

A Data-Driven Approach to Uncovering the Neural Dynamics of Anxiety

by

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Thesis submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Anxiety is a behavioral state induced by low-threat, uncertain situations in which perceived danger is diffuse. The anxiety state is then accompanied by increased vigilance and risk assessment to one's surroundings. Recent studies have shown that the brain regions responsible for encoding anxiety are widely located in the frontal cortex and extended limbic system; however, the network architecture responsible for hypervigilance has yet to be elucidated. Here, I propose to employ a data-driven method of using *in vivo* recordings of electrical activity across multiple brain regions concurrently as mice freely explore classic ethological anxiety-related behavioral assays and are administered pharmacological agents that modulate the anxiety state. Using novel machine-learning techniques, I have generated neural models that reflect the network-level activity engaged during the performance of these tasks. I have then validated the structure of this anxiety network in its ability to generalize to other anxiety-related tasks and models of disease. I anticipate that this strategy will discover an independent network that is correlated with anxiety-related behaviors. Thus, successful completion of the proposed work will lead to a network-level understanding of anxiety. Furthermore, the framework discovered through this study has the potential to facilitate the development of new revolutionary approaches for anxiety disorders.

Dedication

To my extraordinary parents: you taught me to wonder, you instilled in me a desire to be kind, and you trained me to lead a life of authenticity.

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1. Introduction

Anxiety is described as a mental state characterized by an intense sense of tension, worry or apprehension, relative to something adverse that might happen in the future (Saviola et al. 2020). In the rodent literature, anxiety is defined as a temporary behavioral state induced by low-threat, uncertain situations and is accompanied by increased vigilance and risk assessment to one's surroundings (Adhikari. 2014). This emotional state can be an adaptive response driving important behaviors to face possible dangers; but once its considered excessive and unmotivated, this emotional state can be considered an anxiety disorder. However, patients with anxiety disorders display chronically high levels of anxiety and avoidance measures, generally to a debilitating and maladaptive extent (La-Vu et al, 2020). It is estimated that 1 in 13 U.S adults develop a generalized anxiety disorder in their lifetime (Ruscio et al. 2017) and often have comorbidities with several other psychiatric illnesses or cardiovascular disease that greatly reduce their quality of life (Hsin et al, 2018). Anxiety disorders constitute the largest group of mental disorders in western societies, and it has become imperative to not only develop an intimate understanding of anxiety but to create a framework in which to ultimately delineate physiological vs pathological states of anxiety. Preclinical models of anxiety, specifically in rodents, provide a manner to elucidate pathophysiology in a battery of behavioral assays.

The goal of systems neuroscience is to understand how neural activity in the brain is coordinated to produce observable behaviors. While we don't have the benefit of self-report data in our rodent models, we can leverage avoidance behaviors in well-validated ethological tasks to study the neural computations that may underlie these the anxious state.

1.1 Anxiety-Related Rodent Assays

Evaluating risk and reward potential in the execution of motivated behaviors is important in decision-making and requires the activity of positive (approach) and negative (avoidance) valence systems. This delicate balance has been coined “the approach-avoidance conflict” and is used by behavioral neuroscientists to illustrate the internal dispute rodents face between free exploration of novel environments and the avoidance of potential danger. To gauge innate levels of anxiety, behavioral neuroscientists have developed a trove of behavioral assays that exploit the approach-avoidance conflict in rodents. Mice prefer dimly lit, enclosed spaces and are averse to exploring open bright spaces. This movement pattern is described as thigmotaxis as mice prefer to walk along spaces in which their whiskers are in constant contact with a wall or object – presumably to prevent visual detection by predators (La-Vu et al, 2020). Human thigmotaxis relies on similar cognitive and emotional factors to inform their spatial strategy moving along walls e.g. picking a table at the edge of a restaurant rather

than the center (Kallai et al 2007). Enhanced thigmotaxis is observed in anxiety disorders, most notably agoraphobia (Walz et al, 2016).

A major drawback of psychiatric research in animal models is the lack of direct measures of emotional state. Compared to research in humans, where subjects can offer direct report of their emotions, animal research typically relies on measurements of behaviors to infer the underlying emotion. One of the most used anxiety assays is the Open Field Test (OFT), in which rodents are placed in an enclosed square context for 5-10 minutes. The mouse can freely explore the novel context for the allotted time and several behavioral measures are scored to intuit their innate anxiety: time spent in center square vs time spent along the periphery, frequency of entering the center square, latency to enter the center square, and total locomotion. As the center square is considered anxiogenic (generates anxiety), increased evasion of the center square measured by higher latency or decreased frequency to enter is correlated with the higher innate anxiety in the mouse subject. Various factors can also inform anxiety measurements in the OFT such as light levels, specifically with brighter light arenas as more anxiogenic.

The elevated plus maze (EPM) is another classic behavioral task used to study the approach-avoidance conflict. The apparatus consists of an elevated maze with four arms (two open and two enclosed) that are arranged to form a plus (+) shape (Walf et al, 2007). The open arms are avoided and many anxiety-related behaviors such as freezing

and defecation are increased in the open arms of the maze compared to the closed arms (Pellow et al. 1985). The experimental outputs of the EPM are similar to the OFT: time spent in open arms vs closed arms, frequency of entering the open arms, latency to enter the open arms and total locomotion. The more the mouse evades the open arms of the EPM, the higher the innate anxiety the mouse has.

1.2 Validity of Anxiety Assays

Animal models can offer face validity – observable behavioral, anatomical, or physiological responses that are similar to human disease. For example, increased heart rate variability (the variation in time between each heartbeat) is observed in humans with anxiety disorders and mice in anxiogenic environments (Held et al 2021, Gaburro et al 2011). Mouse models of anxiety disorders also have construct validity – mechanisms to induce human disease phenotypes in animals. For instance, low levels of serotonin 1A (5-HT1A) have been observed in humans with anxiety disorders and 5-HT1A knockout mice have increased anxiety and stress responses (Holmes et al. 2003). The EPM and OFT have been used to pharmacologically validate anxiety assays in the preclinical setting. A major disadvantage of animal research is the inability to collect self-report data which is possible in human subjects. Through the use of these ethological tasks, researchers have been able to show that drugs that modulate anxiety in humans have similar effects in rodents. For a treatment to be considered anxiogenic or anxiolytic

(decreases anxiety), it is anticipated that a shift in the anxiety measures (open arm/center square time, frequency and latency to enter open arm/center square) would be evident following drug administration. Previous studies have shown that anxiety behaviors can be influenced by various classes of drugs. Fluoxetine, a selective serotonin reuptake inhibitor (SSRI), inhibits the removal of serotonin from the synapse. Fluoxetine has been shown to be anxiogenic upon acute administration and anxiolytic with chronic administration (Griebel et al. 1998). This time course mirrors their clinical effect, particularly seen in the adolescent population (Harmer et al. 2017).

Benzodiazepines are positive allosteric GABA_A receptor modulators. They are widely prescribed for acute anxiety and panic disorders and have shown to be anxiolytic in rodent models (Griebel et al. 2000). Ethanol interacts with GABA by potentiating GABA_A receptor activity. Ethanol is anxiolytic in mice, similar to alcohol consumption in humans. However, following chronic ethanol administration, acute withdrawals of ethanol have been shown to display an anxiogenic phenotype (Farook et al. 2007).

1.3 Modeling Physiologic vs Pathologic Anxiety

In 1990, Lister and colleagues made the observation that anxiety could be divided in various forms including 'state' and 'trait' anxiety. State anxiety is a temporary reaction to adverse events while trait anxiety denotes a stable personality/temperamental feature. Trait anxiety is thought to belong to a list of

characteristic traits of an individual's personality and it can be associated with different psychopathological conditions and constant high arousal (Saviola et al. 2020). Preclinical behavioral tasks in rodents generally assay the experienced anxiety in a particular moment in time and it is increased by the presence of the anxiogenic stimulus. These assays include exploratory or unconditioned response tests, similar to the EPM, OFT, zero maze and the light/dark choice task (Belzung et al, 2001). On the other hand, 'trait' anxiety does not vary from moment to moment and is considered to an enduring feature of the subject.

'Trait' anxiety models use rodents that were selected for emotional reactivity or utilize genetic manipulations, drug administration, or protocols that exhibit behavioral changes that reflect globally increased anxiety. Many preclinical models of disease take advantage of 'trait' anxiety of the mouse model. Examples of such models include CLOCK delta19 (Bipolar Mania), *Pogz* (autism) and 5HT1A KO (anxious) are genetic manipulations that have been used to correlate abnormal neural activity that may underlie changes anxiety-related behaviors in these mouse models (Cunniff et al. 2020; Roybal et al. 2007; Adhikari et al. 2010). These studies have made incredible advances in our understanding of anxiety-related behaviors and create the backdrop for the bulk of this research.

1.4 Neural Circuits Implicated in Anxiety

Much of our early understanding of emotional substrates in the brain was largely restricted to lesion and inactivation studies. These studies involved physical or chemical inactivation of whole brain regions followed by careful behavioral analysis of the model species. This important work led to the identification of several brain key brain structures believe to encode anxiety: the amygdala (AMY), ventral hippocampus (vHipp), prefrontal cortex (PFC) and the bed nucleus of the stria terminalis (BNST). Many of these regions fall into broad categories sensory processing, emotion generating and emotion modulation regions (Duval et al. 2015). Thankfully, these same regions identified from the rodent literature have been identified using functional imaging in both pathologically anxious and healthy human subjects (Davis et al. 2010).

Further development of sophisticated circuit-level manipulations in rodent models have begun to further dissect the involvement of these brain regions along with their projections to other important brain regions. *In vivo* recordings in rodents suggest that there is an interplay between the mPFC, AMY and the vHipp that affects anxiety (Adhikari et al. 2010). In order to validate this correlative study, optogenetic activation of the BLA-vHipp projection increased anxiety-related behaviors in these tasks whereas inhibition of this projection was anxiolytic in the EPM and OFT (Felix-Ortiz et al. 2013). However, we have learned that the brain is exquisitely interconnected. Studies have yet to focus on the integration of the many brain regions to understand the mechanisms of

emotion. With the rapid development and increasing use of technologies to record large amounts of neural activity, it will require sophisticated analysis methods to deal with such high dimensional data.

1.5 Machine Learning: Data-Driven Method for Neural Network Discovery

Machine learning, first conceptualized in 1959, has become a rapidly growing field in recent years. With applications in all aspects of basic and applied science, machine learning has proven to be incredibly useful in neuroscience, particularly in the analysis of high-dimensional data. Through the use algorithms that can learn from and make predictions based on pattern recognition, machine learning has high potential in the field of systems neuroscience. Supervised machine learning uses data that has known labels and has the goal of learn the relationship between the input data and the provided labels such that the computer can predict the label of a previously unseen data item with accuracy comparable with a human expert (Vu et al, 2018).

While there is mounting evidence that much of the computations involving negative systems are located in the mPFC, vHipp, AMY and their subregions, we chose to take an unbiased, data-driven approach to learning the functional brain network organization underlying anxiety-related behaviors. We hypothesize that the anxiety signal requires a brain-wide network representation. To study brain networks, we use

implanted microwire arrays to record LFPs in awake, freely-behaving mice as they explore the classic anxiety tasks. The electrodes target key brain regions that have been implicated in neural computations associated with anxiety. We then employed a supervised machine learning technique to discover relevant neural dynamics that are shared between the EPM and OFT anxiety tasks as well as under the influence of an anxiogenic drug. This methodology has been used to uncover patterns of rhythmic activity across limbic networks that are correlated with aspects of emotional behaviors (Cunniff et al. 2020; Hultman et al. 2016).

1.6 Experimental Aims

In Chapter 2, we collect LFP data from mice that display anxiety-related behaviors in the EPM, OFT and following acute administration of Fluoxetine (anxiogenic). We then use dCSFA-NMF in each task to in hopes of discovering a network whose activity is correlated with anxiety-related behaviors in each task. Further, we test whether these individual networks generalize to the other tasks for a global anxiety network. In chapter 3, we utilize a multi-task learning scheme in which we combine these 3 datasets to discover a composite network that has the goal of generalizability built into the model. In chapter 4, we aim to validate the composite model using a clinically relevant mouse model of MDD, chronic unpredictable stress, to test how these discovered networks are then modulated in the diseased state.

