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 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)*.

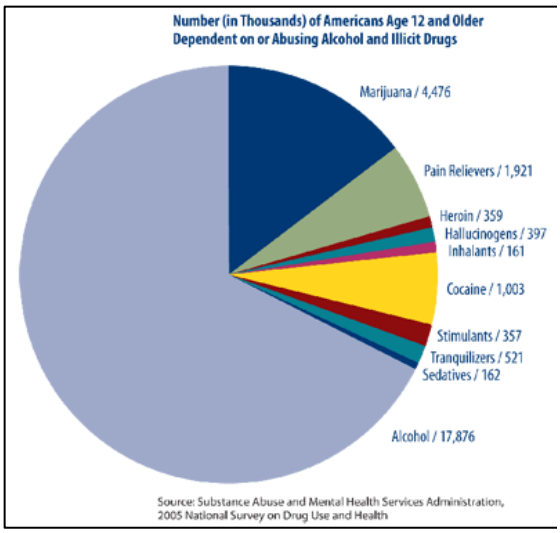

Fig 1: Number (in thousands) of Americans Age>12 that depend on alcohol or drugs; Alcohol= 17,876 while the next highest is Marijuana at 4,476 (Ref 6)

Neznanova et al (2009) explored 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 increases 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.

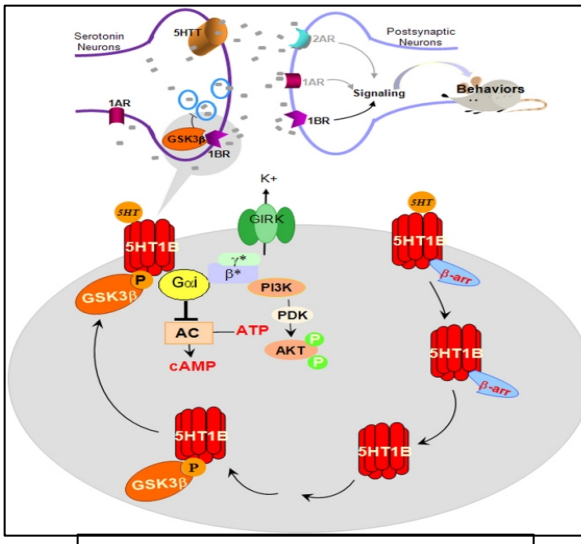

Fig 2: Proposed GSK3B interaction with 5HT1B in Serotonergic (Serotonin) neurons. P-GSK3B binds to 5-HT1B to elicit serotonin release in synapse. (Polter et Li. 2011)

GSK3B also has another region in which phosphorylation can occur known as the Tyrosine 216 site. While ethanol does induce phosphorylation of GSK3B it has not been investigated whether it targets the Tyrosine 216 site. Determining whether ethanol acts to phosphorylate this residue may further our understanding of how GSK3b regulates 5HT1B.

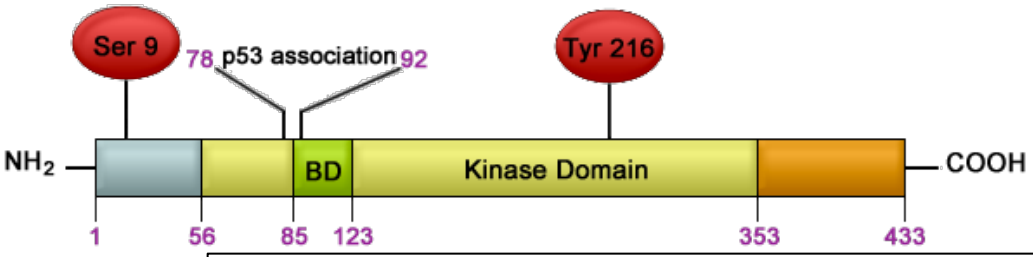

Fig 3:DNA structure of GSK3B; shows Ser9 and Tyr216 sites (Ref 14)

II. Experiment

IIA: General Overview

For this particular experiment four groups of mice will be tested. The first group contains a fully functional unaltered *GSK3B* gene. The second and third groups will have a knockout for the serine9 (replaced with alanine) and tyrosine216 (replaced with phenylalanine) respectively. The mutations will only disable the phosphorylation of those residues but will still leave the GSK3b fully functional. The first three groups will be treated with low doses of alcohol. The final group will be used to compare basal metabolite levels and contain mice with no changes to their

genomes. Mutations will be done by a technique known as site-specific mutagenesis and inserted into mouse embryo with knockouts for GSK3B already in place. Data collection will be done using a technique known as micro dialysis probing as the mice in the alcohol groups are given acute amounts of alcohol (2ug/kg). The data will be quantified using a HPLC machine (discussed below in IIC).

