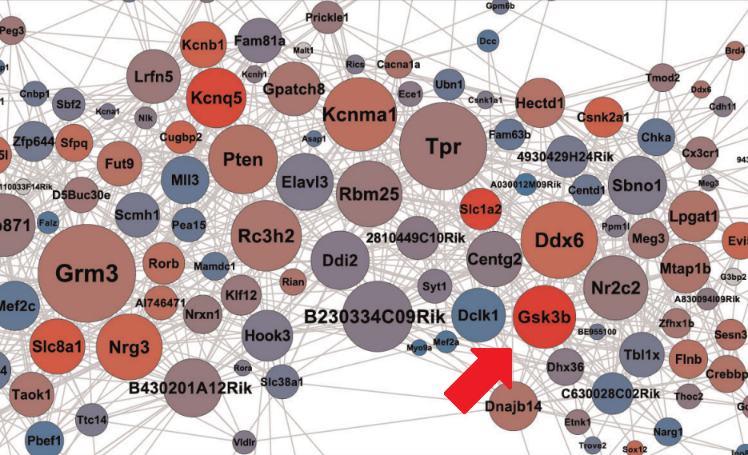
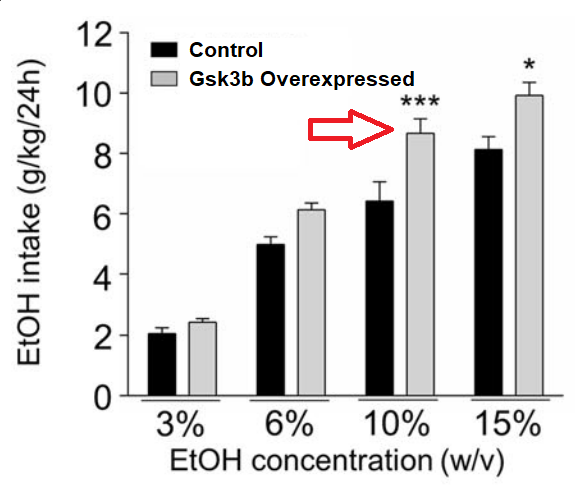
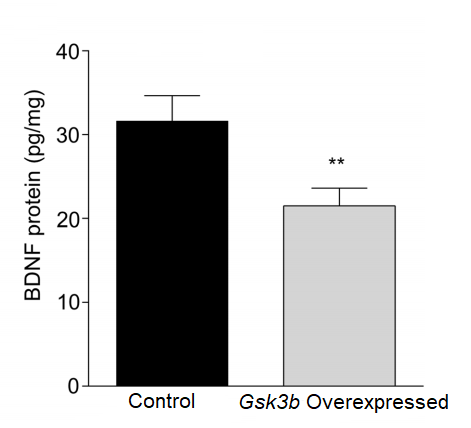
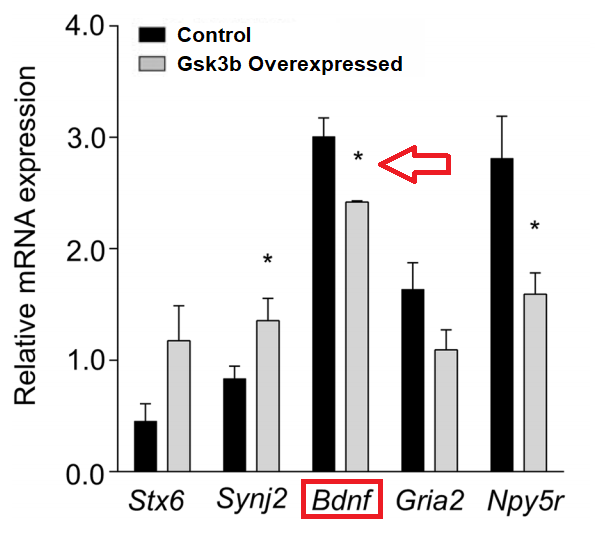
**Determining the Neuron Type Specificity of a   
Potential Mechanism for Alcohol Addiction**

**I. Introduction**

Alcohol use disorders (AUD), taken together, are the fourth leading cause of preventable death in the United States1. This disease is characterized by impulsive craving and consumption of ethanol, despite negative consequences. It is largely heritable, and genetic factors have been shown to account for 40–60% of the risk of developing an AUD2. By understanding the genetic mechanisms and neurobiology that govern alcohol addiction, new therapeutic targets could be identified to treat alcoholism and AUD.