Together, these studies aim to discover a behaviorally relevant network whose activity is correlated with anxiety-related behaviors in classic ethological tasks. More importantly, this work is a strong example of an approachable data-driven methodology that can be utilized in systems neuroscience and further, adapted across species.

2. Training a Network using Individual Anxiety Tasks is Insufficient in Generalizing to Broader Anxiety Contexts

In this chapter, I characterize a data-driven method for discovering brain networks using the elevated plus maze and the open field behavioral tests. Additionally, we utilized an administration of fluoxetine as an anxiogenic agent to cause an anxious state.

2.1 Introduction

To discover the network architecture that is correlated with anxiety-related behavior, we first explored the approach-avoidance conflict in the EPM and OFT. First, implant a cohort of mice with electrode arrays in key brain regions associated with anxiety-related in humans and rodents. These brain regions are shown in **Table 1**. We then trained 3 separate networks (Homecage vs EPM; Homecage vs OFT; and Saline vs. FLX) in hopes of identifying a network that decoded anxiety behaviors within their parent datasets but also possessed network activity that generalizes to the other anxiety tasks. Since each dataset is known to assay anxiety in mice, it is reasonable to assume that the dCSFA-NMF model will discover a brain state that is active in the remaining scenarios. However, as explored in a recent review from the Dzirasa Lab, these machine learning methods may be sensitive to unidentified variables from the training dataset (Walder et al. 2022). Experimental variables such as subject or object novelty, olfactory stimuli, spatial location, and subject velocity can lead to confounding interpretations of

behavioral states. Despite these potential drawbacks, we hypothesize that a global anxiety network can be identified using one of the experimental datasets (EPM, OFT, or FLX).

Table 1: Stereotactic coordinates of the relevant brain regions assayed in this study. Coordinates are all measured from bregma.

	A/P	M/L	D/V
Amygdala (AMY)	-1.4	2.9	-3.85
Nucleus Accumbens (NAc)	1.3	2.25	-4.1
Mediodorsal Thalamus (MDThal)	-1.58	0.3	-2.88
Ventral Tegmental Area (VTA)	-3.5	± 0.25	-4.25
Medial Prefrontal Cortex (mPFC)	1.62	± 0.25	-2.25
Ventral Hippocampus (vHipp)	-3.3	3.0	-3.75

2.2 Important Computational Considerations of Anxiety Tasks

Predicting behavior in the EPM and OFT can be done by first conceptualizing the different regions of interest along with the accompanying anxiogenic stimuli in each task. In the OFT, shown in **Figure 1**, the most anxious animals will spend a majority, if not all, of their time exploring the periphery of the task and completely avoiding the center region. Conversely, the least anxious mice will fully explore the center region with no obvious preference for the center or periphery. This can be said similarly of behavior in the EPM, with the most anxious mice confining their exploration to the closed arms and the least anxious fully exploring the task with no apparent preference. Machine learning applications are best used to discover differences between extremes in the underlying signal – meaning, to distinguish states of high anxiety vs states of low

anxiety. For the purpose of capturing an anxiety network that is relevant to these scenarios, it is best to train an algorithm that can detect the differences in a rodent's brain state between an anxiety-related task (positive class) versus a neutral setting (negative class). We chose our negative class to be a 5-minute recording within the mouse's familiar homecage environment, with the assumption that the animal is either ambivalent or has a positively valence association with the homecage.

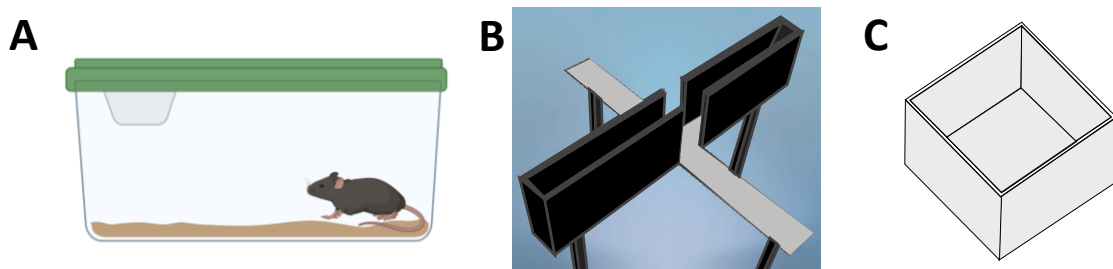


Figure 1. Various contexts to assay anxiety-related behaviors. (A) Familiar homecage environment is the non-anxious state. (B) Classic elevated plus maze (EPM). (C) Classic Open Field Test

The discovered networks must both explain observed patterns in the neural activity data and relate to a specific 'outcome', such as the anxious behavior (Talbot et al. 2020). This framework allows for us to assign the environment of 'Task' (OFT or EPM) without restricting our anxiety state to a particular region of the maze. This distinction is important for 2 reasons: 1) this would be a counter intuitive scenario as only the animals with the least trait anxiety, and the lowest anxiety signal, would be relevant in training our model and 2) we would lose a large amount of relevant data as most animals spend less than 15% of their time exploring the anxiogenic regions of the respective mazes.

2.3 Capturing the Approach-Avoidance Conflict in the EPM and OFT

Rodents will innately avoid the bright lights and open spaces during its exploration of a novel environment. When placed in the EPM, mice spend most of the allotted time within the closed arms and will avoid the exposed environment of the open arm. As expected, rodents spend more time exploring the walled periphery of the open field and avoid its exposed center. The light intensity of the room is an important variable in most free exploration tasks, as it can be used as anxiogenic stimulus and titrate the anxious behaviors (Arrant et al. 2013, Walf). We used 175 and 125 lux in the EPM and OFT, respectively. These light intensities were used to ensure that mice would still explore the open arms and the center region of these tasks. A representative tracing of the behavior is shown in **Figure 2**.

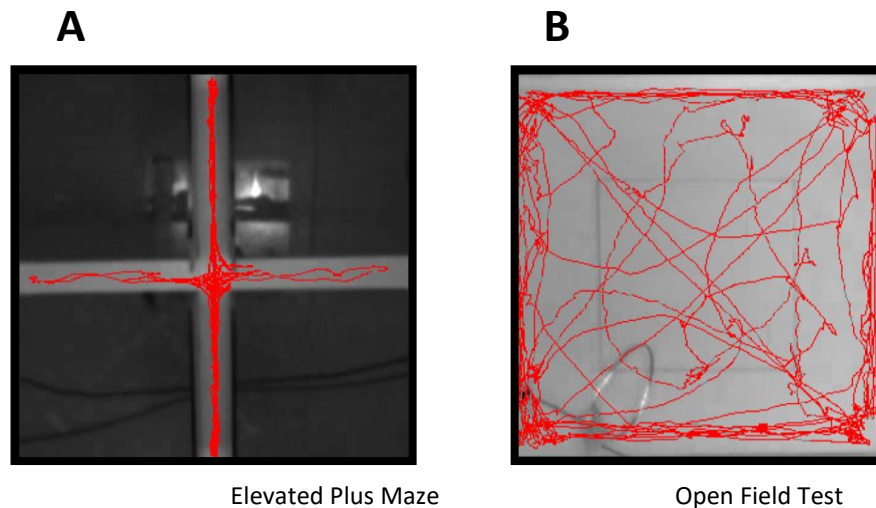


Figure 2. (A) Mice exposed to the EPM spend a majority of tis time within the closed arms of the maze (top and bottom arms) while spending significantly less time in the open arms (left and right). (B) Mice placed in the OFT will spend much less time in the center of the maze as compared to the periphery. Plots of the animal location were analyzed using Ethovision analysis software

2.4 Acute Administration of Fluoxetine is Anxiogenic in the EPM and OFT Assays

There are a wide variety of have clinical psychiatric drugs that have anxiety as a side effect. Stimulants, such as amphetamines and caffeine have been known to exacerbate anxiety in patients suffering from panic disorder (Paz-Graniel et al. 2022). Fluoxetine (Prozac), is a selective serotonin reuptake inhibitor (SSRI) and a widely used antidepressant. Chronic administration (6-8 weeks) of fluoxetine has been shown to decrease depressive and anxiety symptoms in humans; however, children and young adults have been shown to have an increase in depression and anxiety symptoms within the first few weeks of use (Perez-Caballero et al. 2014). These effects have been

extensively studied in rodent models and exhibit predictive validity in anxiety tasks – showing increased avoidance of anxiogenic regions in the EPM and OFT (Ravinder et al. 2011, Marcinkiewicz et al. 2016).

We used an acute administration of fluoxetine as an anxiogenic agent in male C57 mice. Prior to collecting neurophysiological recordings for our dCSFA-NMF analysis, we first piloted using a 20mg/kg IP injection and observed the behavior in the EPM. Mice were randomly assigned to receiving an injection of either saline or fluoxetine (FLX). Following their injection, they were housed in a familiar homecage environment for 30 minutes prior to their exposure to the EPM. As shown in **Figure 3**, mice exhibited an increase in all forms of anxiety-related behaviors (decreased entrances into the open arms, increased latency to first entrance into the open arms, and decreased overall duration spent in the open arms). Additionally, we did not record any alterations in locomotor activity between the saline-treated and fluoxetine-treated mice.

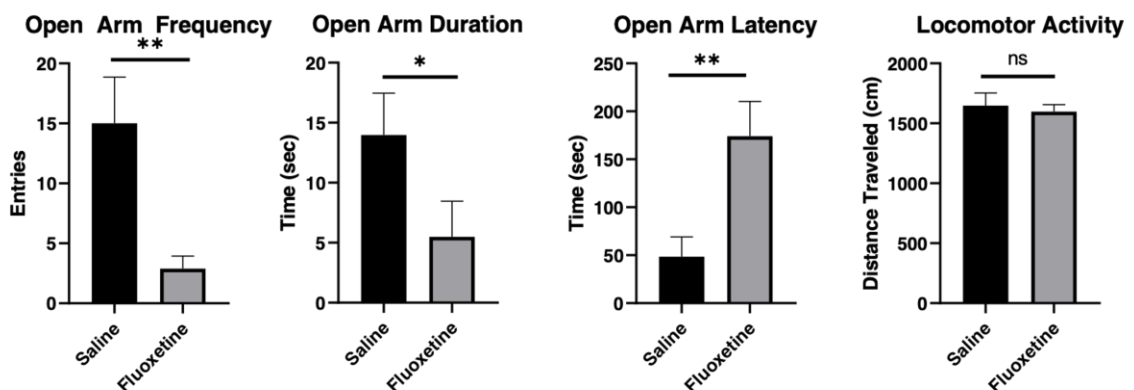


Figure 3. Acute fluoxetine administration increases anxiety-related behavior in the EPM. I.P injection of saline or fluoxetine (20 mg/kg) was given 30 minutes prior to EPM exposure. * = $p < 0.05$; $n = 20$, balanced groups.

2.5 Using dCSFA-NMF to Discover a Network that Correlates to Anxiety-Related Behaviors in the EPM and OFT

To discover the network representation of an underlying anxiety brain state, we performed multisite electrical recordings while mice were exploring the EPM and OFT tasks. Following a 5-minute homecage recording, implanted animals were placed in the OFT for 5 minutes. The location of the experimental mouse was tracked throughout the recording to allow for offline behavioral labeling (location, velocity, and region of interest (center/periphery)). As expected from the behavioral results in **Figure 2**, mice spent substantially more time along the periphery of the OFT than in the center of the task. We collected nearly 175 minutes of data in the OFT from a total of 35 male C57 mice.

