The Power of Diet Intervention: Food as Medicine

Ketogenic Diet as Therapeutic Treatment for Anxiety and Potential Mechanisms Explored

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SIGNIFICANCE

The prevalence of anxiety has risen substantially and steadily in the US since the 1980's. Data from four surveys completed by almost 7 million people in the US concluded that Americans reported higher rates of anxiety in the 2000s-2010s compared to the 1980s-1990s1. It is highly likely that self-reported levels of anxiety may even be under-reported in the US. Currently, with more people seeking treatment for anxiety and similar mental health disorders, there is a significant lack of understanding in both the underlying mechanisms of such behavioral disorders and the treatment options available. Many treatments lead to dependency, tolerance, and uncomfortable side effects, such as weight gain, reduction in libido, and fatigue2. The need for non-pharmaceutical, safe, effective, reliable, and cost-effective treatment options for anxiety is more urgent than ever before.

It is impossible to fully quantify the quality of life and productivity lost to anxiety globally and in the US, but the rise is anxiety globally and in the US is certain and while the impact on one's life can vary, it remains indisputable that chronic prolonged anxiety is on the rise. Anxiety can impact everything from a person's mental wellbeing, family life, work productivity, and academic success to their physical health. The global market for treatment of anxiety disorders and depression is expected to reach $18.6 billion by 2015 with an estimated 275 million people suffering from a form of anxiety1.

Research still has a long way to go in understanding completely the mechanisms underlying anxiety disorders. In addition, symptoms of anxiety can range greatly among individuals. Introducing a pharmacological intervention to aid with symptom relief may or may not work, and even if it does work, it tells us nothing about the underlying root cause of and mechanisms of the disease. A review from Pharmacy and Therapeutics addressing current treatment options and diagnostic approaches for anxiety disorders reveals that despite efforts to find common biological factors to understand to correlate anxiety disorders, researchers have failed to implicate a specific genes or cluster of genes to implicate in anxiety3. Without a mechanistic or etiological understanding, science and medicine remains extremely limited on both how to prevent and how to treat anxiety. It is our goal to aid in getting closer to identifying mechanisms underlying anxiety and to investigate the potential therapeutic potential of ketogenic dietary intervention.

It is important to recognize roadblocks in anxiety research. Firstly, with all preventative approaches, it is impossible to prove a negative. For example, if “x” is done now, “y” will not occur later. It is only possible to show probability of occurrence without the intervention of “x”. Secondly, as mentioned, anxiety can present itself in many different ways, making it difficult to conserve mouse models of anxiety research to human models. In addition, humans have the very complex added variability that comes with advanced cognitive abilities, modern society, social networks, and daily life. While researchers can do their best to find conserved mechanisms to target anxiety treatment, individuals have extreme biological and social variances and one successful treatment approach may not carry over to large diverse samples of humans.

Preliminary research shows that adhering to a ketogenic diet enhances brain function in disease states, such as traumatic brain injuries and Autism Spectrum Disorder4,5,6. A 2019

review analyzed the beneficial effects of exogenous ketone supplementation on psychiatric diseases like bipolar disorder, schizophrenia, depression, attention-deficit/hyperactivity disorder and anxiety7. While the study that the review was referencing observed anxiolytic effects with addition of exogenous dietary ketones, no research has actually studied the ketogenic diet itself as a therapeutic intervention for anxiety disorders8. The goal of our lab is to fill these gaps in anxiety research. Our aim is to identify potential mechanisms for anxiety-like behavior in a mouse model and investigate the therapeutic potential of ketogenic dietary intervention in anxiety treatment.

Dietary intervention is both accessible and affordable for many populations. Without the need for a pharmaceutical company to produce, test and market a drug, the implications for dietary intervention are profoundly rapid and vast as compared to drug intervention. Providing humans with a proven technique to alter their daily food consumption to protect their brains from anxiety and possible reduce anxiety levels at baseline would have a massive impact globally. Even though nutrition has been shown to have an incredible impact on mental health, using dietary intervention as therapeutic treatment is considered a novel approach due in part to the difficulty of operationalizing variables and controlling for confounds9. Our intention is to change this paradigm and reintroduce the potentially powerful therapeutic applications for dietary intervention in mental health.

