLC machine (discussed below in IIC).

IIB: Site Specific Mutagenesis

Site-specific mutagenesis is a technique that is used to mutate a desired site on the gene of interest (Liu et. Naismith, 2008). In practical lab settings usually an order can be placed to get an embryo of a mouse that contains the mutated protein but there are protocols in place to perform site-specific mutagenesis by hand.

Liu et Naismith (2008) developed a one step protocol for this scenario. Essentially, the gene of interest (in this case *GSK3B gene*) will be placed inside a plasmid. A plasmid is a small circular strand of DNA that self replicates. It will be placed along with a primer containing the mutated complementary strand (alanine for serine for the *GSK3B* gene). The primer will integrate itself to the template genome by annealing (combining) to the plasmid DNA. The plasmid can then be cloned using PCR. PCR or polymerase chain reaction is a cloning process that can take a particular DNA sequence or gene of interest and create many copies of that DNA sequence. The plasmid containing the mutated gene can then by inserted into a mouse embryo that has had its original *GSK3B* gene removed/knocked out where it can further integrate with the genome and produce a *GSK3B* protein that can no longer undergo phosphorylation as the embryo grows and develops into an adult mouse (Liu et Naismith, 2008).

IIC: Conventional Intra-cerebral Micro dialysis

Micro-dialysis is the backbone for this experiment. This technique measures the concentration of a free unbound chemical substance in the exterior of any tissue of an organism. For the purpose of the experiment it will be used to measure serotonin concentration within the synapse of serotonergic neurons of the PFC in both wild type and mutated mice. The organism can be anesthetized or in a free movement state while preforming this technique. To reduce the potential introduction of any unnecessary contaminants micro-dialysis will be done while the mouse is awake in the physical experiment. Gardier (2013) explained a very thorough

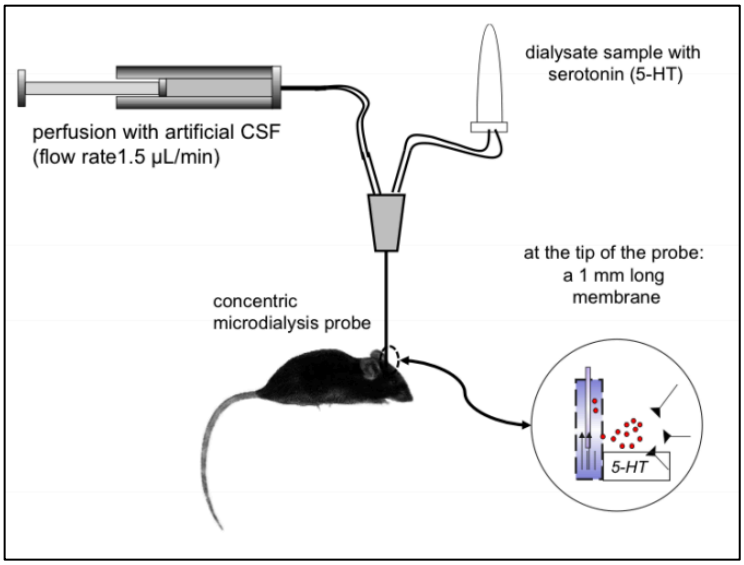

Fig 2: General setup of Conventional intra-cerebral Micro dialysis. (Gardier, 2013)

protocol for micro dialysis in his research article. To begin, a dialysis probe is inserted into the desired brain region. The end of the probe contains a porous membrane, which utilizes the law of passive diffusion: movement of molecules from high concentration to an area of low concentration (Gardier, 2013). In this case molecules such as serotonin are found in high concentration within the synapse and move across the porous membrane into the dialysate tube that is attached to the probe (Fig 2). The contents of the tube can be quantified using high-performance liquid chromatography. HPLC is used to separate and quantify compounds in a mixture. For the purpose of this experiment the means by which this works is irrelevant since a machine itself does quantification and all there is left to do is insert dialysate tube contents into that machine (Gardier, 2013).

III. Discussion

The purpose of this experiment was to investigate the possible pathophysiology of *GSK3B* and ethanol consumption in mice. It is hypothesized that *p-GSK3B* (formed through ethanol phosphorylation of *GSK3B* at the serine 9 site would be able to bind to *5-HT1B* to inhibit serotonin release into the synapse of PFC serotonin neurons under normal conditions. Ethanol however also phosphorylates GSK3B but it may do so at the Tyr216 site, which would cause a different conformational change preventing GSk3B binding with 5HT1B and causing serotonin to build up with in the synapse of neurons. This build up contributes to the euphoria effect and may cause the increased consumption of alcohol that is displayed in mice.

