Activation of Ventral Hippocampus and Paraventricular Nucleus of the Hypothalamus during Anxiety-Related Behaviors

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Abstract

The paraventricular nucleus of the hypothalamus (PVN) and the ventral hippocampus (vHPC) are regions of the brain that play significant roles in regulating memory, emotion, and stress. The PVN has been shown to modulate several stages of the stress response, ranging from the initial verification of the stressful stimulus to the post stress recovery and adaptation processes. The vHPC is involved with regulating neural feedback involved with emotion. Both the PVN and vHPC play significant roles in emotion disruptive disorders, namely anxiety, depression, and post-traumatic stress disorder. This thesis project aimed to investigate and compare the roles of the PVN and vHPC during specific fear and anxiety related behaviors. We examined mice PVN and vHPC activation that occurs during four specific behaviors (head dip, elongation with locomotion, elongation with retraction, and turning) observed in the elevated plus maze (EPM) using calcium imaging. The EPM is a behavioral approach-avoidance model that relies on a mouse's preference towards enclosed spaces and avoidance of open spaces in order to assess anxiety related behavior. We found that the vHPC and PVN activations aligned in direction of change for the elongation with retraction, head dip, and turning around behaviors. The vHPC and PVN activation both increased from the behavior occurring to the post-period for elongation with retraction and head dip behaviors. The vHPC and PVN activation both decreased from the behavior occurring to the post-period for the turning around behavior. The vHPC and PVN activation did not align in terms of change after the elongation with locomotion behavior occurred. The vHPC activation had increased and PVN activation had decreased after the elongation with locomotion behavior occurred.

Introduction

As organisms are faced with threats, the stress response system cascades through a series of neural and physiological changes that initiate distinctive behavioral responses. The stress response system, specifically the hypothalamic-pituitary-adrenal (HPA) axis, enables a release of glucocorticoid hormones called corticosteroids that activates behaviors based on the presence, absence, or anticipation of a threat [1,2]. The manner in which an organism responds and what it acquires from a threat is crucial for its future survival. These behavioral responses have been attributed to specific brain circuits, notably the paraventricular nucleus of the hypothalamus and the ventral hippocampus.

The paraventricular nucleus of the hypothalamus (PVN) modulates the activation of the release of corticotropin-releasing hormones (CRH) within the stress response pathway [3]. During exposure to a stressful event, the organism makes a shift from risk assessment, which involves

verifying the true absence of the threat, to coping, which assists in adaptation to or recovery after a threat. The CRH neurons in the PVN of the hypothalamus are important for both risk assessment, where CRH PVN activity has shown to increase in anticipation of a stressful stimulus, and in coping responses, where the hypothalamic CRH neurons are known to orchestrate the repertoire of individual behaviors that follows a stressful event [4,5].

The hippocampus is another region involved in regulating memory and emotion. In response to stressful stimuli, the hippocampus is especially impacted in terms of reductions to its volume and suppression of overall neuronal proliferation in the area [6]. The dorsal hippocampus and ventral hippocampus have structural and functional differences. The dorsal hippocampus is responsible for general cognitive and information processing, whereas the ventral hippocampus (vHPC) is a compartment of the hippocampus that regulates emotion, stress, and affect [7]. The vHPC, which is our main focus, has been shown to modulate feedback control within the HPA axis. The vHPC controls the expression of behaviors like contextual fear generalization that is a core symptom of post-traumatic stress disorder [8]. The vHPC has also been found within interconnected networks of brain regions that control and regulate the susceptibility to the development of emotion disruptive disorders such as anxiety and depression [9-11].

We hope to understand how the vHPC and PVN circuits correlate with one another and moderate different responses to threat. Localizing these circuits will ultimately advance our understanding of how these regions work together to contribute to stress-related psychopathologies and suggest specific treatment. Using calcium imaging to measure neural activity, we attempted to examine, quantify, and compare the activity of the vHPC and PVN neurons during specific fear- and anxiety-related behaviors that occur in response to potential threats.

The behaviors that were examined were observed in the elevated plus maze (EPM) assay. The elevated plus maze (EPM) is reliant on a mouse's affinity to enclosed spaces (approach) and an unconditioned fear of open spaces (avoidance). Behavioral approach-avoidance models measure the latency to approach or amount of time involved with a potential threat as an indicator of anxiety-related behavior in mice [12-14]. The maze consists of four arms (two open arms and two closed arms) arranged in a plus formation, elevated several feet above the ground. In the EPM assay, we examined three specific response behaviors: *head-dip, elongation, and turning*. The head-dip behavior is a controllable and voluntary risk assessment behavior where a choice between curious exploration and avoidance of a potential dangerous area is made [15]. The elongation response behavior, also known as the stretched-attend posture in other literature, is the elongation of the forepart of an animal's body towards an unknown stimuli [16]. This elongation could be followed by further locomotion in the same direction or retraction of

the body back to the original position. The elongation with locomotion may occur when the mouse is undergoing less fearful risk assessment due to an exploratory-anxiety conflict, whereas the elongation with retraction may be attributed to an ambivalent, more fearful risk assessment during an approach-avoidance conflict [17]. The turning behavior is the execution of a new exploration strategy by the physical reversing of direction. Other kinds of anxiety related response behaviors that can be studied include grooming, rearing, and freezing [18].