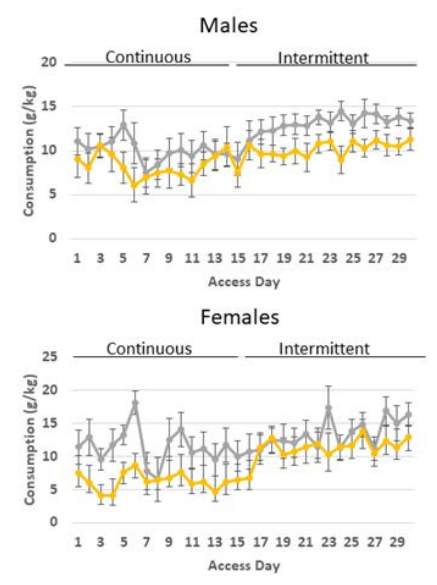
In order to determine the major genes responsible for the heritability of AUD, Wolen et al (2018)3 developed an ethanol responsive gene network by comparing the transcriptional response of genetically diverse mice before and after treatment with ethanol. This analysis identified several key genes that potentially mediate the genetic response to ethanol in the medial prefrontal cortex (mPFC), a region of the brain associated with drug-seeking behavior. One of these genes was *Gsk3b* (Figure 1), encoding Glycogen Synthase Kinase 3 Beta, a multifunctional serine/threonine kinase which is known to be involved in ethanol-induced neurotoxicity4.

**Figure 1: Gene network for acute ethanol.** *Gsk3b* is shown as a hub gene at the red arrow. Red intensity indicate a higher transcriptional response to ethanol and a larger size indicates amount of connectivity to other genes. Figure adapted from Figure 6 of Reference 3.

A follow-up study by van der Vaart et al (2018)5 explored the potential effects of GSK3B in the mPFC. One experiment overexpressed *Gsk3b* in the neurons of mouse mPFC, and measured mean ethanol consumption of the mice at varying concentrations of ethanol. The increase in neuronal *Gsk3b* expression positively correlated with the consumption of ethanol (Figure 2). A second experiment measured the expression levels of synaptic signaling genes using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). One gene in particular, *Bdnf*, coding for Brain-derived Neurotrophic Factor, was found to have decreased expression levels after neuronal overexpression of *Gsk3b* (Figure 3). Protein levels of BDNF were measured by a quantitative Western Blot and that was also shown to decrease (Figure 3). Interestingly, this is a protein that has been shown to be increased in the striatum after the consumption of ethanol, correlating with a decrease in further ethanol intake as a potential negative feedback loop6. In other words, the increase in ethanol consumption after overexpression of *Gsk3b* could be a consequence of a decrease in BDNF protein.

**Figure 2: Ethanol consumption after overexpression of *Gsk3b*.** Black bars show control mice while grey bars show mice with *Gsk3b* neuronal overexpression. Figure adapted from Figure 2A of Reference 5.

**Figure 3: Changes in other genes as a response to *Gsk3b* neuronal overexpression.** Black bars show control mice while grey bars show mice with *Gsk3b* neuronal overexpression. (Left) mRNA expression levels of various synaptic signaling genes. *Bdnf* highlighted in red. (Right) protein levels of BDNF. Figures adapted from Figure 3 of Reference 5.

A separate study by van der Vaart et al (2018)7 attempted to explore the neuronal specificity of the regulatory effects of *Gsk3b* on the response to ethanol. One neuron type in particular, the glutamatergic pyramidal neurons in the forebrain, showed significant changes in the consumption of alcohol upon deletion of the *Gsk3b* gene. These neurons, characterized by the expression of *Camk2a*, Calmodulin Dependent Kinase II Alpha, form the principle output of the mPFC8. Deleting *Gsk3b* in these neurons led to a significant decrease in ethanol consumption (Figure 4).

