**Ethanol tolerance and the effect of polyunsaturated fatty acids on the function of the BK channel**

1. **Introduction**

Alcohol use Disorders (AUD) are a profound and common problem affecting millions of Americans in the U.S. alone. Gene factors as well as environmental factors both play a role in the development of alcoholism [7]. The significant problem is that the cause of alcoholism is not well understood. Ethanol is an intoxicating, energy-yielding molecule produced by alcoholic fermentation from plants with high carbohydrate content such as barley, wheat, corn, and grapes [6]. It is a legal drug that is widely used in society for social and medical benefits. However, ethanol taken in excess amounts is one of the most toxic substances in society in terms of behavioral and physical health. Chronic use of ethanol can lead to alcoholism or alcohol abuse and numerous medical conditions such as heart disease and pancreatitis [5]. Ethanol can also affect the brain chemistry by altering levels of neurotransmitters. These are chemical messenger that transmit the signals throughout the body that control through processes, behavior and emotion. These are many of the reasons why it is important to understand how ethanol affects the human brain and development.

The level of response (LR) to alcohol is strongly predictive of subsequent alcohol abuse [2]. LR is a combination of counteracting responses to alcohol, the level of sensitivity to the drug and the degree to which tolerance develops during the drug exposure. This form of tolerance is known as Acute Functional Tolerance (AFT). Bettinger et al had identified mutated genes which altered AFT and were affecting multiple genes in a genetic pathway that is used to regulate levels of triacylglycerol (TAGs). This indicates that lips-7 plays a significant role of TAGs in the development of AFT in C. elegans. [1].

Eicosapentaenoic Acid (EPA) is a polyunsaturated fatty acid found in fish oils [10]. It is the most abundant fatty acids in the brain and the retina. It is best known for brain development and nervous system function as it is used for the process of neuronal activity. Arachidonic Acid (AA) is an integral constituent of a biological cell membrane, conferring it with fluidity and flexibility, so it is necessary for the function of all cells, especially in nervous system, skeletal muscle, and immune system [9]. AA is obtained from food or by desaturation [9]. It also modulates the function of ion channels, several receptors and enzymes for activation and inactivation [9]. Long chain polyunsaturated fatty acids (LC-PUFAs) are required for AFT in response to ethanol. Raabe et al conducted an experiment to figure out whether polyunsaturated fatty acids are required for ethanol response in *C. elegans*. They looked at mutants lacking subsets of LC-PUFAs and measured the development of acute functional tolerance to ethanol over a 30-minute period exposure [7]. Based on the results, they found that EPA and/or AA is required for AFT. To figure out which of these two fatty acids was necessary for the development of AFT, the authors tested *C. elegans* with a mutation in the *fat-1* gene that encodes for Omega-3 fatty acyl desaturase which is needed for the conversion of AA to EPA. *fat-1* mutants cannot synthesize EPA and several other long-chain fatty acids but can synthesize AA. *fat-1* mutants are not able to develop AFT. This suggests that EPA is required for AFT.

BK channels (big potassium) are large conductance calcium-activated potassium channels also known as Slo1. They are voltage-gated potassium channels that conduct large amounts of potassium ions (K+) across the cell membrane. These channels can be activated by either electrical means, or by increasing calcium (Ca2+) concentrations in the cell. They also help regulate physiological processes, such as circadian behavioral rhythms and neuronal activity [12]. Their function is to repolarize the membrane potential by allowing for potassium to flow outward [12]. BK channels help regulate both the firing of neurons and neurotransmitter release. It plays a dominant role in shaping neuronal activity. Bk channels can be modulated by ethanol [3]. Exposure to ethanol less than 100 mM can disturb the physiology and behavior in alcohol naïve systems [3]. Several minutes of ethanol exposure can lead to an increased BK current. This results from an ethanol interaction that was mapped to the BK channel-forming slo1 protein cytosolic tail domain. The importance of this region in alcohol-induced intoxication has been addressed in Caenorhabditis elegans slo1 mutants [18, 19].