The research in this lab will meet all NIH guidelines for all animal procedures and will not commence until approved by the Animal Care and Use Committee of the National Institute on Aging Intramural Research Program. The work done will contribute greatly to the NIH's mission to improve human health through science and will hopefully empower people by providing them with a tool that they already have (their bodies and their daily food intake) to positively improve their own lives.

INNOVATION

Our study is unique in many ways. First, most clinical research on anxiety focuses on the application of a pharmacological agent to cure a disease. Our study proposes a dietary change, using food as medicine. This study represents a big shift in conventional medicine, and specifically psychiatry to use diet as a means of treatment. In the late 1940s, researchers began analyzing the benefits of a low-fat diet on American's cardiovascular health10. It is now widely recognized that there is no correlation between a low fat diet and improved cardiovascular health or weight loss. It wasn't until recently that research began to recognize that a diet high in fats is in fact healthy and beneficial to the body and brain. Our study aims to join a growing body of research that shows the benefits of high fat on cognitive function and amelioration of anxiety symptoms.11,12

In addition to a novel dietary approach, this study will also utilize a novel approach in inducing stress in the rodents during MRI imaging. We will use an awake mouse imaging methodology by utilizing the restraints in the MRI as a stress-inducer.13 MRI and DTI are generally done under anesthesia or surgical interventions to ensure that the animal's head does not move during the imaging. These techniques however, come with an array of problematic variables. For instance, anesthesia is a vasodilator and can thus have a significant impact on MRI blood-oxygen-level-dependent (BOLD) signaling. Surgical approaches are terminal, so the animal could not be used in future trials. With restraints, not only is it the animal safely immobilized, but restraining is also an innate stressor that will induce activation along the brain circuitry, optimizing the imaging for circuitry and activation in the MRI. This is a relatively new approach to rodent imaging; therefore a picture has been included from a prior study. In this study, the MRI will actually be the stressor to limit confounding variables. We will follow methodology directly from Madularu et. al.

APPROACH

Aim 1: To use an elevated plus arm maze behavioral test to demonstrate anxiety levels in mice on a ketogenic diet and on a standard American Diet compared to baselines before a three-month dietary intervention. We expect to see lower levels of anxiety in mice that are on a ketogenic diet for three months.

In this study, we hope to analyze the effect of diet on mental health, specifically anxiety. Diet has an enormous impact on psychological health, as demonstrated in prior research.14 The ketogenic has been found to have widespread positive benefits on health, yet no study has been done to demonstrate the impact of a ketogenic diet on anxiety.15,16 In the first experiment, we will demonstrate that a ketogenic diet will have anxiolytic effects on the mice by spending more time on the exposed arm of an elevated plus arm maze than mice on a Standard American Diet. This experiment will be the first step in an effort to offer a non-invasive, non-pharmacological therapeutic option for people with anxiety.

Our method of measuring level of anxiety behaviorally will be with an elevated plus maze. This task involves a four-arm, elevated maze with two enclosed arms and two open arms. An elevated plus maze takes advantage of the approach-avoidance conflict, as opposed to other stressful stimuli like electric shocks, loud noises, or predator odors.17 Mice have a natural affinity for dark, confined spaces (approach) and a natural aversion to light, open areas (avoidance). Mice with lower anxiety will feel more comfortable venturing into the open arms, whereas anxiety-driven mice tend to stay in the enclosed arms. This maze is inexpensive, effective, time efficient, and simple with few confounding variables to skew the results.

This study will use young adult male mice that are 2-3 months old. The strain will be C57BL/6J based on a study that had success with these mice adapting to a ketogenic diet.5 There will be 9 mice per diet group. The mice will initially be group housed to acclimate the mice to their surroundings for 1 week and then transferred into single housing during the experiment with ad libitum access to food and water. The housing room will be maintained with a standard 12-hour light/dark cycle at 22 ± 1°C. This protocol is based on methodology from Huang et. al.5

The mice will first undergo baseline testing on the elevated plus arm maze. The mice will then be fed either a standard American Diet or a ketogenic diet for three months. It is important to allow for three months to ensure that the mice in the ketogenic diet reach nutritional ketosis and allow for the body to adjust to the diet. Nutritional ketosis occurs when the brain switches from the default mode of utilizing glucose in the brain as fuel to break down fats for fuel.18 As a result, the body releases ketones, which can be tested in the blood to ensure that the appropriate level of nutritional ketosis has been reached. Ketones will be measured three hours post-prandial using a precision Xtra glucose and ketone monitoring system according to the manufacturer's instructions to measure circulating levels of β-hydroxybutyrate and glucose in the blood.16 The nutritional content of the ketogenic diet will consist of 10% protein and 90% fat. The nutritional content of the Standard American Diet will breakdown into 10% protein, 80% carbohydrates, and 10% fat. Food intake and body weight will be taken every other day at the same time.