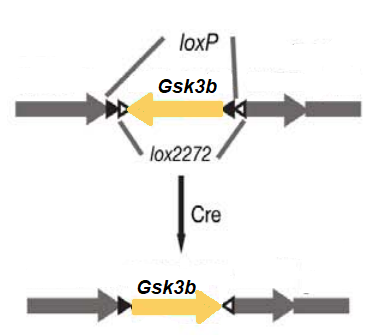
To explore the potential mechanism behind *Gsk3b* and *Bdnf* interactions, it is important to understand whether or not this process is dependent on a certain cell type. Since Gsk3b has already been shown to have an effect in the Camk2a+ neurons in the forebrain, this cell type is a likely candidate to look into to understand the pathway of *Gsk3b* and *Bdnf*. The purpose of this experiment is to determine whether or not this potential mechanism of the regulation of alcohol addiction is specific to the glutamatergic pyramidal neurons in the mPFC.

**Figure 4: Overexpression of *Gsk3b* in Camk2a+ neurons decreases ethanol consumption in mice.** Grey lines show control mice while yellow bars show mice with *Gsk3b* deleted. Figure obtained from Figure 5.15 of Reference 8.

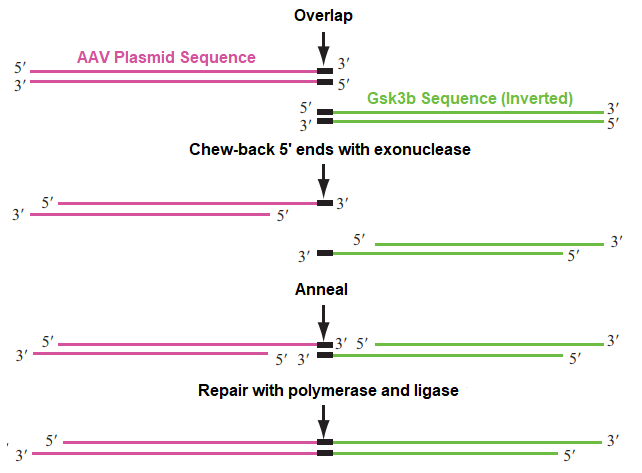
**II. Experiment**

This experiment will aim to measure the expression levels of *Bdnf* in the mPFC of genetically identical (C57B6J) mice upon overexpression of *Gsk3b* in only *Camk2a* expressing neurons. Knowing that overexpression of *Gsk3b* in all neurons correlates with a decrease in *Bdnf* expression, this experiment will allow for the determination of whether this effect is fully, partially, or not dependent specifically on glutamatergic pyramidal neurons in the forebrain. *Bdnf* expression levels in these mice will be compared against expression in control wild-type mice and against mice with *Gsk3b* overexpressed in all neurons. If the downregulation of *Bdnf* is fully dependent on *Camk2a* expressing neurons, the experimental levels of *Bdnf* expression should match exactly what was seen in the general overexpression experiment by van der Vaart et al. (2018)5.

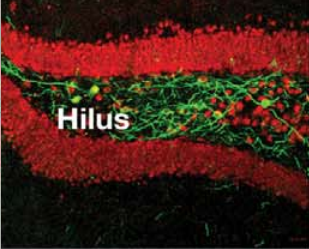
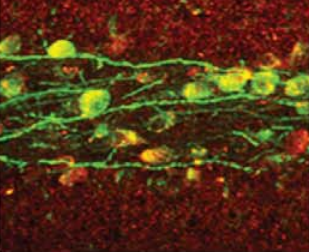
II.A. Cell-type specific overexpression of *Gsk3b*

To achieve overexpression of *Gsk3b* in only Camk2a+ neurons, a viral vector (Adeno-Associated Virus, AAV) containing an inverted open reading frame of *Gsk3b*, will be injected into the mPFC of mice brains. This means that DNA encoding the inverted sequence of *Gsk3b* will be added to all the cells, where it is unable to be expressed due to the lack of a start codon in the right position. However, this inverted open-reading frame will lie in between two pairs of short DNA segments, known as loxP and lox2272 sites (Figure 5). When in the presence of the enzyme Cre-recombinase, the *Gsk3b* open-reading frame will undergo inversion, followed by excision of one copy of each lox site to prevent re-inversion9. The result is a *Gsk3b* open-reading frame that is only functional in the presence of Cre-recombinase. This type of system is known as the double-floxed inverse orientation, or DiO. *Gsk3b* in this vector will be driven by an *hSyn* (human synapsin I) promoter, which has been shown to be preferentially active in all cortical neurons10.