Methods

Animals

Male and female Crh-IRES-Cre hemizygous mice were bred from pairs of homozygous Crh-IRES-Cre mice (*B6(Cg)-Crhtm1(cre)Zjh/J, Jax #012704*) and C57BL/6 mice (both obtained from The Jackson Laboratory). Mice were housed under a 12 h light/dark cycle with ad libitum access to food and water. All experiments were conducted in accordance with the U.S. National Institutes of Health Guide for the Care and Use of Laboratory Animals and the institutional Animal Care and Use Committees at University of California, San Francisco.

Surgical Procedures and Behavioral Experiments

Experimental mice and experimental data were collected previously and provided by Kheirbek Lab members Victoria Turner and Rachel O'Sullivan.

At 8-12 weeks old, mice were injected unilaterally with AAV1-syn-jGCaMP7s-WPRE in the ventral hippocampus (vHPC) and Cre-dependent AAV1-syn-FLEX-jGCaMP7s-WPRE (Addgene) in the paraventricular nucleus of the hypothalamus (PVN) at the following stereotaxic coordinates: *PVN* AP -0.6, ML -0.2, DV -4.9 (64 nL), DV -4.8 (32 nL), DV - 4.7(128 nL) from bregma; *vHPC* AP -3.20 ML +3.35, DV -3.85 (32 nL), DV -3.75 (96 nL), -3.65 (32 nL) from cortical surface. During surgery, mice were anesthetized with 1.5% isoflurane and then head-fixed in a stereotaxic frame (David Kopf). Craniotomies were made with a round 0.5-mm drill bit (David Kopf), and a Nanoject II syringe (Drummond Scientific) was used with a pulled glass pipette to inject virus. Following viral injection and a diffusion period of 10 minutes, the viral injection pipette was withdrawn and a fiber optic photometry cannula (Ø 400 um, Doric Lenses) was implanted over each of the two brain regions (*PVN* DV -4.60 from bregma, *vHPC* DV -3.75 from cortical surface) and secured with dental cement. All mice were administered lidocaine, meloxicam, and buprenorphine during surgery and allowed to recover for 5 weeks and habituated to handling before experiments began.

On the day of each experiment, mice were habituated outside the colony in a quiet room for 1-2 hours. *Elevated plus maze.* Animals were first recording in their home cage for 5 min. Animals were then transferred to the arena (150 lux) and remained in the arena while photometry recording took place for the duration of the experiment. Animals were recorded in the elevated plus maze assay before footshock to assess innate anxiety responses prior to any experience of shock.

Recordings were performed using Synapse software (TDT) with an RZ5P processor (TDT) and optical components (LED drivers, LEDs, photoreceivers) from Doric Lenses. Two sets of excitation LEDs at 465 nm and 405 nm were sinusoidally modulated and relayed through respective filtered fluorescence minicubes (Doric). All signals were acquired at 10 kHz and downsampled to 10 Hz during analysis to match the behavioral video recorded at 10 Hz.

Behavioral Coding

The behavioral coding was performed on The Observer XT (Noldus), a platform that allows for efficient data integration and behavioral coding in a quantitative manner [19].

Videos of the behavioral sessions were input into The Observer XT (Noldus) and each behavior (head-dip, elongation, and turning) was assigned a unique key. Each observation was reserved for a separate mouse. The elongation response behavior had an additional modifier that would separate between the elongation response with locomotion and elongation with retraction responses. As each behavior was manually observed, the respective key and/or associated modifier assigned to the behavior would be selected. The output data included a holistic compilation of the coded behaviors, including the type of response alongside the timestamp at which that behavior occurred.

The timestamp at which each behavior was coded associated with a single frame. For elongation with locomotion, the timestamp is the frame at which the mouse finishes elongating fully and the first back leg starts to move forward. For elongation with retraction, the timestamp is the frame at which the mouse finishes elongating fully and is in the process of retracting. For head dip, the timestamp is the frame at which the mouse's head is stationary as it is over the edge. For the turning behavior, the timestamp is the frame at which the mouse first starts to change direction.