The elevated plus maze will be performed under 200 Lux illumination at the same time of day each trial. Mice will be gently placed in the central area with its nose facing one of the two closed arms. Movement will be tracked for five minutes via an overhead camera. Time spent on the open arms, the number of times the mouse places a limb in the exposed arm

area, and the total distance travelled on the exposed arms will be quantified.5 This elevated plus maze set-up will be the same for both baseline testing and three months later with the same mice.

Statistical analysis for these results will involve a t-test with a significance of $p = 0.05$, given the smaller population size. A t-test will show the statistical significance between the two diets and the level of anxiety shown in the behavioral test.

Stress, in any study, is limited by its very nature of being a psychological disorder. There is so much variance in how stress is exhibited in different species. We aim to stay as simple as possible in the elevated plus maze to reduce confounding variables. Behavioral tests are incredibly susceptible to environmental influences, so consistency will be a top priority in terms of handling the animals and time of testing, among others. One caveat is that the elevated plus maze is generally thought to have a one-trial tolerance (OTT), characterized by a reduction in anxiolytic-effects due to exposure to the stimuli more than once. However, this is ameliorated with the three-month long interval between trials. A prior study demonstrated that OTT subsides in as little as 28 days.19

Aim 2: will attempt to visualize differences in fMRI scans in rodents that performed on the behavioral test in aim one. fMRI will be taken for both groups (ketogenic and non-ketogenic) immediately after behavioral test is completed.

The rationale of our study in similar as stated above, to see the effects of diet on mental health, and analyze this through three different methods. In addition to measuring the effect of the ketogenic diet on anxiety in a behavioral paradigm, we also want measure differential activation of brain regions implicated in anxiety. Our brain regions of interest implicated in

anxiety include the amygdala, hippocampus and prefrontal cortex.20 In the ketogenic diet group, we expected to see decreased activation of the amygdala and hippocampus and increased activity in the prefrontal cortex, indicative of decreased anxiety.20,21

For this experiment, we will put the mice from Aim 1 in an fMRI to measure activation in the amygdala, hippocampus and prefrontal cortex. The fMRI data will be collected on the immediately following elevated plus maze testing. The mice will be restrained in the fMRI to induce a stress response in the mice. They will not be habituated in order to maintain the stress response. Mice will be lightly anesthetized for under one minute using isoflurane in order to aid in the setup procedure. The mice will then undergo 10 minute episodes of functional scanning. This induction of anxiety in the scanner will allow us to measure anxious responsiveness in the brain while minimizing confounding variables. We will follow methodology from Madularu et. al. to perform the awake imaging technique in rodents using an MRI.13

We expect that mice fed a ketogenic diet versus a typical American diet will have decreased anxiety. We identified certain brain regions that we hypothesize will have increased or decreased activation after being on a ketogenic diet based on a review analyzing the neurobiology of anxiety disorders.22 We hypothesize that areas of the brain that are implicated in anxiety disorders will be less active, such as the amygdala. In addition, we expect to see increased activation in the prefrontal cortex, an area of the brain implicated in extinguishing fearful, anxiety-provoking memories and regulating mood. We also expect to observe increased activation in the hippocampus, given the inhibitory GABAergic projections onto the amygdala involved in the hypothalamic stress-response system.

Statistical analysis on MRI data will be analyzed with three t-tests to see the relationship of diet and level of activation in the brain. The level of activation will be operationalized as representative of anxiety. A p value will be set at 0.05 and is appropriate for our smaller sample size. For the parametric modeling involved in fMRI research to fit voxel size, a statistician will develop this model to establish the best model activation for our paradigm.

Specific Aim 3: will utilize diffuse tensor imaging (DTI) to discern white matter tract differences between the mice on each diet. Immediately following the behavioral experiment, DTI will be performed. We expect that mice that are in nutritional ketosis will show decreased fractional anisotropy in DTI tests as compared to mice who are not in ketosis, showing a potential mechanism for changes in anxiety on a ketogenic diet.