A similar procedure was followed for our neurophysiological recordings in the EPM. Implanted animals were allowed to explore their familiar homecage environment for 5 minutes prior to being placed into the EPM. Behavior was video recorded for behavioral labeling from an overhead camera. Light intensity in the room was adjusted to 175 lux (recorded from the ends of both open arms) by two overhead lights. Similar to the OFT animals, mice exhibited the approach-avoidance conflict in the EPM as they spent a majority of the recording time exploring the closed arms of the EPM and the

avoiding the open arms. We collected roughly 230 minutes of data in the EPM from a total of 45 male C57 mice.

dCSFA-NMF is a supervised machine learning approach designed to be both descriptive (discovers brain activity measures that are integrated across seconds of time) and predictive (discovers networked patterns of brain activity that encode external behavioral variables) (Talbot et al., 2020). To retain biological interpretability in the network structure, we focused the analysis on specific features of LFPs: Power (strength of oscillatory amplitudes across 1-56Hz frequencies), LFP synchrony (a measure of coordinated activity between 2 LFPs) and LFP Granger synchrony/directionality (estimation of information transfer direction between 2 LFP signals). In addition to providing the architecture of the anxious brain state, the dCSFA-NMF model yields an activity score for the discovered network, which indicates the strength at which that network is represented during each one-second segment of LFP (Talbot et al. 2020, Mague et al. 2022). This is particularly useful in studying anxious behaviors as it is difficult to intuit how the anxious brain state will modulate behavior when freely exploring a task. A schematic of the dCSFA-NMF model is shown in **Figure 4**.

Local field potentials are synched with the behavioral labels in the video recordings and sectioned into 1-sec bins. The signals are then represented by their LFP features (power, coherence, and granger causality) at the second timescale before an encoder maps these features to a lower dimensional latent space. These latent spaces are

then set to reconstruct the original data by the decoder with the objective to minimize the reconstructive loss. Once the parameters that reliably minimize reconstruction are set, the AUC (Area Under the Curve) of the Receiver Operator Characteristic (ROC) is determined based on the ability to predict anxious vs non-anxious using logistic regression.

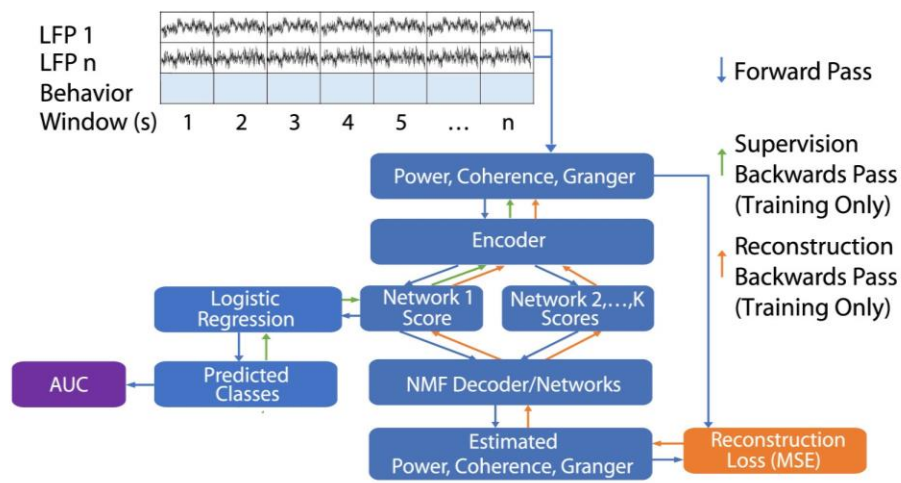


Figure 4. Schematic of the dCSFA-NMF machine learning model used to discover an anxiety brain state.

Since the dCSFA-NMF framework is a supervised model, the behavioral labels were the 5-minute homecage recording for our negative (non-anxious) class and the 5 minute in-task (EPM or OFT) label for our positive (anxious) class. We split the data into a training set and validation set using roughly 60% and 10% of our mice, respectively. Having this split allowed us to first train a model using most of our data and evaluating its performance on a much smaller selection of the data. 30% of the data was completely

withheld from the analysis and saved for a final test set. The goal of our network discovery is two-fold: 1) to identify a brain state in our training set that generalizes in new subjects in the same task (Validation set) and 2) to assess if our individual brain states generalize to the other anxiety task.

2.6 Using dCSFA-NMF to Discover an Ectopic Anxiety Signal Using Acute Administration of Fluoxetine

To capture the anxiety network associated with acute fluoxetine administration, we designed an experiment that followed the case-crossover design, commonly used in pharmacoepidemiology (Delaney et al. 2009). Each mouse would receive an injection of saline and fluoxetine, separated by a 2-week washout period **Figure 5**. This experimental design allows for within-subject comparisons to be made. Mice were randomly assigned to the saline treatment group or the fluoxetine treatment group and a 5-minute homecage recording was collected prior to receiving the assigned drug treatment. Drug recordings all occurred within their familiar homecage environment for one hour. As seen in **Figure 3**, mice who received fluoxetine will produce a behavioral phenotype that resembled high anxiety 30 minutes post injection. By recording for an hour, we intended to use the activity scores from our supervised network to record the emergence of the anxiety brain state. In the dCSFA-NMF model, we assigned the fluoxetine case as our

positive (anxious) class and the saline case as our negative (non-anxious) class. We collected over 1000 minutes of data from a total of 10 male C57 mice.

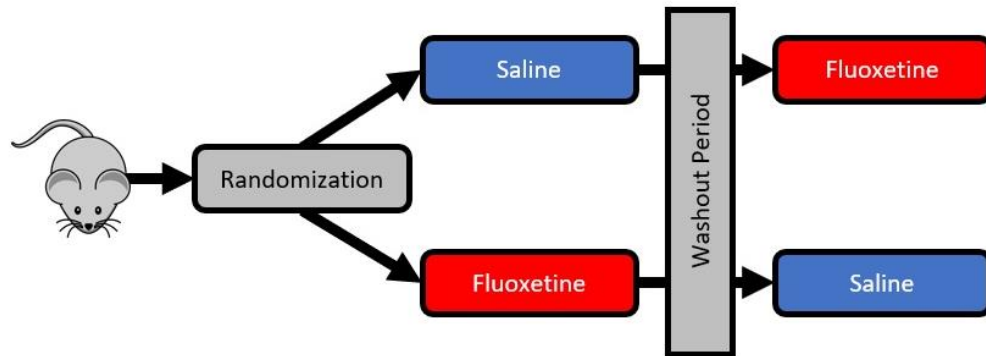


Figure 5. Crossover drug design used classically used in pharmacoepidemiology studies. Mice were randomly assigned to receiving either a saline or fluoxetine injection prior to electrophysiology recording. A 2-week washout period was chosen to ensure that FLX was completely eliminated from the bloodstream.

2.7 Individually Trained “Anxiety” Networks Do Not Generalize Across Tasks

Our goal was to discover a network that encoded anxiety in mice. If correct, the network activity should reflect the following scenarios: 1) whether the mouse is present in its homecage or within an anxiety task (EPM or OFT), 2) distinguish whether an animal received an injection of saline or fluoxetine, 3) the network activity should reflect anxiety-related behaviors on an individual mouse basis and 4) the discovered network should generalize to other anxiety tasks. To achieve these criteria, we trained 3 separate models: an EPM network (homecage vs EPM), an OFT network (homecage vs OFT) and

a fluoxetine network (saline vs fluoxetine). A flowchart of this process is shown in **Figure 6**.

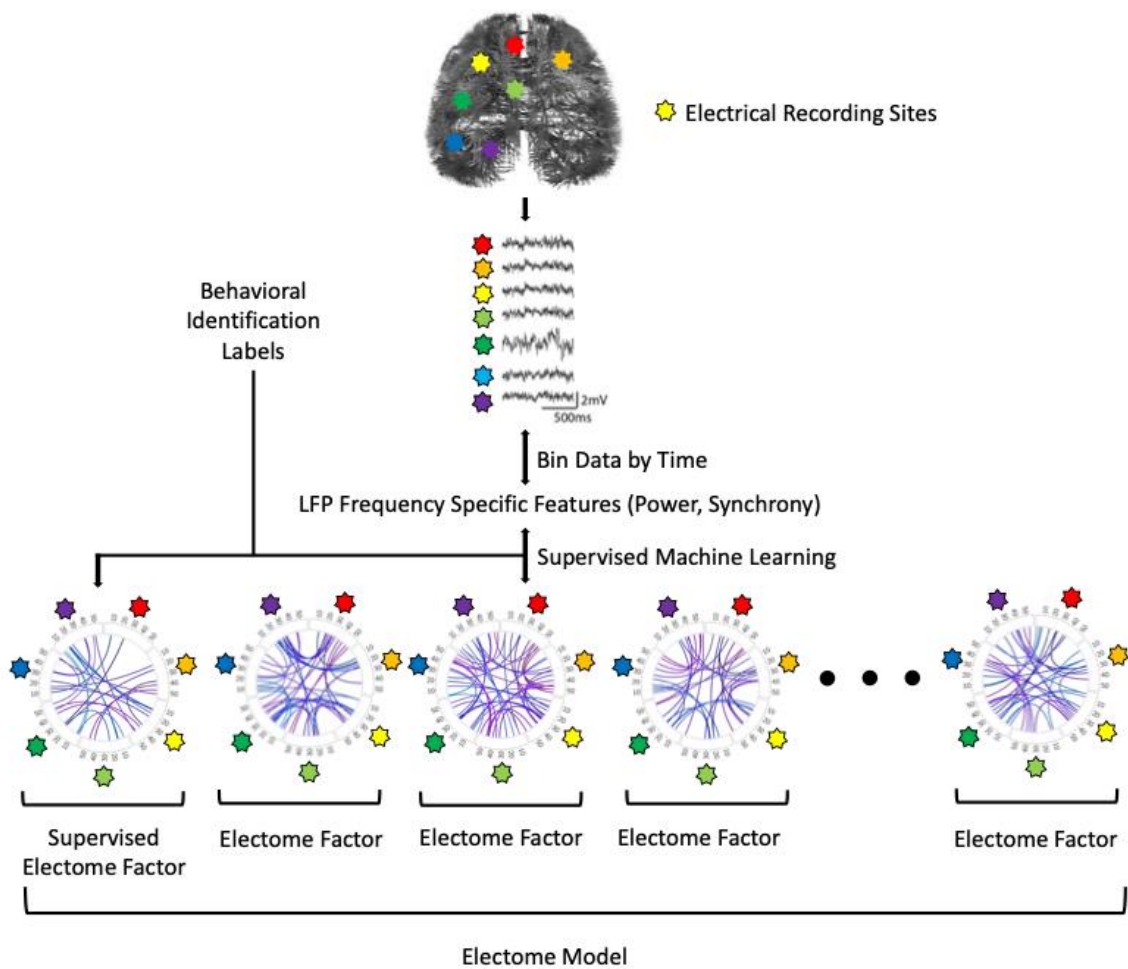








Figure 6. Simplified LFP recordings from regions implanted are collected and separated into time windows. LFP frequency specific features are calculated for each time window. Behavioral scoring identifies the relevant windows and are binned together according to the relevant labels. dCSFA-NMF the supervised machine

learning algorithm, is applied to the data to identify both the supervised network factors and the unsupervised networks.

ROC determines the performance of the model by determining the tradeoff between the true-positive rate versus the false-positive rate. Area under the ROC curve (AUC) summarizes model performance – results larger than 0.5 indicates the model has identified a network related to the behavioral classes. As shown in **Table 2**, each network trained received a near perfect training AUC close to 1.0. Additionally, when predicting window labels within the validation set (30% of the collected data), each individual model did exceptionally well and can distinguish the positive class from the negative class in the validation set within the same experiment (shown in the shaded boxes along the diagonal).