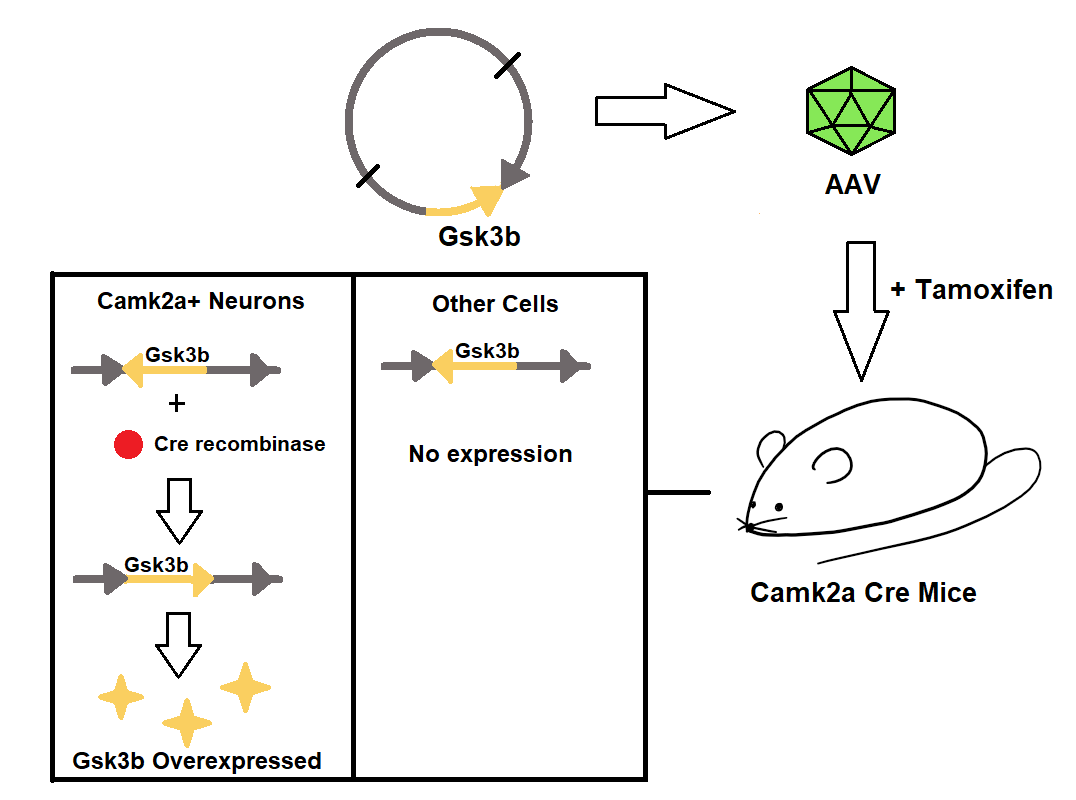
**Figure 5: Inversion of Gsk3b in Vector.** In the presence of Cre, the sequence between loxP and lox2272 sites is inverted back into a functional orientation, available for gene expression. Figure adapted from Figure 1A of Reference 11.

This construct will be synthesized using a pre-existing DiO plasmid, with the open reading frame of *Gsk3b* replacing the pre-existing one through the use of Gibson Assembly. This molecular biology technique can take virtually any two DNA sequences and attach them into a single molecule of DNA11. It involves primers that only partially anneal to the region of interest, creating products with single stranded overhangs. By generating PCR products of each segment with complementary overhangs, both PCR products will fuse into a single product (Figure 6). The resulting plasmid will contain the *Gsk3b* open reading frame in reverse as long as the single stranded overhangs matched the correct ends of the plasmid.

**Figure 6: Gibson Assembly Method.** By adding on an overlapping region in two pieces of DNA, they can be attached together through this method. Figure adapted from Figure 15.4 of Reference 10.