The control group gives us a baseline for comparison. The Ser9 mutated mice and control would show buildup of serotonin in the synapse as a result of *p-GSK3B* binding to *5-HT1B* in the wild type mice. However this would have to coincide with high levels of serotonin within the synapse of the Tyr216 mutated mice as well in order for the result to support the stated hypothesis. This would be an enormous breakthrough considering we have just identified a means by which GSK3B regulates neurotransmission in the presence of alcohol.

However, due to variability it is possible to see multiple negative results. This would include no significant difference between the each of the groups.

Alternative negative findings, such as low levels of serotonin within the synapse of Tyr216 mutant samples, would be compelling counter evidence against this hypothesis as well. This would suggest that ethanol phosphorylation does not impact the Tyr site at all and effects GSK3B some other way perhaps through the regulation of a different receptor protein. Another interesting finding, though negative, would be if there were very minimal serotonin concentration within the synapse of the wild type mice. This would completely dispel my hypothesis but it can serve as a means of further study. If this result was to be observed it could mean that the presence of ethanol is not only acting on *GSK3B* but also preventing *5-HT1B* activation by acting on a separate molecule. Perhaps looking at if *5-HT* receptors maybe modulated by other protein receptors such as *AKT* (another protein kinase in the brain) rather than solely focusing on *GSK3B*. While this may seem to be a tangent

its worth as a note for further study. In fact this may even be an uncontrollable factor, which can limit the effectiveness of my experiment as well.

Like any experiment there are several possible pitfalls. Perhaps the most glaring source of error in my experiment would arise during site-specific mutagenesis. This technique is often prone to errors including mutation of a different gene or inability to inhibit *GSK3B* itself if not done properly. The obvious answer is to be as careful as possible while handling the plasmid and embryo but the actual process itself such as proper annealing of the primer to the template strand is beyond my control. If I were to be extremely picky the best possible alternative would be to scrap the hands on protocol and opt for a set of pre-prepared embryos.

Although there are concerning weaknesses (that I have already addressed) in this proposal the idea itself still holds tremendous value. *GSK3B* is a multifaceted kinase receptor that facilitates many regulatory pathways within the brain. As such, understanding how *GSK3B* works is not only important for alcoholism but other neurological and cognitive pathways as well. This study gets us one step closer to understanding the mechanics behind this mysterious protein.

IV. References

1) Polter, Abigail M. et al. "Functional Significance of Glycogen Synthase Kinase-3 Regulation by Serotonin." Cellular signalling 24.1 (2012): 265–271. PMC. Web. 10 Nov.
2) Polter, Abigail M., and Xiaohua Li. "Glycogen Synthase Kinase-3 Is an Intermediate Modulator of Serotonin Neurotransmission." *Frontiers in Molecular Neuroscience* 4 (2011): 31. *PMC*. Web. 14 Nov. 2017.
3) Zhou W, Chen L, Paul J, Yang S, Li F, et al. (2012) The Effects of Glycogen Synthase Kinase-3beta in Serotonin Neurons. PLoS ONE 7(8): e43262. doi:10.1371/journal.pone.0043262
4) Liu, Huanting, and James H Naismith. "An Efficient One-Step Site-Directed Deletion, Insertion, Single and Multiple-Site Plasmid Mutagenesis Protocol." *BMC Biotechnology*, vol. 8, no. 91, 4 Dec. 2008, doi:10.1186/1472-6750-8-91.
5) Neznanova, O. et al. "Acute Ethanol Challenge Inhibits Glycogen Synthase Kinase-3 Beta in the Rat Prefrontal Cortex." *The international journal of neuropsychopharmacology / official scientific journal of the Collegium Internationale Neuropsychopharmacologicum (CINP)* 12.2 (2009): 275–280. *PMC*. Web. 26 Nov. 2017.
6) Prescription Drug Abuse: A Fast-Growing Problem | NIH MedlinePlus the Magazine." *U.S National Library of Medicine*. U.S. National Library of Medicine, n.d. Web. 05 June 2016. <https://www.nlm.nih.gov/medlineplus/magazine/issues/fall11/articles/fall11pg21.html>. (Citation for Figure 1)

7) Lovinger, D.M. (1997) Serotonin's role in alcohol's effects on the brain. *Alcohol Health Res World* **21**, 114–120.
8) Gardier, Alain. (2013). Antidepressant activity: Contribution of brain microdialysis in knock-out mice to the understanding of BDNF/5-HT transporter/5-HT autoreceptor interactions. *Frontiers in pharmacology*. 4. 98. 10.3389/fphar.2013.00098.
9) Bax B et al. The structure of phosphorylated GSK-3 beta complexed with a peptide, FRATtide, that inhibits beta-catenin phosphorylation. Structure 2001, 9, 1143–115210.1016/S0969-2126(01)00679-7.