IIB: Site Specific Mutagenesis

Site directed mutagenesis is a process in recombinant DNA technology that allows the user to preform a mutation on one specific amino acid in a given polypeptide sequence. There are multiple mutagenesis protocols circling the scientific community but the most common one, which I wish to exploit, is known as the quick-change protocol for site directed mutagenesis. To preform this technique a plasmid containing the GSK3B gene along with two primers (forward and reverse) with the desired point mutation must be obtained.

Creation of Plasmid:

Before the protocol can be initiated the GSK3B gene must be retrieved and inserted into a vector such as a plasmid. While PCR can be used in order to generate the target plasmid, for convenience purposes pSP72 GSK3B plasmids will be purchased from addgene.org. This plasmid contains the gene required for the expression of GSK3beta and will serve as the template for mutagenesis (Fig 4).

Fig 4: pSP72 Plasmid containing GSK3B (Ref 11)

Quick Change protocol w/PCR from Ref 12:

Once the pSP72 plasmid has been retrieved, the quick-change protocol will be initiated. Thomas Kunkel made the parent protocol in 1985. Researchers often use derivations of this protocol but the actual manual for the kit was found online in which is what this experiment will go off of. This protocol will be run once for each of my experimental groups: Tyr216 knockout and Ser9 knockout mice. This experiment relies on PCR cloning to generate enough of the desired mutated genes into a plasmid form.

Actual Genome at ser9 site: 5' CCC AGA ACC ACC TCC TTT GCG GAG AGC 3'
Mutation T for G: Alanine (TCC to GCC)
Forward Primer w/mutation: 3'GGG TCT TGG TGG CGG AAA CCC CTC TCG 5'
Reverse Primer w/Mutation: 5' GCT CTC AGA AAA GGC GGT GGT TCT GGG 3'

Actual Genome at Tyr216 site: 5' CCC AAT GTT TCG TAT ATC TGT TCT CGG 3'
Mutation T for G: Phenylalanine (TAT to TAG)
Forward Primer w/Mutation: 3' GGG TTA CAA AGC ATC TAG ACA AGA GCC 5'
Reverse Primer w/Mutation: 5' CCG AGA ACA GAT CTA CGA AAC ATT GGG 3'

Fig 5. Primer sequence construction

5 µl of 10× reaction buffer
X µl (10 ng) of dsDNA template
X µl (125 ng) of oligonucleotide primer #1
X µl (125 ng) of oligonucleotide primer #2
1 µl of dNTP mix
3 µl of QuikSolution
ddH_2O to a final volume of 50 µl
n add
1 µl of *PfuTurbo* DNA polymerase (2.5 U/µl)

Table 1: Solution contents for Quick Change (Ref 12)

The protocol calls for two primers, one forward and one reverse, to be placed in conjunction with the pSP72 plasmid. As the figure depicts the forward primer is synthesized by complementary base pairing from the 5' to 3' end while the reverse primer is synthesized via complementary base pairing from the 3' to 5' end. The synthesized primers will have the point mutation that changes the serine 9 residue to alanine and tyrosine 216 to phenylalanine. Each primer will contain the mutated nucleotide sequence at the center, as requested by the protocol, and will span a length of 12 nucleotides on either side. Figure 5 shows the primers to be created. Genome of GSk3B was found on NCBI genbank page (Ref 11).

Once 125ng of each the primers along with a contents illustrated in Table 1 are placed, PfuTurbo DNA polymerase and all four types of deoxyribonucleotide triphosphate's (four bases of DNA) will be added to the solution. The reaction itself will be carried out in in a thermal cycle allowing for the slow denaturation of the template plasmid that is necessary for each of the primers to anneal with the plasmid (Table 2) (Ref 12).