The DiO system has been employed by Andrews-Zwilling et al (2012)12 to express their gene of interest, eNpHR3.0 in hilar GABAergic interneurons of the dentae gyrus. They created a DiO plasmid with an inverted eNpHR3.0 open reading frame to make the expression Cre-dependent. They then expressed *Cre* only in hilar GABAergic interneurons through the use of an enhancer sequence specific to this cell type (Dlx-I12b). With this construct, they were able to achieve expression of their vector in only the hilar GABAergic interneurons (Figure 7). This is evidence that cell-type specific gene expression can be achieved with a DiO system as long as *Cre* is expressed in a cell-type specific manner.

**Figure 7: Regiospecific Expression using DiO Plasmid.** Green represents eNpHR3.0. (Left) Red represents NeuN marker staining for all neurons. (Right) Red represents a marker for GABA. Figures obtained from Figure 1D and 1H of Reference 11.

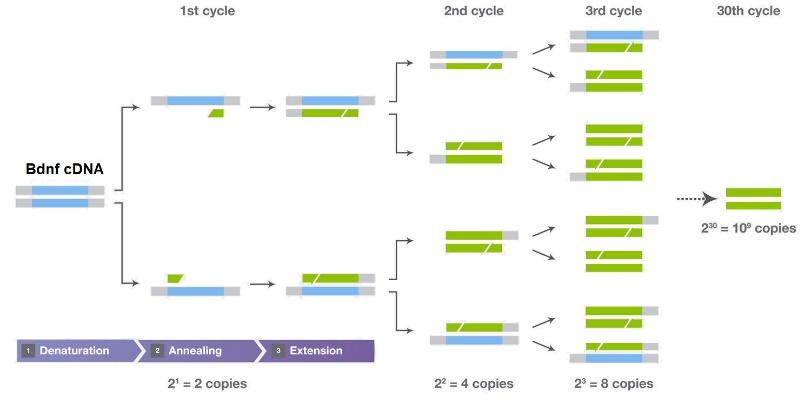
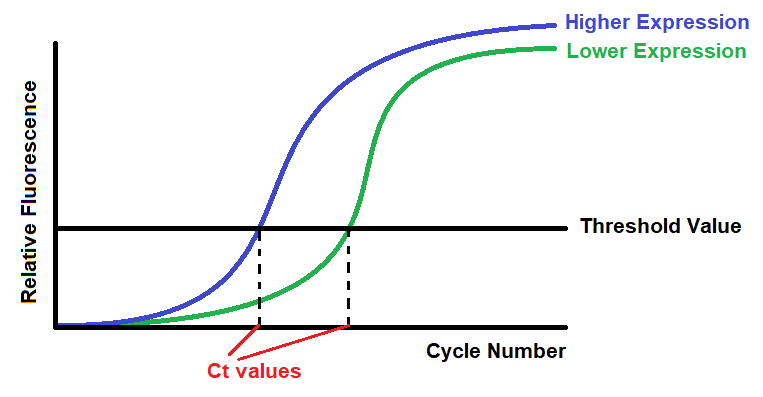
Using the DiO system for *Gsk3b*, expression can be limited to only the glutamatergic pyramidal neurons in the forebrain through the use of a *Camk2a* promoter. This can be achieved through a transgenic mouse line, where the *Cre* gene has been inserted after the *Camk2a* gene in the mouse genome13. These mice will only express *Cre* in cells that are also expressing *Camk2a*, which therefore means that the injected *Gsk3b* will only be inverted and expressed in these cells, causing overexpression of *Gsk3b* in Camk2a+ neurons (Figure 8). We will use CreER(T2), a type of Cre-recombinase fused with an estrogen receptor that will only be active in the presence of tamoxifen. With this particular Cre-recombinase, we can control the time at which overexpression is induced by controlling when tamoxifen is administered, allowing for more precise control.

**Figure 8: Process for the over-expression of *Gsk3b* in Camk2a+ neurons.** A plasmid is created with an inverted *Gsk3b* open reading frame through the use of Gibson assembly. The vector is packaged into adeno-associated virus and injected into transgenic mice with the *cre* gene inserted after the *Camk2a* gene. Upon injecting with tamoxifen, Cre-recombinase is activated in Camk2a+ neurons, inverting the *Gsk3b* open reading frame via loxP/lox2272 recombination. The *Gsk3b* gene is now in the correct direction for transcription only in the Camk2a+ neurons.