Table 2: Performance of the individually trained EPM, OFT and FLX networks. Each network performed well above chance within their validation sets. The EPM and OFT networks can distinguish between the positive and negative classes above chance while there identified dynamics that project into the FLX dataset. None of the individually trained networks generalized across all three of the datasets

	Projection Dataset		
	EPM (HC v Task)	OFT (HC v Task)	FLX (Sal v FLX)
EPM Network 	AUC = 0.81 ± 0.03	AUC = 0.67 ± 0.02	AUC = 0.49 ± 0.04
	Projection Dataset		
	EPM (HC v Task)	OFT (HC v Task)	FLX (Sal v FLX)
OFT Network 	AUC = 0.56 ± 0.01	AUC = 0.84 ± 0.06	AUC = 0.48 ± 0.02
	Projection Dataset		
	EPM (HC v Task)	OFT (HC v Task)	FLX (Sal v FLX)
FLX Network 	AUC = 0.5 ± 0.02	AUC = 0.5 ± 0.01	AUC = 0.65

Our next goal is to test for generalizability from these three networks. With the use of ‘out of training subjects’ in a different paradigm, we inquire whether the brain state in question can discern between the positive and negative class of another paradigm. In other words, can the trained EPM network (row 1 of **Table 2**) predict when an animal is exploring its homecage (negative class) or the OFT (positive class)? Can it discern between saline and fluoxetine in the FLX dataset? With the goal of discovering a global anxiety dataset, we assert that these conditions must be met. Surprisingly, both the EPM and OFT networks perform well in generalizing between the networks (EPM Model \rightarrow OFT dataset, AUC = 0.67) and (OFT Model \rightarrow EPM dataset, AUC = 0.56). With the goal of performing above chance (0.5), it is reasonable to assume that there are shared dynamics in these exploration-based tasks and that the supervised network identified by dCSFA-NMF was successful. However, both the EPM and OFT networks failed to generalize to the FLX experimental condition. The fluoxetine network, on the other hand, performs very well within its own experiment (Validation AUC = 0.65), yet fails to generalize into either the EPM or OFT tasks (AUC \sim 0.5). Due to the unconstrained training goal of saline vs. fluoxetine, the network could simply identify more prominent neural changes occurring under the influence of fluoxetine such as locomotion, sensation, sensation, etc.

By training individual models from the EPM, OFT and FLX experiments, we were unable to identify a global brain state that generalizes across all three anxiety-based scenarios.

3. Using a Multi-Task Learning Scheme to Train a Composite “Anxiety” Network

In this chapter, I characterize an alternative training methodology that leverages the data collected from the EPM, OFT and FLX datasets to discover a single composite network. This approach, known as multi-task learning, has been utilized in a multiple of machine learning applications due to its added benefit of driving generalization amongst its training sets. In order to evaluate the results of this technique, we retain the same validation and test sets as described in Chapter 2.

3.1 Introduction

The network discovery scheme outlined in Chapter 2 did not successfully fulfill our goals in discovering a global anxiety network as it failed to generalize across all anxiety tasks. Given the success of the dCSFA-NMF model in discovering a social appetitive network and a depression vulnerability network (Mague et al. 2022, Hultman et al. 2018), it is unlikely that the algorithm is unable to identify the dynamics shared across the EPM, OFT and fluoxetine administration. When utilizing machine learning approaches, it is important to choose a training method to better align with the

hypothesis being tested (Vu et al. 2018). We hypothesize that by applying a multi-task learning approach, we will discover an anxiety network that will generalize across the EPM, OFT and FLX datasets and correlate with anxiety-related behaviors within these tasks.

3.2 Model Training: Employing Multi-Task Learning to the EPM, OFT and FLX Datasets

The dCSFA-NMF model uses the features (power, coherence, and directionality) of the LFP signal from each brain region to train a network to classify between the supervised labels. This classification task is attempting to differentiate between 2 experimental conditions (anxious and non-anxious). In order to drive generalization between the EPM, OFT and FLX datasets, altered the input labels that are provided for the dCSFA-NMF algorithm. We combined all the positive label features (EPM, OFT and FLX) to create a single ‘anxious’ positive label. Similarly, we combined all the negative label features to create an aggregate ‘non-anxious’ negative label. We then used we trained a network that would classify between these two new behavioral conditions. A schematic of this approach is shown in **Figure 7**.

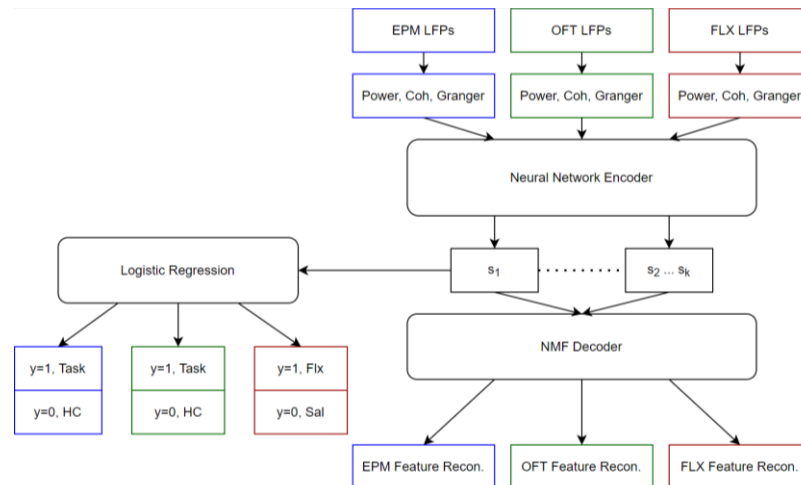



Figure 7. A flowchart of the multi-task learning scheme used to discover an anxiety network across 3 training datasets.

By sharing the same neural network encoder, the shared features across all three datasets undergo dimensionality reduction to make the data more biologically interpretable. The innovation of this method is that the scores are then passed through a logistic regression scheme that ensure the shared features can still classify the original classes from the individual tasks. In other words, the multitask learning algorithm drives the ability of the network to generalize by identifying the shared features that are inherent in all 3 datasets. Then, those shared features must also satisfy the condition of being able to distinguish homecage vs. EPM, homecage vs. OFT and saline vs fluoxetine.

3.3 Composite Network Successfully Generalizes Across Anxiety Tasks (Validation Sets)

We utilized multitask learning to force dCSFA-NMF to identify dynamics that were common between the EPM, OFT and FLX experiments. We tested whether the composite network we learned in the training set would generalize to the validation mice – a new set of mice that were not a part of model training. The performance of the network in the validation set is shown in **Table 3**. The validation AUCs indicate that our composite network performs well above chance across the three anxiety experiments.

Table 3: Performance of the trained composite network using the EPM, OFT and FLX datasets. The composite network performed well above chance for all three-training classification, as indicated by the validation AUCs. EPM Model (Training Set n = 21; Validation Set n = 5); OFT Model (Training Set n = 16; Validation Set n = 3); FLX Model (Training Set n = 6; Validation Set n = 1)


	Projection Dataset		
	EPM (HC v Task)	OFT (HC v Task)	FLX (Sal v FLX)
Composite Network 	Val AUC = 0.74 ± 0.02	Val AUC = 0.80 ± 0.06	Val AUC = 0.75

3.4 Composite Network Successfully Generalizes into Anxiety Test Sets

Since the composite network performed well in the validation set, we then evaluated its performance on the remaining mice in the test set. With the increase in sample size, it confirms that the composite network is activated in the EPM, OFT and FLX recordings **Table 4**. The network activity score, which indicates the strength of the network during each one-second LFP window, illustrates that the convergent brain state

holds the same correlation across each dataset **Figure 8**. Each positive class successfully has a higher network activity score than the corresponding negative class.

Table 4: Performance of the trained composite network using the EPM, OFT and FLX datasets. Discriminatory performance is retained in the test sets. EPM projection (Test Set n = 19); OFT projection (Test Set n = 16); FLX projection (Test Set = 2)

	Projection Dataset		
	EPM (HC v Task)	OFT (HC v Task)	FLX (Sal v FLX)
Composite Network 	AUC = 0.73 ± 0.02	AUC = 0.79 ± 0.02	AUC = 0.62 ± 0.11

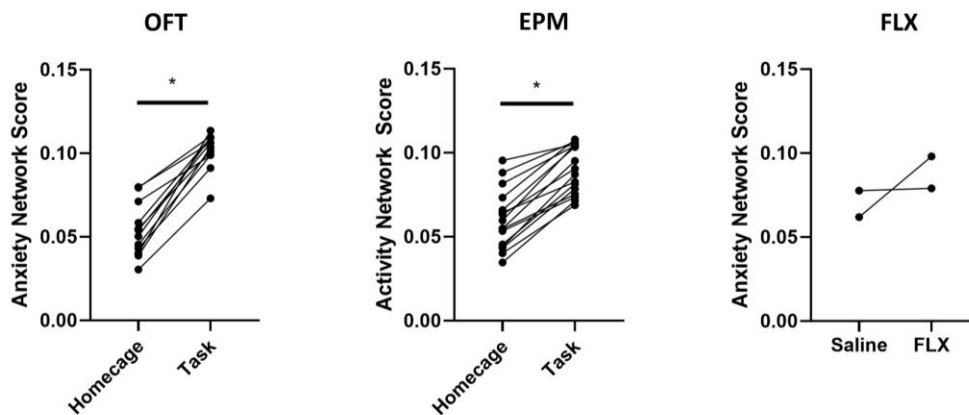


Figure 8. Composite network score from the test sets have the same correlations across all experiments anxiety tasks. * indicates $p < 0.05$. One-Tailed, Non-Parametric Wilcoxon Rank-Sum Test (Paired). EPM projection (Test Set n = 19); OFT projection (n = 16); FLX projection (n = 2)

3.5 Composite Network Activity is Associated with Anxiety-Related Behaviors in the EPM and OFT

As indicated by AUC values above 0.5, the discovered composite network using the multitask learning scheme converges across the EPM, OFT and FLX experiments. While this is exciting, it is important to evaluate the behavioral relevance of the network. The dCSFA-NMF framework will identify any neural dynamics that are shared amongst the datasets and correspond to the behavioral labels provided (anxious and non-anxious). However, the network could easily identify aspects of these behavioral tasks that are evident from the environment and not the emotional state of the mouse; these include: environmental novelty, changes in light intensity, increased locomotion, etc. In fact, it is more likely that the factors explaining most of the variability are commonly not the most predictive of an outcome (Jolliffe, 1982).

First, we wanted to exclude that this network's performance is based on mouse velocity. Local field potential activity is sensitive to changes in velocity, particularly hippocampus activity in the theta range (Buzsaki, 2002). We regressed the composite network performance in both the EPM and OFT to the corresponding average velocity per mouse. The network AUC is not correlated to locomotor activity in the exploratory tasks **Figure 9**.

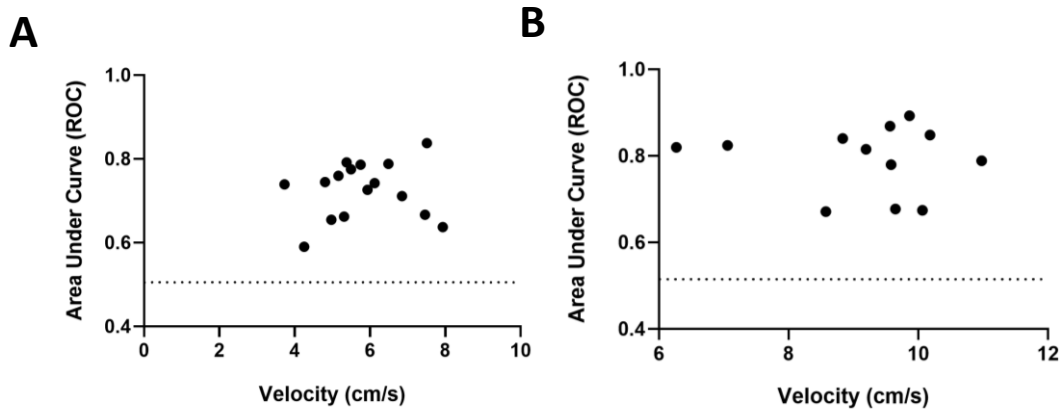


Figure 9. Network performance is not due to locomotor activity in the (A) EPM and (B) OFT tasks. Average velocity (cm/s) was obtained following behavioral analysis in Ethovision. Spearman correlation: (A) $p = 0.2931$; $n = 16$. (B) $p = 0.4955$; $n = 12$.

Once locomotor activity was ruled out, we investigated whether network performance in each task was correlated with anxiety-related behavior on the individual mouse basis. Extending this approach, we regressed against frequency of entrances into the anxiogenic regions of the mazes (open arm/center square) as well as duration of time spent in those regions **Figure 10**.

