**Whether the Ethanol effect of BK channel is affected by the presence of EPA**

1. **Introduction**

Alcohol use Disorders (AUD) are a profound and common problems across 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 understanding the cause of alcoholism is not well understood by many. 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 response to alcohol, the level of sensitivity to the drug and the degree to which tolerance develops during the drug exposure. The tolerance is known as Acute Functional Tolerance (AFT). Bettinger et al discovered that the lips-7 gene was the first indication that lipids in C. elegans were important for AFT. They had identified mutated genes, which altered AFT, that were affecting multiple genes in a genetic pathway that is used to regulate levels of triacylglycerol (TAGs). This indicates that there’s a significant role for TAGS in the develop of AFT [1].

Eicosapentaenoic Acid (EPA) is an omega-3 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 and chain elongation of the plant-rich essential fatty acid [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 EPA or AA is 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 out 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 and triggers the activation of the BK channel [12]. BK channels help regulate both the firing of neurons and neurotransmitter release. It plays a dominant role in shaping neuronal activity and is strongly affected by ethanol. Bk channels can be modulated by ethanol [3]. Ethanol less than 100 mM exposure of calcium-gated BK channels 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 ethanol interaction that is 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].

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 known to alter lipid raft structures and it may be that it affects the BK channel in the membrane (Raabe et al). This will be directly tested in order to see if the presence of EPA as a component of a lipid bilayer can alter the activity of the BK channel. I can then hypothesize that in order for AFT to occur, the BK channel may be relocated to an EPA-rich domain of the cell membrane where the activity of the BK channel 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 a regular fatty acid, 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 lipid 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 fatty acids respectively [14]. This is how EPA, AA, and regular fatty acid will be inserted into the bilayer. 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.



 All fatty acids listed above will be ordered from Avanti Polar Lipids (Alabaster, AL) [14].

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]. *Hslo* cDNA gene is a cell line which will be expressed in order to create the BK channel. HEK 293 membrane fragments were isolated (using a protocol for COS cells (Sun et al 1994).[16] Then the HEK 293 cells will be infected with *hslo* cDNA and grown to converge and form pellets, and then the cells will resuspend on ice in 10mL of buffer: 30mM KCI, 2mM MgCl2, 10mM HEPES (buffering agent), and 5 mM EGTA (chelate agent, pH 7.2) [15] The cell suspension will be forced through a 27-gauge needle 4 times and then ultrasonic vibration will be used at 30% maximum power for 30 seconds, twice. [15] The suspension will be layered on a 20 to 38% sucrose density gradient and centrifuged at 25,000 rpm for 60 minutes at 4 degrees Celsius. [15] Then a syringe will be used to collect the band at the 20 to 38% interface, and it will be diluted with bi-distilled H20, and centrifuged in a 50.2 Ti rotor at 45, 000 rpm for 50 minutes at 4 degrees Celsius. The resulting pellet will be resuspended in 200 ul of buffer: 250 mM sucrose and 10 mM HEPES, Ph 7.3.[15] All the aliquots will be stored at -80 degrees Celsius until used.

The Bk channels will be incorporated by dropping .5 to 1 ul of the membrane preparation and pipetting into the artificial bilayer [14]. A small amount is used so that only 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 clam method is used.

To step up patch calm, 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 thus all the ions that flow when the BK channel opens must into the pipette. The resulting electrical current will be measured with an ultra-sensitive electronic amplifier connected to the pipette. This arrangement will the cell-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, voltage sensors will allow the channel gates to open and calcium will flow inward. After less than one millisecond, the BK channel will inactivate. When an inactivation gate swings up and blocks the pore, the poor will remain blocked even during prolonged depolarization [17]. The pore will then close, and the inactivation gate will open as the membrane potential return to resting levels. This patch clam method will be used for all three test cases for the comparison of BK channel activity between EPA present vs. AA present vs. fatty acid 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 all the artificial membrane. Crowley et al have already tested this part of the experiment in their paper [14].



For the last part of the experiment is testing the effect of ethanol to the control fatty acid, EPA, and AA in the BK channel. When ethanol Is added, the exposure usually leads to an increased BK current [14]. 50 mM of ethanol will be added [14]. (How will it be added?) This is to check and see whether the control fatty acid, EPA, or AA has the most electrical movement using the patch clamp method. A figure below is shown below of an increase in electrical activity when ethanol is added for all tests.

 

1. **Discussion**

The overall expected result based on the experiments above is that BK channel should have more electrical activity in the artificial membrane containing EPA, which should cause the electrical flow to be faster. This means there will be more opening and closing 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 the tolerance will high. This may suggest that this is how AFT will be working.

One huge pitfall from this experiment is that when comparing the membrane with EPA vs. membrane with AA vs. membrane with short fatty acids, the BK channel might not produce activity in the EPA or AA lipid membranes. We do know that the BK channel works with a short lipid membrane based on Crowley et al’s experiment [14]. If EPA in the membrane inactivates the BK channel, then there is no way we can 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 couple 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 short fatty acids. Another limitation is that this could also affect the fluidity. 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.

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