II.B. Measuring expression levels of *Bdnf*

To measure expression levels of *Bdnf*, we will use Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR). This molecular biology technique starts by taking all of the mRNA transcripts in a sample and synthesizes the complementary sequence as DNA (cDNA) to preserve their stability and quantity. Then, by using forward and reverse DNA primer sequences that target a section in the *Bdnf* transcript, the region encapsulated between the two primers can be amplified with a three step cycle:

1. Denaturation: All double stranded DNA is separated into individual strands at high temperatures.
2. Annealing: The DNA primer sequences will hybridize to their corresponding regions onto the single stranded DNA.
3. Extension: DNA polymerase binds and completes the reaction in the presence of deoxynucleotides.

By repeating this cycle, the segment of DNA between the two primer sequences is amplified (Figure 9). Quantification of the PCR products can be done by using a double stranded marker such as SYBR Green I, a compound that increases in fluorescence upon intercalation into double stranded DNA. After every cycle, more DNA is produced, and the amount of fluorescence given off by the compound will be recorded. The amount of fluorescence is then plotted against the cycle number. After fluorescence increases to a certain threshold, the cycle producing that fluorescence is recorded as the cycle threshold (Ct) value. This can then be compared between samples. Lower Ct values represent greater fluorescence earlier in the process, indicating more starting product, and therefore higher expression. (Figure 10). By comparing Ct values, the relative expression levels between samples can be quantified.

**Figure 10: RT-qPCR example.** Fluorescence can be measured after every cycle of PCR. Samples with higher levels of expression (blue) will reach the threshold fluorescence level earlier and produce lower Ct values.

**Figure 9: Description of the three steps of a Polymerase Chain Reaction.** Blue represents template DNA and Green represents newly synthesized DNA. Figure obtained from Thermo Fisher Scientific - PCR Basics.

II.C. Comparisons against controls

The *Bdnf* expression levels from the Camk2a+ neuronal overexpression will need to be compared against at least two other controls. These will be a replication of the *Gsk3b* neuronal overexpression experiment described above (Figure 3). A control with no additional *Gsk3b* expression will be used to measure basal expression levels of *Bdnf*. Separately, a control with *Gsk3b* expressed in all neurons will also need to be prepared. This will be done by injecting a viral vector with the *Gsk3b* gene into the mPFC of mice. The gene in this vector will also be driven by an *hSyn* promoter in order to achieve specificity for cortical neurons. *Bdnf* expression levels will be measured in this condition as well, where it should appear lower than in the no gene control. These two controls will then be compared to *Bdnf* expression levels in the Camk2a+ neuronal overexpression condition.

**III. Discussion**

Should this experiment proceed as expected, differences between the expression levels of *Bdnf* should be clear between these different overexpression conditions. If, hypothetically, the decrease in *Bdnf* is dependent specifically on *Gsk3b* overexpression in Camk2a+ neurons, we would expect to see the same decrease in expression between the Camk2a+ neuronal overexpression condition and the general neuron overexpression condition. If this effect is dependent on more than just the Camk2a+ neurons, the decrease in *Bdnf* should only be a fraction of what is observed after general neuronal overexpression. Similarly, if *Gsk3b* induced downregulation of *Bdnf* is not at all dependent on Camk2a+ neurons, no decrease in *Bdnf* expression should be seen, and it should match what is observed in the control condition.

These data would give valuable insight into the molecular mechanisms of how *Gsk3b* downregulates *Bdnf* expression. If *Bdnf* expression downregulation can be correlated to a particular neuron type, then the molecular mechanism behind it should be limited to a specific process of that cell type. Conversely, if the downregulation is not limited to Camk2a+ neurons and is consistent across other neurons, then mechanism would likely be controlled by a common process between all those neuron types.