Photometry and Behavioral Analysis

The processing of the neural data was performed using custom Python code. The Tucker-Davis Technologies (TDT) Python API was used for reading data into Python and interacting with the Synapse software [20]. Preprocessing involved downsampling the signal to a new frame-rate of 10 Hz to match the behavioral session that was recorded at that frame-rate. The UV signal was then fitted to the GCamP signal in order to calculate the dF/F value. The dF/F value is the change in fluorescence for each time point. The following formula was used to calculate the value: $dF/F = (F - F0)/F0$. The F0 value was calculated by fitting a line to a plot of GCamP against UV, then multiplying the UV against the coefficients of the fitted line. This value was determined to be the 'fitted UV', which was used as the F0. The dF/F value was calculated by: (GCamP signal - fitted UV)/fitted UV. Further preprocessing included taking the z-score of the dF/F value and trimming the first 3 seconds and the last 3 seconds of the recording. The trimming ensured the removal of any unwanted artifacts that would clutter the signal at the beginning and ending of the recording. Average and standard deviation values were calculated for each type of behavior. The timestamps at which each behavior occurred was also matched to the PVN and vHPC recordings in order to determine any recurring organization or frequency of behaviors to one another.

Statistical Analysis

To measure the averages, standard deviation, and frequency of each behavior, the Tucker-Davis Technologies (TDT) Python API was utilized.