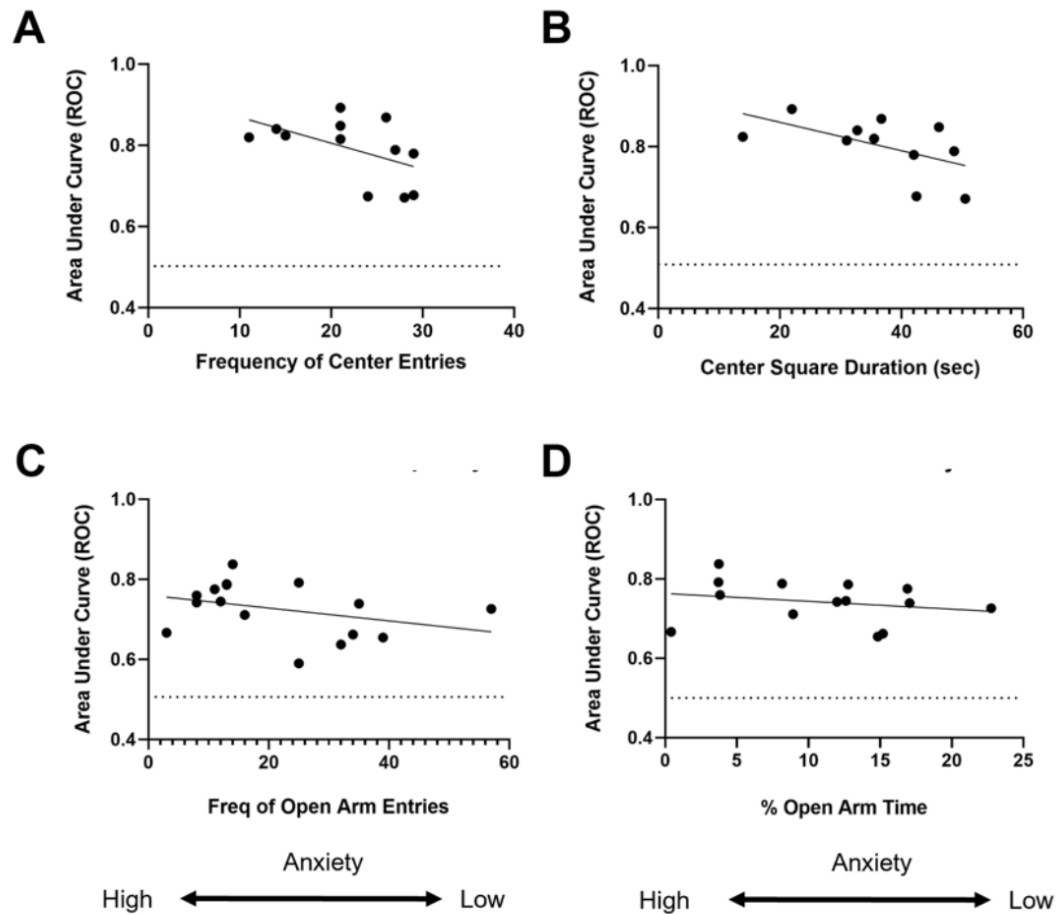


Figure 10. Network performance is correlated with various anxiety-related behaviors in the OFT and EPM tasks. In both the EPM and OFT, the network achieves a higher AUC for the more anxious animal as evident by their behavior (relative scale is included at the bottom). (A) Decoding AUCs per mouse is correlated with its frequency of entering the center region in the OFT; $p = 0.0288$; $n = 12$. (B) AUCs per mouse is correlated with time spent in the center region; $p = 0.0409$; $n = 12$. (C) AUCs per mouse strongly trends with frequency of open arm entries; $p = 0.0675$; $n = 16$; (D) AUCs per mouse strongly trends with open arm time; $p = 0.0975$; $n = 15$. Non-parametric Spearman correlation

To obtain a more granular perspective of the network utilization during the tasks, we identified the average network scores within the safe and anxiogenic regions

of the maze. Interestingly, not all mice displayed an increased network score within the open arms of the EPM or the center square of the OFT (**Figure 11**). If the activity of the composite network somehow reflects the subjective anxiety experienced within the EPM and OFT, the difference in network score between the safe and anxiogenic zone of the maze may dictate the overall anxiety-related behaviors in the task.

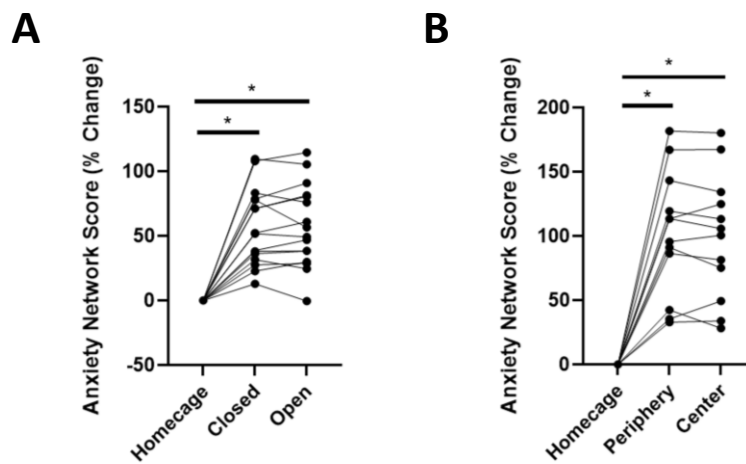


Figure 11F. (A) Network activity during homecage and EPM recordings (n = 19) (B) Network activity during homecage and OFT recordings (n = 16). * = p<0.05 using Friedman's test, followed by two-tailed sign-rank test.

Critically, the discriminatory strength of the composite network was correlated with time spent in the anxiogenic zone of the EPM and OFT. This suggests that our multi-task learning strategy successfully biases dCSFA-NMF to learn a network that integrates anxiety to learn a network (**Figure 12**). These correlations are a window into where the animals are experiencing higher anxiety.

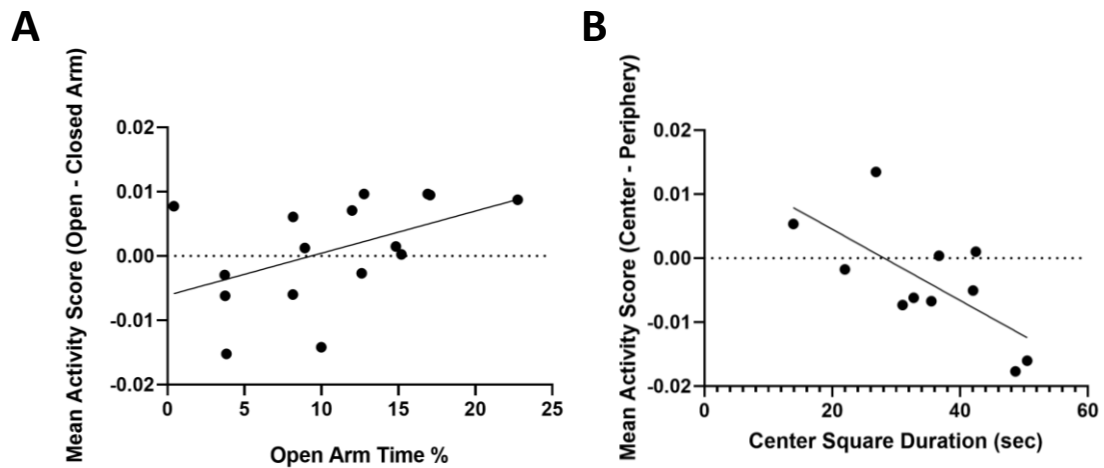


Figure 12. Differences in network activity within the ‘safe’ and anxiogenic zones of each task is correlated with the time spent in the anxiogenic regions of the maze. (A) Correlations observed in the EPM. The most anxious animals have a higher network score in the closed arm instead of the open arm. $p = 0.0085$; (B) Correlations observed in the OFT. The most anxious animals have a higher network score in the periphery of the task. $p = 0.0844$; Non-Parametric Spearman correlation, one-tailed. **3.6 Network Activity Scores Can be Used for Tracing the Anxiety Brain State in FLX Drug Recording Sessions**

To further interrogate the results of the FLX projection, we can plot the composite network activity score for each 1 second from the drug recording test set. Interestingly, both the saline and fluoxetine network scores diverge the saline scores decrease over time (habituation) and the fluoxetine scores increase over time (drug effect). Tracing is shown in **Figure 13**. Following the acute fluoxetine administration, mice are generally placed in their homecage for 30 minutes prior to being tested in the EPM or OFT. After 30 minutes, 1800 sec, the difference between the network scores is roughly maximum. This ectopic anxiety signal may drive the increased anxiety-related behaviors seen in **Figure 3**.

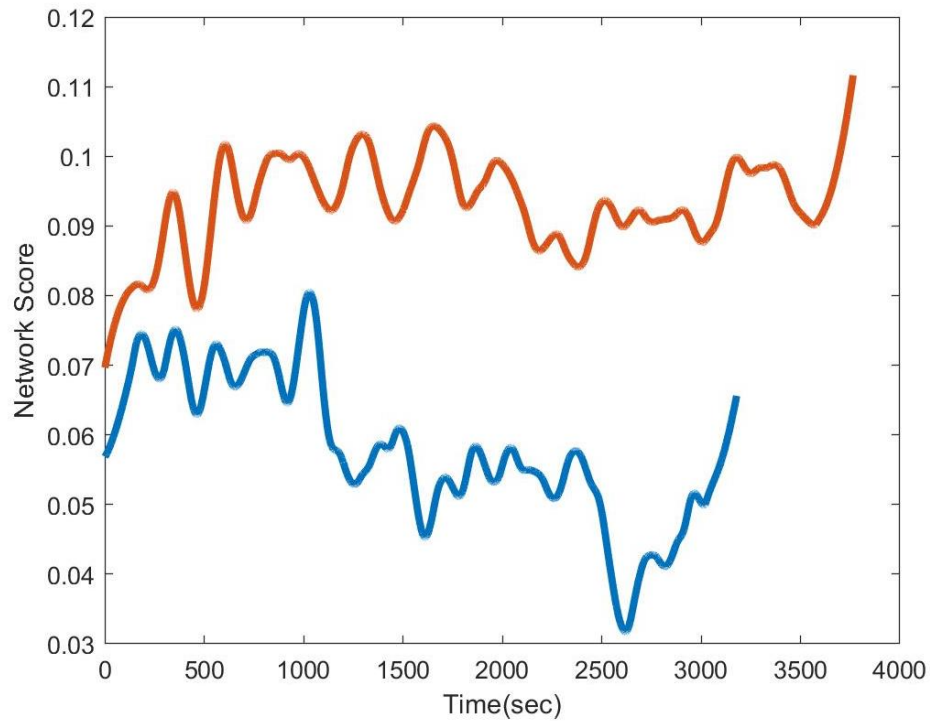


Figure 13. Representative plot of the saline and fluoxetine tracings from a test set mouse. Fluoxetine (red) steadily increases in the beginning of the recording until it levels off just after 1000 sec. Saline (blue) doesn't begin to decrease until after 1200 seconds.

After finding that the composite network reflects an anxious brain state in that generalizes across 3 separate anxiety tasks, we explored the spatiotemporal dynamics that make up the supervised network. The anxiety network mapped to LFP theta (3-12 Hz) power across all recorded brain regions. Additionally, the network also included distinct theta synchrony across all implanted brain regions. The architecture of the discovered brain state is mainly confined to the low frequencies. Power, Coherence and Directionality features are shown in **Figure 13** and **Figure 14**.