It is important to understand that with this experimental design, since only one neuron type is being studied, if *Bdnf* expression is downregulated but not to the extent of the general overexpression, it can be hard to interpret if the underlying mechanism is involved with all neurons, or with a specific subset of them. Determining which of these cases is more likely would have to be done in subsequent experiments that explore other neuronal types. The benefit of having a DiO plasmid for *Gsk3b* is that to overexpress in a different neuron type, all that needs to be changed is the specificity of *Cre*, which can be altered through the use of a different mouse line. This experiment, therefore, serves as a powerful first step to understanding this potential mechanism contributing to alcohol addiction. It will also be an important experiment to evaluate the potential of *Gsk3b* as a possible drug target to treat alcohol use disorders.

**References**

1. Stahre, M., Roeber, J., Kanny, D., Brewer, R. D., Zhang, X. (2014). Contribution of Excessive Alcohol Consumption to Deaths and Years of Potential Life Lost in the United States. Preventing chronic disease. 11, E109. doi:10.5888/pcd11.130293
2. Kendler, K. S., Neale, M.C., Heath, A.C., Kessler, R.C., Eaves, L.J. (1994) A twin-family study of alcoholism in women. Am J Psychiatry 151, 707–715.
3. Wolen, A. R., Phillips, C. A., Langston, M. A., Putman, A. H., Vorster, P. J. ... Miles, M. F. (2012) Genetic Dissection of Acute Ethanol Responsive Gene Networks in Prefrontal Cortex: Functional and Mechanistic Implications, PLoS One, 7(4):e33575, doi:10.1371/journal.pone.0033575
4. Chen, G., Bower, K. A., Xu, M., Ding, M., Shi, X ... Luo, J. (2009) Cyanidin-3-glucoside reverses ethanol-induced inhibition of neurite outgrowth: role of glycogen synthase kinase 3 Beta. Neurotox Res 15, 321–331. doi:10.1007/s12640-009-9036-y
5. van der Vaart, A. D., Meng, X., Bowers, M. S., Batman, A. M., Aliev, F. ... Miles, M. F. (2018). Glycogen synthase kinase 3 beta regulates ethanol consumption and is a risk factor for alcohol dependence. Neuropsychopharmacology. 0:1-11, doi: 10.1038/s41386-018-0202-x
6. Logrip, M. L., Barak, S., Warnault, V., Ron, D. (2015). Coritcostriatal BDNF and alcohol addiction. 1628. 60-67.
7. van der Vaart, A. D. (2018). Molecular Brain Adaptations to Ethanol: Role of Glycogen Synthase Kinase-3 Beta in the Transition to Excessive Consumption. (Doctoral dissertation). Retrieved from VCU Electronic Theses and Dissertations (Number 5510).
8. Warthen, D. M., Lambeth, P. S., Ottolini, M., Shi, Y., Barker, B. S. ... Scott M. M. (2016). Activation of Pyramidal Neurons in Mouse Medial Prefrontal Cortex Enhances Food-Seeking Behavior While Reducing Impulsivity in the Absence of an Effect on Food Intake. Front Behav Neurosci 10, 63. doi:10.3389/fnbeh.2016.00063
9. Schnütgen, F., Doerflinger, N., Calléja, C., Wendling, O., Chambon, P., Ghyselinck, N. B. (2003). A directional strategy for monitoring Cre-mediated recombination at the cellular level in the mouse. Nature Biotechnology. 21(5). 562. doi:10.1038/nbt811
10. Nathanson, J. L., Yanagawa, Y., Obata, K., Callaway, E. M. (2009). Preferential labeling of inhibitory and excitatory cortical neurons by endogenous tropism of AAV and lentiviral vectors. Neuroscience. (161)2, 441–450. doi:10.1016/j.neuroscience.2009.03.032
11. Gibson, D. G. (2011). Enzymatic Assembly of Overlapping DNA Fragments. Methods in Enzymology. 497, 349–361. doi:10.1016/B978-0-12-385120-8.00015-2
12. Andrews-Zwilling, Y., Gillespie, A. K., Kravitz, A. V., Nelson, A. B., Devidze, N. ... Huang, Y. (2012). Hilar GABAergic Interneuron Activity Controls Spatial Learning and Memory Retrieval. PLoS One. 7(7), e40555. doi:10.1371/journal.pone.0040555
13. Madisen L., Zwingman, T. A., Sunkin, S. M., Oh, S. W., Zariwala, H. A... Zeng, H. (2010). A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. Nat Neurosci. 13(1), 133–140. doi:10.1038/nn.2467