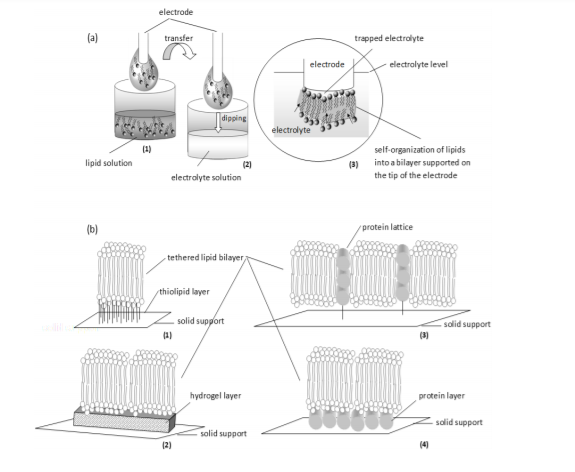
EPA’s and AA’s membrane fluidity may have a possible effect on the BK channel when ethanol is added. Both are unsaturated fatty acids, so they have lots of double bonds which makes them kinked or bend. This will increase membrane fluidity meaning the lipids will be loosed instead of packed.

One of the proposed mechanisms in which EPA is thought to control AFT is through the effects of membrane structure or function [7]. EPA is also known to alter lipid raft structures and it may be that it affects the BK channel in the membrane [7]. The BK channel will be directly tested in order to see if the presence of EPA as a component of a lipid bilayer can alter the electrical activity. AA will also be included in comparison to EPA for testing the membrane fluidity, as it is possible that the BK channel affect by ethanol could be the fluidity of the membranes. More specifically, if EPA and AA do not have an effect on the membrane fluidity, then I can then hypothesize that in order for AFT to occur, the BK channel in an EPA-rich domain of the cell membrane where the electrical activity will be less affected by ethanol.

1. **Experiment**

Major components that we will need to test the hypothesis are a synthetic artificial phospholipid bilayer membrane containing EPA-containing phosphatidylcholine, artificial phospholipid bilayer membrane containing AA-containing phosphatidylcholine, phospholipid bilayer containing POPC, BK channel, and ethanol.

In order to generate an artificial phospholipid bilayer one of the methods Siontorou et al have used is the dipping method. The first step is to dip a metal (Ag, Pt, Ni, etc.) wire into a lipid solution. A small drop of the solution will stick to the edge of the wire [8]. There will be three phospholipids solutions used in this experiment for testing: 1. For the control part of the experiment, chloroform solution containing 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) /1-palmitoyl-2-oleoyl-glycero-3-phosphocholine (POPC), 2. For testing EPA, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) /1-O-hexadecyl-2-(5Z,8Z,11Z,14Z,17Z-eicosapentaenoyl)-sn-glycero-3-phosphocholine, 3. For testing AA, chloroma solution containing 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) /1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine. Each solution will have a 3:1 ratio of DOPE: other phospholipids respectively [14]. This is how EPA, AA, and POPC acid will be inserted into the bilayer. All of the phospholipids will be ordered from Avanti Polar Lipids (Alabaster, Al) [14]. The second step is transferring the metal wire into an electrolyte solution. The lipid drop will be forced to self-organize into a bilayer at the tip of the wire. Last step is tethering. Either thiolipids or hydrogels can be used as an anchoring layer. Proteins can be used as a lattice or as a layer. A figure is provided below for visualization.



For the next part of the experiment is getting the BK channel. I will be using BK Channel *hslo* from a human embryonic kidney (HEK) 293 membrane preparation [15]. The *hslo* cDNA will be expressed in the HEK293 cell line. HEK 293 membrane fragments were isolated using a protocol for COS cells (Sun et al 1994).[16] The first step is that HEK 293 cells will be transfected with *hslo* cDNA (Ahring had already done this) [15]. This means that the cDNA (DNA without introns) will be expressed to form BK Channel proteins in the cell membrane of the HEK 293 cells. The HEK 293 cells will then be formed into pellets and put them into buffer [15]. The cells will be concentrated to get rid of all the excess media that is keeping them alive, so this results in forming small pellets of the cells. Then the cell pellets will be resuspended in buffer to keep BK channel proteins from degrading. The next step will be to lyse the cell pellets using a 27-gauge needle multiple times to turn them into smaller pieces. Ultrasonic vibration is also used to rupture the cell pellets even further. Next, the suspension will be layered on sucrose density gradient and centrifuged. The sucrose density gradient is used to separate the heavy molecules from the small molecules, so that the middle layer, or band, will form the membrane containing the BK channels. A syringe will be used to collect the band and the band will be diluted with bi-distilled H20 and then centrifuged. This step is used to get rid of sucrose. Finally, the resulting pellets will be resuspended to maintain the state of the membrane. The Bk channels will be incorporated by using a small amount of the membrane preparation and will be pipetted into the artificial bilayer [14]. Only a small amount will be used so that 1 BK channel will be inserted rather than many. The next step is to make sure that the BK channel will function in the artificial membrane. In order to do this, I have to figure out the electrophysiology of the BK channel in which I measure the channel’s electrical activity. A patch clamp method is used.