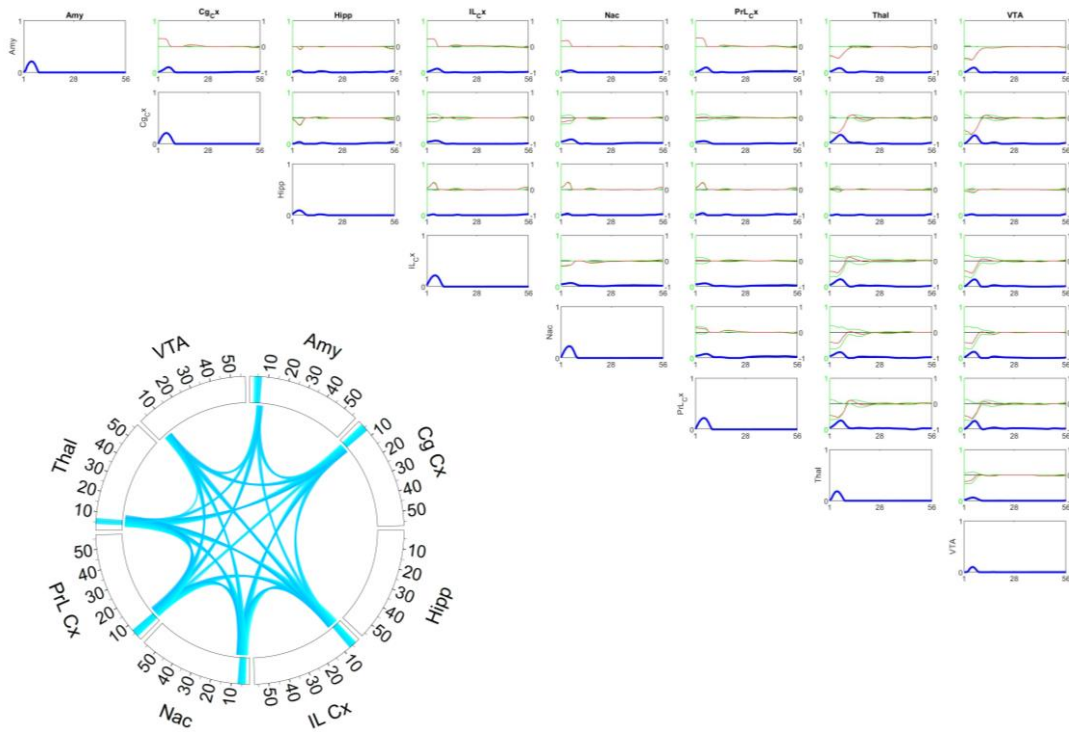


Figure 14. Power and synchrony measure that define the anxiety brain state. Brain areas are shown along the top and left, identifying power and synchrony contributions for the supervised network. Amplitude values reflect the relative contribution of the LFP spectral energy observed at each frequency. The offset between the two non-normalized synchrony functions for each brain pair ($A \rightarrow B$ and $B \rightarrow A$) are shown in green with the true signal in red. Positive values correspond to frequency at which the area listed along the top leads the area listed on the left. Negative spectral offsets correspond to the frequencies at which the area listed on the left leads the area listed on the top. The circle plot is a graphical summary in which the outside 'wheel' represents the significant power features at various frequencies denoted by the brain region. The 'spokes' connecting those power features are the significant pair-wise coherence measurement between regions.

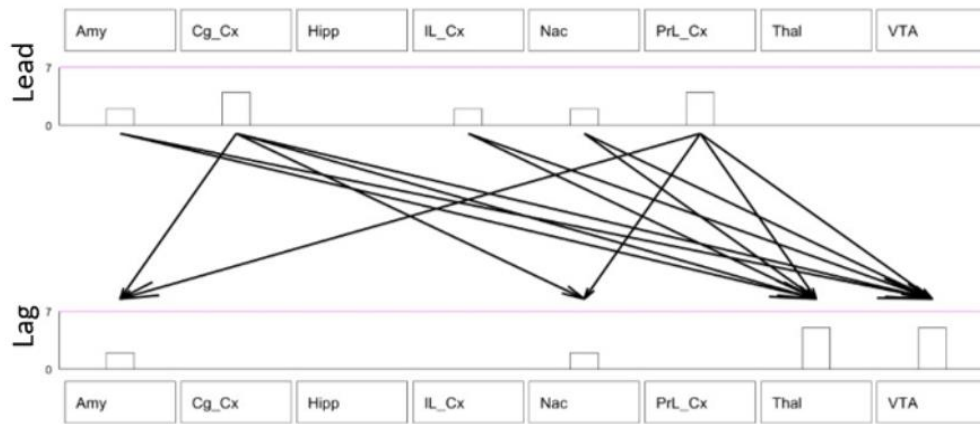


Figure 15. Granger offset measures were used to quantify directionality within the anxiety network. Directionality was observed across the theta frequency (3 – 12 Hz) with a spectral density threshold of 0.01.

4. Anxiety Network Predicts Prior Stress Exposure in both Familiar and Task Conditions

In this chapter, I demonstrate how the discovered composite network can be used to predict trait anxiety in a validated preclinical model of major depressive disorder, chronic unpredictable stress. We hypothesize that our composite anxiety network will show increased network activity in an anxiety task when compared to the age-matched controls.

4.1 Chronic Unpredictable Stress Induces an Anxious Phenotype

Chronic unpredictable stress (CUS) is a widely validated preclinical model used to quantify the effect of chronic stress on behavior. In this paradigm, test mice are repeatedly exposed to 2 stressors per day, one occurring during the light phase of their circadian rhythm cycle and the other during the dark phase. The stressors are summarized in **Table 5**. Stressors were chosen according to a pseudo-random schedule with the caveat that no stressor was repeated on successive days. CUS lasts for 8 weeks and at the conclusion of the repeated stress exposure, test mice develop a behavioral syndrome that is reminiscent of Major Depressive Disorder (MDD): metabolic dysfunction, anhedonia- and increased anxiety-like behaviors (Monteiro et al. 2015). Taken together, the CUS paradigm provides a context to assay anxiety network activity post-stress.

Table 5: Aversive stressors used in the CUS protocol.

Chronic Unpredictable Stress	
Light Phase Stressor	Dark Phase Stressor
Cold Temperature Exposure (4°C) – 1 hr	Wet, Saturated Bedding
Physical Restraint Stress	Complete Bedding Removal
Circadian Rhythm Disruption, Lights Off – 12 hr	45° Cage Tilt
Equilibrium Disruption (Plate Shaker)	50% Decrease in Cage Size
Soiled Rat Cage Bedding	Strobe Light Exposure – 12 hr
	Access to Food and Water Removed
	Circadian Rhythm Disruption, Lights On – 12 hr

A total of 20 8-week old C57 male mice underwent electrode implantation surgeries with appropriate post-operative recovery. Mice were group housed with 5 mice to a cage and cages were randomly assigned to receiving the CUS paradigm or the control group. Control mice were housed in the mouse colony under normal conditions but were handled by the experimenters for daily to avoid a handling confound. Following the 8-week stress paradigm, mice were habituated to the experimental room prior to a 5-minute homecage recording and a 5-minute exploration of the EPM. A total of 12 animals (6 mice per group, balanced) were tested in the EPM. Animal loss occurred during the post-operative period and several mice were excluded from analysis due to poor electrode signal.

Mice that underwent the CUS stress paradigm exhibited an increase in anxiety-related behaviors in the EPM. CUS animals displayed a marked decrease in time spent in the open arms, a decrease in open arm entries and a decrease in overall locomotor

activity when compared to the control group. No statistical differences were seen in the latency to entering the open arm. Results are summarized in **Figure 15**.

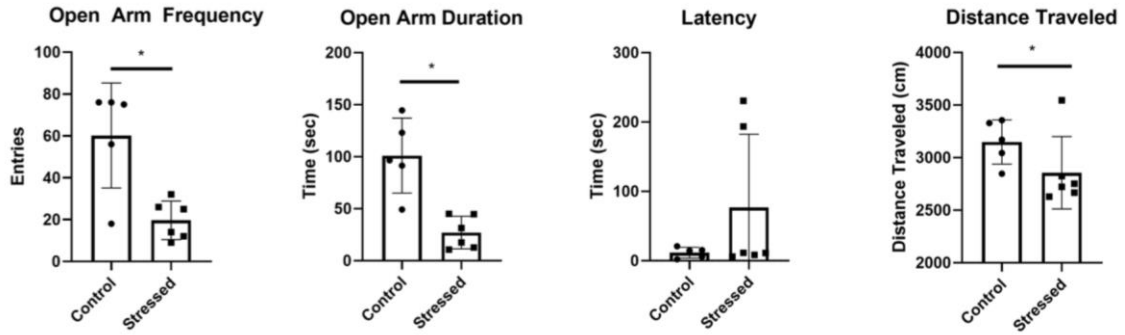


Figure 16. Chronic unpredictable stress causes an increase in anxiety-related behaviors observed in the EPM. * indicates $p < 0.05$. One-Tailed, Non-Parametric Wilcoxon Rank-Sum Test

4.2 Network Utilization Predicts Prior Exposure to Unpredictable Stress

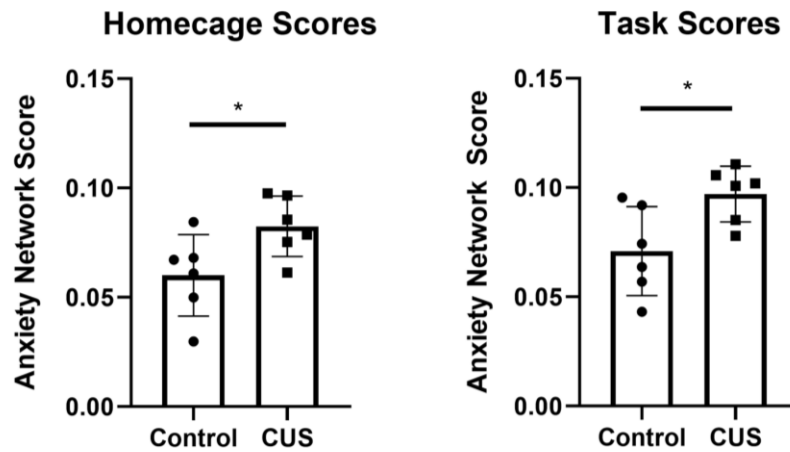


Figure 17. Mice that Underwent CUS have a higher anxiety network activity score when compared to age-matched controls.

5. Future Directions/Conclusions

Previous work in understanding the spatiotemporal dynamics of anxiety has included extensive work in dissecting hippocampal input to the medial prefrontal cortex. This circuit has been shown to have increased theta coherence when an animal is taken from a familiar environment and placed within the elevated plus maze. Further, increases in mPFC and vHipp theta power is present in each compartment of the open field and EPM when compared to the familiar arena (Adhikari et al. 2010). Using both optogenetic stimulation and inhibition, experimenters were able to demonstrate the utility of this projection as stimulation increases anxious behavior and inhibition decreases these behaviors in both the EPM and OFT tasks (Padilla-Coreano et al. 2019). These findings have been further replicated in various mouse models of disease.

Interestingly, theta coherence between the ventral hippocampus and medial prefrontal cortex was found to be necessary for working memory. By disrupting hippocampal input to the prefrontal cortex, rodents show deficits in a t-maze working memory task (delayed non-match-to-sample) (Sigurdsson et al. 2010; Spellman et al. 2015). Using a mouse model of the 22q11.2 microdeletion, a high penetrance risk allele for psychotic disorders in humans, mice also show dysfunctional communication between these brain regions as well as impaired behavior in the working memory task. Long-range hippocampal-prefrontal coherence demonstrates relevance across species in

rats (Wang et al. 2006), further establishing the very same neural activity as a biomarker of episodic memory (Eichenbaum, 2017).

This begs the question: *Is vHipp-mPFC theta coherence an underlying computation of anxiety or working memory?* Further work will be necessary to attempt to answer this pointed question; however, it cannot understate the clear evidence that supports the involvement of this circuit in these behaviors. Since the dorsal hippocampus is known for spatial representation of the environment, it is possible that communication between the ventral subregion and the prefrontal cortex creates a 'valence' map that is important for motivated movement, either for reward motivation (T-Maze context) and avoidant motivation (EPM context) (Bannerman et al. 2004).

Using an model for autism, *pogz*, researchers from the Sohal group further investigated the behavioral significance of the vHipp-mPFC circuit. Using a data driven method along, they found similar follow-up results that show the necessity of this projection. They then followed up with whole cell patch clamping to identify the cell types in the mPFC that are engage in exploratory and avoidance behavior. Pyramidal cells from the vHipp will have long range projections to either inhibitory PV interneurons or excitatory pyramidal neurons in the mPFC. The current hypothesis is vHipp projections have 2 distinct cell type targets: PV interneurons and pyramidal neurons. It is postulated that the mPFC PV interneurons are responsible for anxiety related behavior (evident by the EPM and OFT) while the mPFC pyramidal neurons are

responsible for exploration and working memory (observed in T-Maze). However, it must be stated that these findings have only occurred in models of autism and psychotic disorder and have yet to be replicated in WT mice. Causal manipulation of cell-type specific projections has yet to be established at this time.