Pfu DNA polymerase is the DNA replicating enzyme associated with the hyperthermophillic archea *Pyrococcus furiosus*, and is used extensively for PCR amplification due its unusually large thermo stability. Pfu turbo is a mix of normal cloned Pfu and polymerase enhancing factors. It is used to enhance both the yield of the PCR and the ability to clone longer and more complex vectors and DNA sequences. As the primers elongate nicks will be formed in the DNA strand which will be ligated together later.

Cycling Parameters for the QuikChange XL Method

Segment	Cycles	Temperature	Time
1	1	95°C	1 minute
2	18	95°C	50 seconds
		60°C	50 seconds
		68°C	1 minute/kb of plasmid length
3	1	68°C	7 minutes

Table 2: Thermal Cycling (Ref 12)

After the two mutant daughter strands have been generated the parental template is digested by the addition of an enzyme known as DPN1. DPN1 is a nuclease that targets and breaks down methylated DNA sequences. Since only the parental strands have been methylated this allows the user to isolate the synthesized mutated strands. The isolated plasmids will be placed in a solution containing ultra-competent cells to allow for DNA transformation and subsequent ligation to reseal the nicks in the strands. These cells are altered E. Coli whose cell walls are more permeable to DNA transformation. However, to continue with the experiment the competent cell is not needed but the plasmid inside is. Alkaline lysis will be preformed using a kit to isolate the plasmid of interest for insertion into an embryo of mice allowing for the synthesis of transgenic mice (Mulhardt et Beese, 2007). The lysis involves the

addition of select solutions from the kit to lyse the cell and separate the plasmid DNA from the chromosomal DNA for future use.

At the conclusion of the second round of quick change two different plasmid types will have been synthesized one for tyr216 knockout and another for ser9 knockout.

Creation of Transgenic Mice:

Transgenic mice are mice that have been altered at the germ line by introducing a cloned gene into a fertilized ova or embryonic stem cells. The previously generated mutant DNA will be inserted through a microinjection, done via an inverted microscope equipped with Hoffman micromanipulators, into newly fertilized ova that already have a gsk3b knockout that was received from my lab (Griffiths et al., 2000). The main advantage to using this technique as opposed to others is that it would allow the use of DNA from a variety of species and not just the one being grown in an embryo. This is what allows us to reference the human gsk3b for primer synthesis earlier in fig 5 (Buy, 1995).

The injection requires that the DNA be isolated from its plasmid structure or else it would risk contamination of the young zygote (Cho et al., 2017). Since alkaline lysis already isolated the plasmids the DNA is ready to be injected.
The injection itself will take place during very early stages of fertilization when the two pro-nuclei exist. The injected DNA can then easily integrate itself within the genome of the growing embryo with minimal complications. The embryo will then by injected into a surrogate and grown into fully-grown adult mice when testing can commence.

<u>IIC: Conventional Intra-cerebral Micro dialysis</u>

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 protocol for micro dialysis in his research article. To begin, a dialysis probe is inserted into the

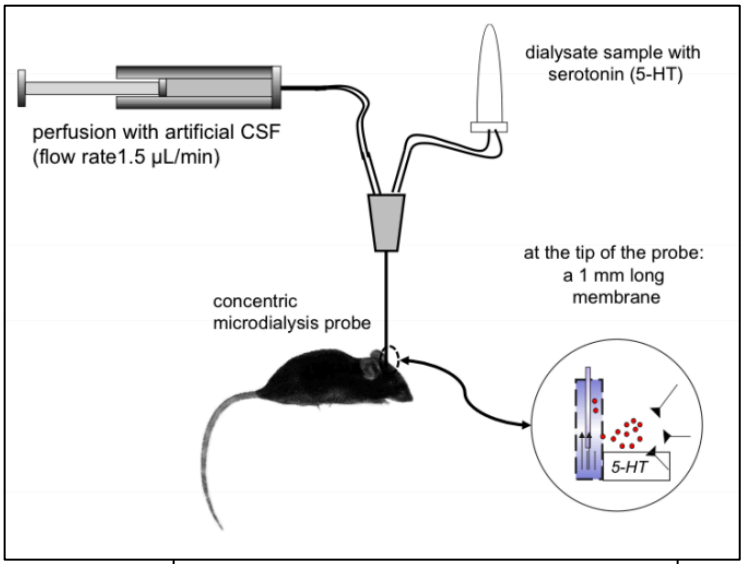

Fig 6: General setup of Conventional intra-cerebral Micro dialysis. (Gardier, 2013)

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 6). 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 affects 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

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