Whereas MRI is effective at analyzing the activation in certain brain regions, DTI is an incredible approach to look at brain circuitry. In this experiment, we will examine the effects of diet on white matter tracts in the brain by analyzing levels of anisotropy between the amygdala, hippocampus, and prefrontal cortex. We predict there will be decreased fractional anisotropy between these brain regions in mice that have been fed a ketogenic diet. This hypothesis is based on evidence that circuitry dynamics are mediated by anxiety and fear, in which magnetic resonance diffusion tensor imaging can detect.20 In a prior study using DTI, mice have been shown to have microstructural alterations induced by stress in the hippocampus.21Understanding the underlying mechanism behind anxiety and the impact that diet can have on those mechanisms is crucial to developing a potential therapeutic intervention.

Prior studies have shown that anxiety can cause impairments to the prefrontal cortex, amygdala, and hippocampus using DTI in humans.22 Non-invasive brain imaging is

essential to understanding how treatments influence pathology. Despite evidence that demonstrates that a ketogenic diet is beneficial to one's mental health, there is no definitive research to understand the underlying neurological impact it is potentially having on stress pathways in the brain.

Immediately following the elevated plus arm maze, we will perform MRI and DTI in awake mouse imaging by using restraints to induce a stress response13. In order to visualize this circuitry in mice, we will use the same mice from Aim 1. All animals will be imaged prior to diet intervention for baseline testing and then will be imaged following three months of dietary intervention of either Ketogenic Diet or Standard American Diet. This ensures consistency throughout the experiment. Mice will be lightly anesthetized for less than one minute using isoflurane in order to aid in the setup procedure. The mice will then undergo 10 minute episodes of functional scanning. Protocol for this experiment will be used from Madularu et. al.13

Refer to Aim 1 for detailed information about mouse model and experimental controls. Statistical analysis will be used to determine regional comparisons in tissue properties so that pathways can be visualized and compared for statistical significance. Each scan will be segmented into the ROI's (regions of interest) for the present study, the hippocampus, amygdala and prefrontal cortex, and the pathways between them. DTI datasets will be able to create visualizations of average pathway activation and compare statistically to determine significant differences or not. As discussed, we expect to see reduced activation of stress behavior pathways in mice adapted to a ketogenic diet. If it is true that DTI appears statistically significantly different between ketogenic diet mice and control mice, with confounding variables minimized, then the present study will suggest potential pathway associated mechanisms for adaptation to ketogenic dietary intervention.

The primary caveat to this approach is that differences in DTI may suggest pathway-associated mechanisms for adaptation to ketogenic dietary intervention, but differences will not be able to identify where or what in the pathway is specifically being affected. DTI cannot differentiate between types of neurons or synapses so further studies will need to be done investigating these stress pathways in more detail23.

A t-test will be performed to see differences between the ketogenic diet and control (American Diet) group on fractional anisotropy. The p value will be set at 0.05. At the end of our experiment, all the values will be correlated together to see if decreased anxiety in each paradigm matches between aims.

References

1. Twenge JM. Time Period and Birth Cohort Differences in Depressive Symptoms in the U.S., 1982–2013. Soc Indic Res. 2015;121(2):437-454. doi:10.1007/s11205-014-0647-1

2. NIMH »Mental Health Medications. https://www.nimh.nih.gov/health/topics/mental-health-medications/index.shtml. Accessed December 4, 2019.

3. Bystritsky A, Khalsa SS, Cameron ME, Schiffman J. Current Diagnosis and Treatment of Anxiety Disorders. Pharm Ther. 2013;38(1):30-57.

4. Elamin M, Ruskin DN, Masino SA, Sacchetti P. Ketogenic Diet Modulates NAD+-Dependent Enzymes and Reduces DNA Damage in Hippocampus. Front Cell Neurosci. 2018;12. doi:10.3389/fncel.2018.00263

5. Huang J, Li Y, Wu C, et al. The effect of ketogenic diet on behaviors and synaptic functions of naive mice. Brain Behav. 2019;9(4). doi:10.1002/brb3.1246

6. Ruskin DN, Fortin JA, Bisnauth SN, Masino SA. Ketogenic diets improve behaviors associated with autism spectrum disorder in a sex-specific manner in the EL mouse. Physiol Behav. 2017;168:138-145. doi:10.1016/j.physbeh.2016.10.023