The animal studies strongly implicate the vHipp-mPFC network in cognitive process and emotional regulation associated with psychiatric disorders such as schizophrenia, anxiety disorders, and PTSD (Jin et al. 2015). Physical or functional disruptions in the vHipp-mPFC circuit might be a form of pathophysiology that is common to many psychiatric disorders and may not be specific to anxious behaviors (**Figure 17**). Additionally, the circuit's susceptibility to stress and its relationship with the extended limbic system from the amygdala only strengthens involvement in the pathophysiology of disease. This further illustrates the difficulties in establishing a relevant link between human and rodent behavior, particularly in the context of disease, altered physiology is likely present across the entire brain.

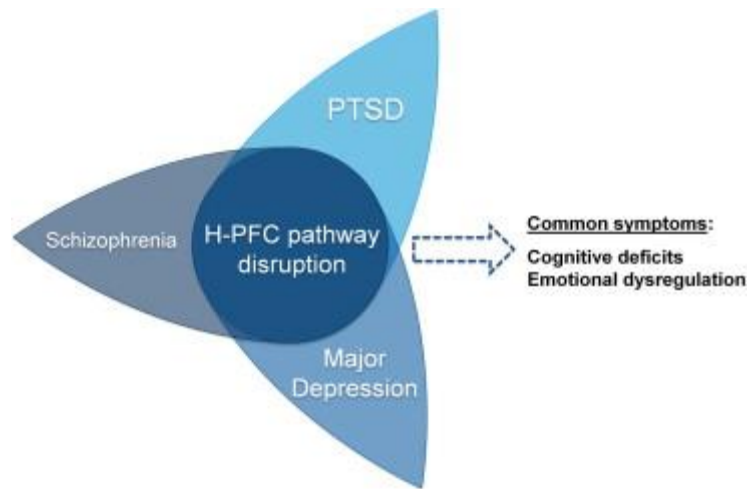


Figure 18. Proposed significance of the vHipp-mPFC pathway dysfunction in the context of disease. (Adapted from Godsil et al. 2013).

We assert that through the collection of large amounts of pertinent data and the use of un-biased analysis methodologies, we may gain traction in comprehending emotional behaviors and their underlying neural activity. This stance is based on mounting evidence in both systems neuroscience and data science in that the underlying neurodiversity of humans will require enormous amounts of emotional, cognitive, and resting state activity in various contexts, somewhere on the scale of thousands of individuals (Marek et al. 2022). Our initial findings suggest that the ventral hippocampus does not play a large role in anxious behavior is surprising given its relevance in the literature. We believe that this region's involvement decreases as we increase the number of anxiety-related tasks that are included in the model training. We propose that training solely on the EPM or OFT would illustrate the importance of the

hippocampus in these exploration-based contexts. The addition of the pharmacologically induced anxiety with acute fluoxetine administration into our multi-task learning approach effectively removes the exploration aspect relevant in ethological tasks; instead, identifying global dynamics that are shared amongst other anxiety-inducing scenarios. Furthermore, we would be able to ascertain the networks whose role are involved in the many top-down computations involved in anxious behaviors: stimulus detection, interpretation, response initiation, and finally, the observable response. It is quite possible that the global network that we discuss in this thesis play a role in only one of these computations.

Future research using this line of reasoning would be appropriate as there exists distinct categories of anxiety disorders, all with differing anxious stimuli (e.g. exploration-based ⊗ agoraphobia; experience-based/learned ⊗ PTSD & specific phobias) (Walz et al. 2016). However, it is important to emphasize the dangers of this line of reductive scientific reasoning as the DSM is based on syndromic classifications that are constantly being updated (Steimer et al. 2011). The diagnosis of psychiatric disorders will eventually include a more nuanced understanding of behaviors, comorbidities and underlying genetic and epigenetic factors which, in turn, will require analysis methodologies that can handle high dimensional data.

In this work, we identified a network representation that was associated with anxiety phenotypes in mice. That network showed higher maximum activation in

anxiety-related tasks when compared to the familiar homecage environment. Activity scores of the network is correlated with the anxiety behaviors elicited in the EPM and OFT tasks. Additionally, network performance can distinguish between animals that received an anxiogenic drug, fluoxetine, from those that received a saline injection. And lastly, not only does this network generalize out of experiment, but also can predict above chance whether an animal was exposed to CUS, a chronic stress protocol that has been shown to increase anxiety in C57 males.

It is important to emphasize that we have established an association between this brain state and anxiety phenotypes but have not yet established a causal relationship. Innovations such as Channelrhodopsin could lead to causal testing and elucidate the microcircuits involved in emotional processing, recognizing that anxiety requires the recruitment of a distributed array of interlinked neural circuits from across the brain (Calhoun et al. 2015). Future work could begin to clarify the causal role of the identified anxiety network by measuring whether disrupting communication between the identified brain regions and evaluating the impact on anxious behaviors.

This data-driven methodology has its merits in systems neuroscience. The resulting anxiety network that we have identified has converged on the dynamics identified in previous publications, namely the NAc \rightarrow VTA (Guangjuan et al. 2022; Dzirasa et al. 2011). Furthermore, the framework utilized in this work has the benefit of being hypothesis-driven and can be applied to answering scientific questions that have

high clinical relevance. Theta frequency band which has been shown to modulate behavior in exploration-based anxiety tasks. In our model, there is significant prefrontal output (Cg_Cx, PrL_Cx and IL_Cx) which has been of notable interest in anxiety-related tasks as well as cognitive flexibility.

The Dzirasa Lab is uniquely equipped to tackle questions regarding large-scale distributed network organization and behavioral output. The lab has introduced the concept of *Electome* networks a moniker for “the electrical connectome”. These networks currently are fully investigated brain state representations that have also been manipulated for causal validation. At this time, a social appetitive and stress vulnerability electome have been identified and rigorously test across many behaviors, drug effects, and genetic models. More work is required a fully establish an anxiety electome network that generalizes across new mice, experiments, and multiple disease models. However, successful completion of this goal could evaluate the relative importance of genetic/epigenetic factors and life events in determining increased or decreased vulnerability to anxiety disorders such as early life stress and pregnant stress models.

A recent study from the Pisapia group made the hypothesis that at the structural level, trait anxiety in humans may be related to volume alterations in limbic regions such as the amygdala, parahippocampal gyrus and cingulate regions. Trait anxiety, on the other hand, is a result of activity at the functional level, particularly in how these

regions communicate during decision-making tasks and as a compensatory response to cognitively demanding tasks (Saviola et al. 2020). The approach outlined in this thesis support the idea that anxiety is a complex phenomenon in which functional changes are taking place and upon further research can eventually advance our knowledge neural structure-function relationships that may be the key to fully understanding emotional regulation in health and disease.

To complete the project outlined in this thesis document, further evaluation of the composite network would need to be conducted. First, more animals would need to be implanted for electrode recordings of the fluoxetine administration. While we were able to identify network dynamics that achieved impressive out-of-sample prediction for our validation sets, a more balanced multitask learning scheme would be achieved with balanced datasets. Additional optimization of the network training would also be beneficial. Currently, the model is trained with 20 networks as it is the chosen default in first-pass application of these methods. There exists a number of networks that would optimally balance complexity (explaining the variance) with parsimony (a principle that the simplest representation of the behavior is the preferred representation). This was evident in the previously published social-appetitive network which only required 6 networks to strike that balance (Mague et al. 2022)

For the EPM, the analysis used in this thesis do not explore the center square. The center square of the EPM square is considered to be an ambiguous region of the task

(Walf et al. 2007) and most analysis of the EPM is constricted to locomotion in the open and closed arms. The elevated zero maze, another anxiety-related ethological task, was originally developed to remove the center square as a rodent engages in approach-avoidance conflicts (**Figure 18**). Analyzing the network scores in both contexts would shed light on the relevance of the center square of the EPM as well as validating our discovered anxiety brain state in another task.

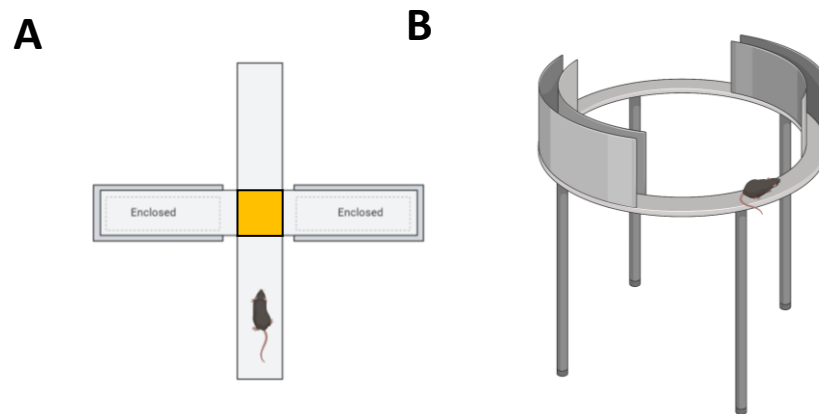


Figure 19. Schematics of the elevated plus maze and the elevated zero maze. (A) the analyses discussed do not interpret the center region of the EPM task (shown in yellow). (B) The use of the elevated zero maze as it omits the ambiguity of the center square.

To establish the composite anxiety network as an official *electome*, the use of chemogenetic or optogenetic manipulations to disrupt our network representation with behavioral read-out would further validate this contribution. We have collected data from a cohort of mice that have been implanted using our complete multisite recording electrodes as well as receiving optogenetic activation of ventral hippocampus terminals in the lateral hypothalamus (LH) (Jimenez et al. 2018). This projection has been shown to

alter behavior in the elevated plus maze and should influence the network functional connectivity when activated. While the initial analysis of our network does not have high dependence on the vHPC-LH circuit, we anticipate network-level side effects as perturbation of these cells can impact the activity of downstream neurons (Allen et al. 2015).

Lastly, exploring the utilization of our composite anxiety network in as many anxiety-related tasks and manipulations would not only validate the network but establish this data-driven approach. We have collected local field potential recordings from the same brain regions to project into the network space. The results of this network analysis would allow us to investigate the strength of our network across genotype and sex along with how it may drive the observed avoidance behaviors following environmental manipulations and with anxiolytic drug administration (**Table 6**).

Table 6. Outline of the experiments and their effects on anxiety related behaviors. These experiments have been collected and will be used to validate the network architecture proposed.

Experiment	Manipulation/Strength	Effect
EPM and OFT, Balb/c mice EPM and OFT, CLOCK delta19 mice	Cross-Genotype Analysis Physiologic (N/A)	↓ Anxiety-Related Behaviors
Chronic Social Defeat Stress (Homecage Recording, EPM and FIT)	Environmental Manipulation	↑ Anxiety-Related Behaviors
Fear Conditioning (Cued/Contextual Recall)	Environmental Manipulation	↑ Anxiety-Related Behavior
Optogenetic Stimulation of vHipp-LH Circuit	Direct Circuit Manipulation	↑ Anxiety-Related Behaviors
Diazepam Administration	Pharmacologic Manipulation	↓ Anxiety-Related Behaviors

Appendix A: Methods and Materials

Animal Care & Use

C57BL/6J (C57) mice were purchased from Jackson Labs at 8 weeks of age. All mice were group-housed, 3-5 mice per cage, in the Duke University Division of Laboratory Animal Resources facilities on a 12-hour light/dark cycle and maintained in a humidity- and temperature-controlled room with water available *ad libitum*. Elevated plus maze, open field test, and fluoxetine administration were conducted with approved protocols from the Duke University Institution Animal Care and Use Committee and were in accordance with the NIH guidelines for the Care and Use of Laboratory Animals. Studies were conducted using mice that were 12-20 weeks old.