To step up patch clamp, a hollow glass tube such as a micropipette or patch pipette with a very small opening will be used to make tight contact with the tiny area of the artificial membrane [17]. After the application of a small amount of suction to the back of the pipette, the seal between pipette and membrane will become so tight that no ions will flow between the pipette and the artificial membrane, so when the BK channel opens, the ions will flow through it and into the pipette. The resulting electrical current will be measured with an ultra-sensitive electronic amplifier connected to the pipette. This arrangement will use the membrane-attached patched clamp recording method [17]. A record of the current flowing through the BK channel will reveal when the channel is in an open or closed state. When the membrane potential is depolarized (triggers the BK channel), voltage sensors will allow the channel gates to open and potassium will flow outward. After less than one millisecond, the BK channel will inactivate. This patch clamp method will be used for all three test cases for the comparison of BK channel activity between EPA present vs. AA present vs. POPC membrane (for control). It should be producing some electrical activity such as the one pictured below, which proves that there is BK channel activation happening in the artificial membrane. Crowley et al have already tested this part of the experiment in their paper [14].

The last part of the experiment is testing the effect of ethanol on BK channels in the POPC- (control), EPA-, and AA-containing phospholipid membranes. When ethanol Is added, the exposure usually leads to a frequent opening of the channel[14]. 50 mM of ethanol will be added into the electrolyte’s solution using a pipette [14]. This is to check and see whether the POPC-, EPA-, or AA-containing membrane has the most electrical movement using the patch clamp method. A figure below is shown below of a possible increase in electrical activity when ethanol is added for all test cases except for EPA .



1. **Discussion**

Based on my hypothesis, that EPA can reduce the effect of ethanol on BK channels, the overall expected result based on the experiments above is that BK channel should have reduced activity in the presence of ethanol in the artificial membrane containing EPA compared with the two other membrane types. This means there will be less opening of the BK channel with EPA enrich in the artificial membrane compared to AA and the control tests. This will cause the activity of the BK channel to be less affected by ethanol meaning that tolerance to ethanol might be produced through an interaction between EPA and BK channels. This may suggest that this is how AFT will be working.

In terms of the comparison between EPA and AA for membrane fluidity, if both have the same affect, meaning if both have the same affect on BK channels with or without ethanol, then this might simply be an effect on membrane fluidity. If EPA and AA have different effects on BK channel activity with or without ethanol, then it suggests that there is something else more specific about EPA or AA that matters for altering BK channel activity.



One huge pitfall from this experiment is that when comparing the membrane with EPA vs. membrane with AA vs. membrane with POPC, the BK channel might not produce activity in the EPA- or AA-containing lipid membranes. We do know that the BK channel works with DOPE:POPC (3:1) membrane based on Crowley et al’s experiment [14]. If EPA or AA in the membrane inactivates the BK channel, then it is unlikely that we will be able to test the ethanol activation of the BK channel when EPA or AA is present. We would not be able to test if ethanol decreases activity of the BK channel because the BK channel would have already been shut down. We need the BK channel to be activated in order to test if ethanol increases or decreases activity of the BK channel. This will be a technical outcome which will prevent the completion of the experiment.

There will be a couple of limitations of this experiment. We will be using a simple artificial lipid bilayer system instead of an actual bilayer system from a cell. This will be used for one including EPA, one for AA, and another for POPC. In order for EPA to change the BK channel function, it could be the direct interaction with channel itself or changing the membrane fluidity which is somehow altering the channel’s function. Lastly, another limitation of this experiment is that hexadecyl is used instead of hexadecanoyl for one of the phospholipids. This is because I want all the lipids to come from the same source (Avanti Polar Lipids (Alabaster, AL)). This will keep the reagents consistent throughout the experiment. The company does not make palmitoyl EPA phosphocholine either.

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