7. Kovács Z, D'Agostino DP, Diamond D, Kindy MS, Rogers C, Ari C. Therapeutic Potential of Exogenous Ketone Supplement Induced Ketosis in the Treatment of Psychiatric Disorders: Review of Current Literature. Front Psychiatry. 2019;10. doi:10.3389/fpsyt.2019.00363

8. Ari C, Kovács Z, Juhasz G, et al. Exogenous Ketone Supplements Reduce Anxiety-Related Behavior in Sprague-Dawley and Wistar Albino Glaxo/Rijswijk Rats. Front Mol Neurosci. 2016;9. doi:10.3389/fnmol.2016.00137

9. Firth J, Marx W, Dash S, et al. The Effects of Dietary Improvement on Symptoms of Depression and Anxiety: A Meta-Analysis of Randomized Controlled Trials. Psychosom Med. 2019;81(3):265. doi:10.1097/PSY.0000000000000673

10. Berge L, F A. How the Ideology of Low Fat Conquered America. J Hist Med Allied Sci. 2008;63(2):139-177. doi:10.1093/jhmas/jrn001

11. Sussman D, Germann J, Henkelman M. Gestational ketogenic diet programs brain structure and susceptibility to depression & anxiety in the adult mouse offspring. Brain Behav. 2015;5(2):e00300. doi:10.1002/brb3.300

12. Hernandez AR, Hernandez CM, Campos K, et al. A Ketogenic Diet Improves Cognition and Has Biochemical Effects in Prefrontal Cortex That Are Dissociable From Hippocampus. Front Aging Neurosci. 2018;10. doi:10.3389/fnagi.2018.00391

13. Madularu D, Mathieu AP, Kumaragamage C, et al. A non-invasive restraining system for awake mouse imaging. J Neurosci Methods. 2017;287:53-57. doi:10.1016/j.jneumeth.2017.06.008

14. Tapsell LC, Neale EP, Satija A, Hu FB. Foods, Nutrients, and Dietary Patterns: Interconnections and Implications for Dietary Guidelines12. Adv Nutr. 2016;7(3):445-454. doi:10.3945/an.115.011718

15. Salberg S, Weerwardhena H, Collins R, Reimer RA, Mychasiuk R. The behavioural and pathophysiological effects of the ketogenic diet on mild traumatic brain injury in adolescent rats. Behav Brain Res. 2019;376:112225. doi:10.1016/j.bbr.2019.112225

16. Roberts MN, Wallace MA, Tomilov AA, et al. A Ketogenic Diet Extends Longevity and Healthspan in Adult Mice. Cell Metab. 2017;26(3):539-546.e5. doi:10.1016/j.cmet.2017.08.005

17. Walf AA, Frye CA. The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. Nat Protoc. 2007;2(2):322-328. doi:10.1038/nprot.2007.44

18. Shilpa J, Mohan V. Ketogenic diets: Boon or bane? Indian J Med Res. 2018;148(3):251-253. doi:10.4103/ijmr.IJMR_1666_18

19. Schneider P, Ho Y-J, Spanagel R, Pawlak CR. A Novel Elevated Plus-Maze Procedure to Avoid the One-Trial Tolerance Problem. Front Behav Neurosci. 2011;5. doi:10.3389/fnbeh.2011.00043

20. Taylor JM, Whalen PJ. Neuroimaging and Anxiety: the Neural Substrates of Pathological and Non-pathological Anxiety. Curr Psychiatry Rep. 2015;17(6):49. doi:10.1007/s11920-015-0586-9

21. Liu Y, Cheng A, Li Y-J, et al. SIRT3 mediates hippocampal synaptic adaptations to intermittent fasting and ameliorates deficits in APP mutant mice. Nat Commun. 2019;10(1):1-11. doi:10.1038/s41467-019-09897-1

22. Martin EI, Ressler KJ, Binder E, Nemeroff CB. The Neurobiology of Anxiety Disorders: Brain Imaging, Genetics, and Psychoneuroendocrinology. Psychiatr Clin North Am. 2009;32(3):549-575. doi:10.1016/j.psc.2009.05.004

23. Ayling E, Aghajani M, Fouche J-P, van der Wee N. Diffusion tensor imaging in anxiety disorders. Curr Psychiatry Rep. 2012;14(3):197-202. doi:10.1007/s11920-012-0273-z