Elevated Plus Maze

The elevated plus maze (EPM) has been previously described (Walf et al. 2007). The EPM consists of four cross-shaped arms (30.5cm length× 30.5cm width, at 91.4cm height from floor) and a 5cm×5cm central region. Two ‘closed’ arms are surrounded on three sides by walls of 16.5cm height and the other two ‘open’ arms are surrounded by a short piece of tape approximately 1 mm in height. Mice were habituated to the behavioral room for two hours, 24 hours before testing. Following a one-hr habituation period on the test day, mice were placed in the center region of the elevated plus maze facing a closed arm. Neural recordings were obtained for ten minutes, and the location

of the mice was captured by overhead video camera. All EPM testing was performed at 175 lux.

Open Field Test

The open field test (OFT) has been previously described (La-Vu et al. 2020). This task consists of a square (35 cm x 35 cm) box where the mice are allowed to explore this novel environment for 5 minutes. The 'center square' is the innermost third of the box and is considered the anxiogenic zone of the task. Similar to the EPM, mice were habituated to the behavioral room for two hours, 24 hours before testing. Following a one-hr habituation period on the test day, mice were along the periphery of the context. Neural recordings were obtained for 5 minutes, and the location of the mice was captured by overhead video camera. All OFT testing was performed at 125 lux.

Chronic Unpredictable Stress (CUS)

The CUS behavioral paradigm was adapted from Monteiro et al. 2015. 8-week-old C57 mice were split into an experimental CUS group and a control group that was subjected to gentle handling twice a week. Briefly, CUS were exposed to 2 aversive stressors a day – one during the light cycle (7AM-7PM) and one during the dark cycle (7PM – 7AM). The stressors were as follows: restraint – mice were placed in a plastic cone of ~50mL volume with opening in both sides for breathing for 1 hr; shaking – group-housing cage was placed on an orbital shaker for 1hr at 150 rpm; overnight illumination – mice were exposed to regular room light during the 12 hr dark cycle;

inverted light cycle – mice were exposed to dark cycle room conditions during the light cycle and light conditions during the dark cycle; tilted cage – homecages were tilted at a 45 degree angle for 1 hr; strobe – mice were placed in a room with a strobe light during the light cycle; wet bedding – cage bedding was saturated with water for 1 hr; soiled rat bedding – cage bedding was replaced with used rat cage bedding for 1 hr; cold exposure – mice were placed in a 4°C refrigerator for 1 hr; and missing bedding – homecage bedding was completely removed from the cage for 12 hr. Stressors were presented in pseudo random order in a unpredictable fashion. Body weight was monitored once a week to ensure mice didn't lose more than 10% body weight during CUS.

Drug Administration

For the behavioral fluoxetine experiments, mice were randomly assigned to receiving either an injection of Fluoxetine or Saline 30 minutes prior to being placed on the EPM. Fluoxetine (Sigma) was made up in 0.9% NaCl to a concentration of 1mg/mL and then injected at 10mL per kg for a final concentration of 10 mg/kg, i.p. (Marcinkiewicz et al. 2016). Physiologic saline injection was injected at 10 mL per kg as well. Animals were habituated to i.p injection for 1 week prior to behavioral testing. Fluoxetine only has an 8 hr half-life in mice but a lengthy washout period was chosen to ensure no traces of the drug remained (Oh et al. 2009).

For electrophysiologic recordings of the acute fluoxetine network, animals followed a standard pharmacological crossover design with a 2-week washout period.

On testing day, animals were allowed to habituate to experimental room for 1 hour. LFPs were recorded for 5 minutes while the animals explored their homecage. Mice were randomly assigned to receiving a fluoxetine or saline injection prior to starting the recording system for 1 hour. Following a wash-out of 2 weeks, animals underwent another 1 hour recording, receiving the cross over drug (either Saline or Fluoxetine). At the conclusion of each drug recording, mice were placed in the OFT for 10 minutes to serve a 'behavioral read-out' for locomotor effects.

Electrode Implantation Surgery

The electrode implantation surgery procedure has been described previously (Dzirasa et al. 2011; Carlson et al. 2017). Mice were anesthetized with 1.5% isoflurane, placed in a stereotaxic device and metal ground screws were secured in above 3 locations: anterior cranium (midline), cerebellum (midline) and laterally, roughly half-way between the former locations. Thirty-two tungsten microwires were arranged in array bundles designed to target prelimbic cortex (PrL), nucleus accumbens shell (NAc), mediodorsal thalamus (mdThal), ventral hippocampus (vHipp), and ventral tegmental area (VTA), all on the left hemisphere. Bundles were centered on stereotaxic anterior-posterior and medial-lateral coordinates measured from bregma, and dorsal-ventral coordinates were measured from the dura (**Table 1**). Implanted electrodes were anchored to round screws using dental acrylic. To mitigate pain and inflammation related to the procedure, all animals received carprofen (5mg/kg, s.c.) injections once

prior to surgery and then once every 24 hours for three days following electrode implantation.

Histological Confirmation

Histological analysis of implantation sites was performed at the conclusion of experiments to confirm electrode placement. Animals were perfused with 4% paraformaldehyde (PFA) and brains were harvested and stored for 24 hours in PFA. Brains were then cryoprotected with sucrose and frozen in OCT compound prior to being stored in -80C. Brains were sliced at 35 um using a cryostat and stained with either DAPI (AbCam) or cresyl violet (Sigma) using standard protocols. Slices were imaged at 4x and 10x magnification, specifically looking for electrode tracks in the brain regions outlined in **Table 2**. If electrodes tracked missed their target, the animal's recorded LFP data was removed from further analysis.

Neural Electrophysiological Data Acquisition and Video Recording

Mice were carefully connected via M and Mu headstages (Blackrock Microsystems) without the use of anesthesia and placed in the homecage to habituate to the weight of the headstage. Prior to all neurophysiological recordings, mice were recorded in a homecage environment for 5 minutes. Neuronal activity was sampled at 30kHz using the Cerebus acquisition system (Blackrock Microsystems). LFPs were bandpass filtered at 0.5-250Hz and sampled at 1000Hz. 60 Hz noise cancellation algorithm was applied to remove outlet electrical artifact in the recordings.

Video recording occurred from above using the OptiTrack camera system (NaturalPoint) and the accompanying NeuroMotive software. Mouse position was tracked offline using the Bonsai, an open-source computer vision software used in behavioral neuroscience. Mouse position (x,y) was recorded from each video and aligned to the electrophysiological records on a per second basis to align with the 1s window output of dCSFA. Arena was traced using custom MATLAB code which also calculated task regions and velocity.

Determination of LFP Features: Power, Coherence, and Granger Coherence

LFPs were averaged across wires within the same region to yield a composite LFP measure. For LFP Power, a sliding Fourier transform with Hamming window was applied to the averaged LFP signal using a 1 second window and a 1 second step. Frequencies were analyzed from 1-56Hz. Coherence was calculated from the pairs of averaged LFPs using magnitude-squared coherence. Spectral Granger causality features were calculated using the multivariate granger causality (MVGC) MATLAB toolbox. The non-stationary data required a high pass, so a high pass Butterworth filter with a stopband was applied.

Discriminative Cross-Spectral Factor Analysis – Nonnegative Matrix Factorization (dCSFA-NMF)

To apply our Supervised Cross-Spectral Factor Analysis – Nonnegative Matrix Factorization (CSFA-NMF) model, which fully described elsewhere (Talbot et al., 2020),

we consider each window of data to be an independent stationary measurement. This implies that the relevant dynamics happens at the scale of windows, so the extracted network scores are all that is needed for later analysis. In this work, we choose a 1 second window because this balanced fine-grained behavior with enough length of signal to estimate the relevant LFP features. Prior work has shown relative robustness to windows between 0.5s to 5s in similar methods (Ulrich et al., 2015), so we expect similar results for similar window lengths.

For each window of data, we have the generated features, consisting of spectral power features, coherence features, and exponential granger features, totaling distinct features per window. Using the subscript to denote window and state that there are total windows. We describe the preprocessed data as (the -dimensional non-negative domain) and the observed behavioral label, where the binary indicates an anxious or non-anxious behavioral label. To briefly described this model, we set up an objective function to learn the different network factors, the network factor scores are given by the multi-output function, and the relationship between the network factor scores and the behavioral labels is given by . This equation has a mean squared error loss on the behavior labels. In practice for binary behavioral labels, we instead use a binary cross-entropy loss. The relative importance of reconstructing the observed data and the importance of the predictive task were balanced by choosing the hyperparameter. This represents a novel method to fit an NMF model using supervised autoencoders and requires the user to

choose a parametrization for σ . In our method, this is simply set to an affine function following by a non-linearity, $\sigma(x) = \text{softplus}(ax + b)$, where the parameters of the function are a and b and the softplus means an element-wise operation of the operation $\text{softplus}(x) = \log(1 + \exp(x))$, which maps a real number to the non-negative space. This function can vary in complexity to allow greater model complexity, but we found that this function was sufficient in practice. Because this objective function follows a supervised autoencoder structure, a common deep learning structure, we can implement this technique in Tensorflow (Abadi et al., 2016) using the ADAM algorithm for learning (Diederik and Ba, 2014).

A benefit of using this structure for learning is that performing statistical inference from new data is fast and straightforward. In factor models, one typically must set up an optimization algorithm to find the maximum a posteriori estimate. However, in our supervised CSFA-NMF framework, we can calculate the network scores on new data simply by calling the function, allowing easy portability, and facilitating future real time applications.

Hyper-Parameter Selection

The proposed dCSFA-NMF procedure requires us to choose several different settings in the algorithm, which was done with a cross-validation procedure where complete mice from the training set were left out. The hold-out mice, as described earlier in the thesis, were not used for hyperparameter selection and represent a true blind test set. Specifically, we must choose the number of factors K , the importance of the

supervised task λ , the relative importance of the power features, coherence features, and exponential Granger features, and the parameterization of the mapping function.

We had dual goals in our analysis: reconstructing the original data well, which is to say that the learned networks describe the neural measurements well and predict the behavioral task well. The reconstruction error was evaluated by the Mean Squared Error on the validation mice, and the performance on the behavioral task was evaluated by the mean AUC on the validation mice. Greater emphasis was placed on the behavioral task, so for each candidate number of networks K , we used the cross-validation procedure to choose the settings that maximized the mean AUC.

Data Projection/Validation Testing

After a particular network had been trained, the learned feature representation can be applied to novel data by ‘projecting’ the new data onto the original set of factors. We projected LFP data recorded from new mice and/or new paradigms into our initial learned network model. We then performed direct comparison across conditions (e.g., behavioral conditions, CUS stressed vs. controls) using the median network activity score for each condition per mouse. Activity scores were compared using non-parametric statistics, or parametric statistics a Box–Cox transformation was applied the raw data. To further enable evaluation of the robustness of our findings, the decoding strength (area under the curve of the receiver operating characteristic, which considers

the activity scores for all the transformed time windows) was also provided in the main text alongside the statistical results obtained through direct comparisons of the median activity scores.

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Biography

Dalton Hughes attended the University of Maryland, Baltimore County and majored in Chemical Engineering and graduated in 2014. During his 5 years at the university, he worked with Dr. Jennie Leach studying biomaterial application to nervous system – specifically studying the signaling pathways involved in a cell’s ability to sense its environment. It was in this lab that ignited a passion for neuroscience research. Since matriculating to the Duke MD/PhD program, he has emersed himself in the medical sciences. He has been co-authored on several publications including:

- *Regulation of sensorimotor gating via Disc1/Huntingtin-mediated Bdnf transport in the cortico-striatal circuit* (Molecular Psychiatry, 2022)
- *Brain-wide electrical dynamics encode individual appetitive social behavior* (Cell, 2022)
- Prefrontal cortex reactivity underlies trait vulnerability to chronic social defeat stress (Nature Communications, 2014)
- *Grief: An epidemic within an epidemic* (American Journal of Hospice and Palliative Medicine, 2020)

Additionally, Dalton has received the following fellowships, awards and distinctions:

- Duke University Wakeman Fellowship
- Ruth K Broad Fellowship
- Society for Neuroscience, Neuroscience Scholars Program
- F30 NRSA Award (NIMH)
- Burroughs-Wellcome Fund: Graduate Diversity Enrichment Program Fellow