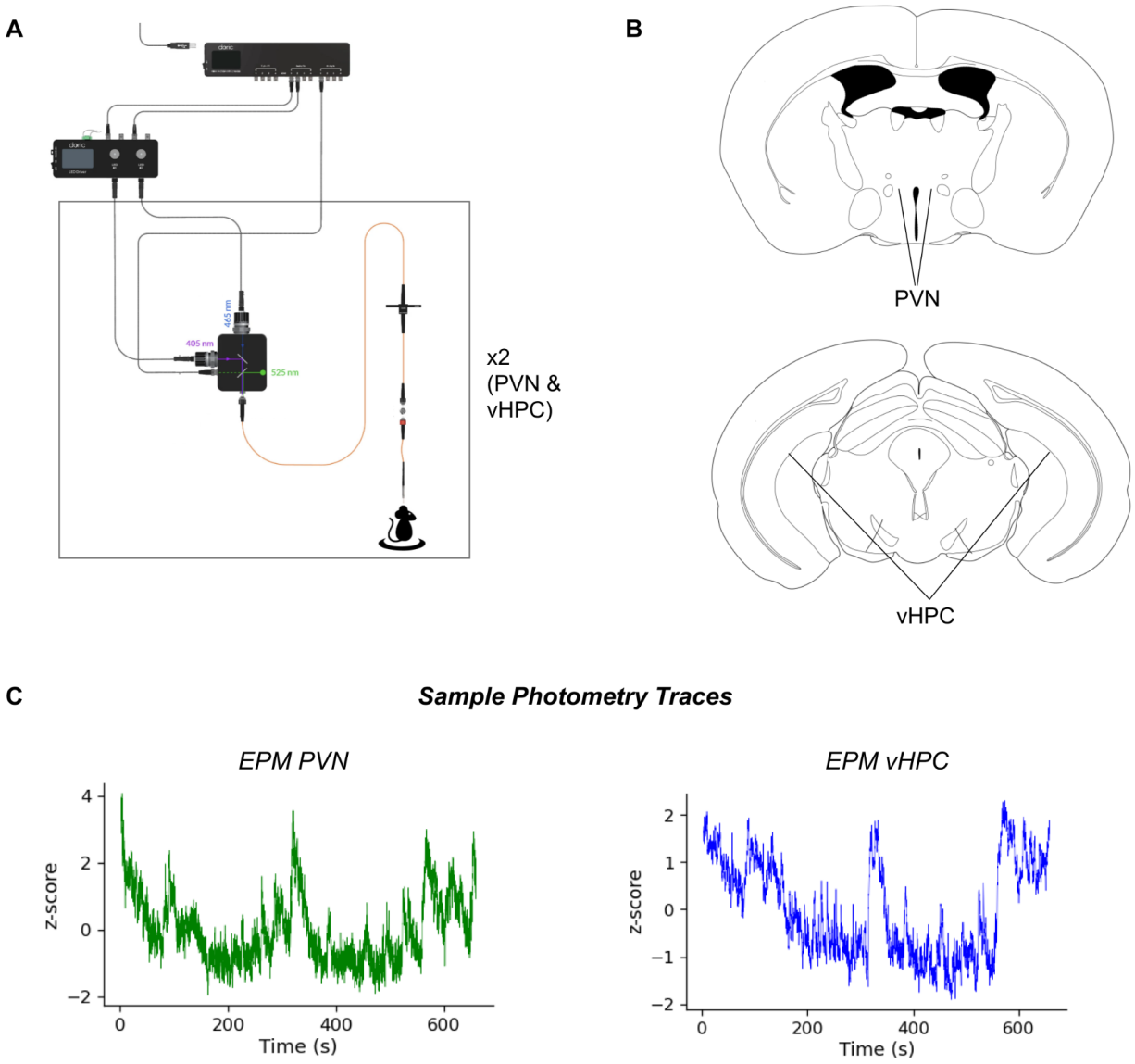


Figure 1: Experimental setup and localized brain regions **A)** The photometry design and setup: Two sets of excitation LEDs at 465 nm and 405 nm were modulated and relayed through respective filtered fluorescence Doric mini cubes. This setup was repeated for both vHPC and PVN separately. **B)** Coronal view of the brain highlighting the location of the PVN and vHPC. **C)** EPM sample photometry traces of the PVN and vHPC recordings respectively. Both signal recordings have the beginning and ending artifacts trimmed.

Table 1: Mouse Exploratory and Stress Response Behaviors

Behavior	Elongation & Retraction	Elongation & Locomotion	Head-Dip	Turning Around
Assay	*Elevated Plus Maze*	*Elevated Plus Maze*	*Elevated Plus Maze*	*Elevated Plus Maze*
Description	Elongation of the forepart of an animal's body towards an unknown stimuli while maintaining a safe distance from the possible threat, followed by retraction of the body.	Elongation of the forepart of an animal's body, followed by a locomotion bout.	A controllable, voluntary risk assessment behavior where a choice between exploration and avoidance of a potential dangerous area is made.	Execution of a new exploration strategy by reversing direction.

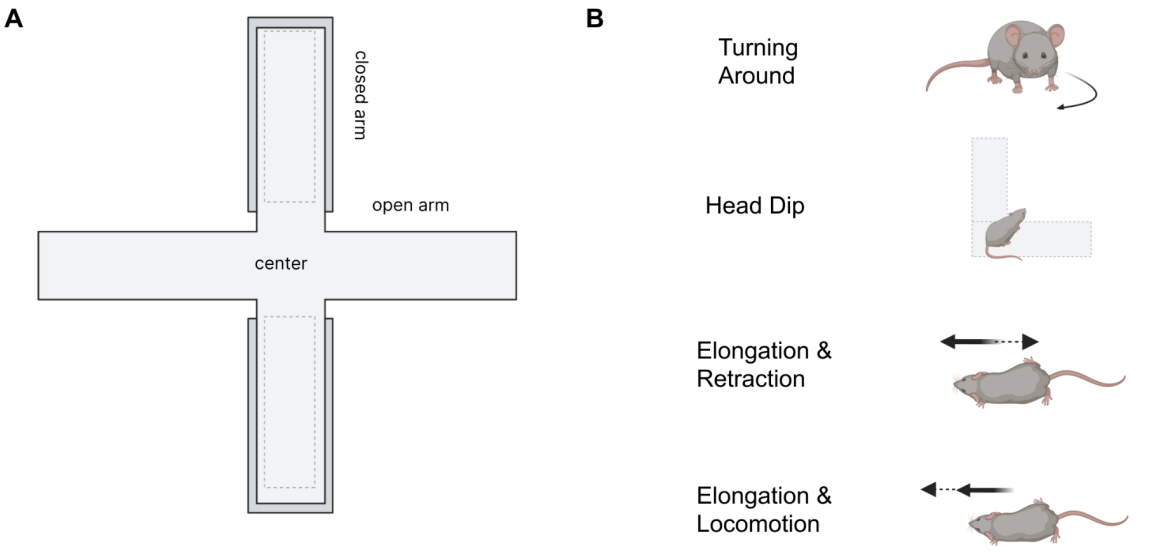


Figure 2: EPM Behaviors and Experimental Design **A)** The design of the elevated plus maze (EPM). There is a center, two open arms, and two closed arms. The maze is arranged in a plus formation and elevated a few feet above the ground. The vHPC and PVN activity were recorded as the mouse interacted with the maze. **B)** The EPM response behaviors that were studied include: turning around, head dip, elongation with retraction, and elongation with locomotion, which are depicted and labeled in the diagrams.

Results

Table 2: EPM Behaviors Frequency and Mean

	Average time of First Occurence	*Average time of Last Occurence*	*Average Number of Occurences*
Elongation & Retraction	39.0375 s	348.775 s	4.25
Elongation & Locomotion	105.68 s	344 s	3.25
Head Dip	14.95 s	434.675 s	9.375
Turning Around	40.55 s	303.85 s	10.5

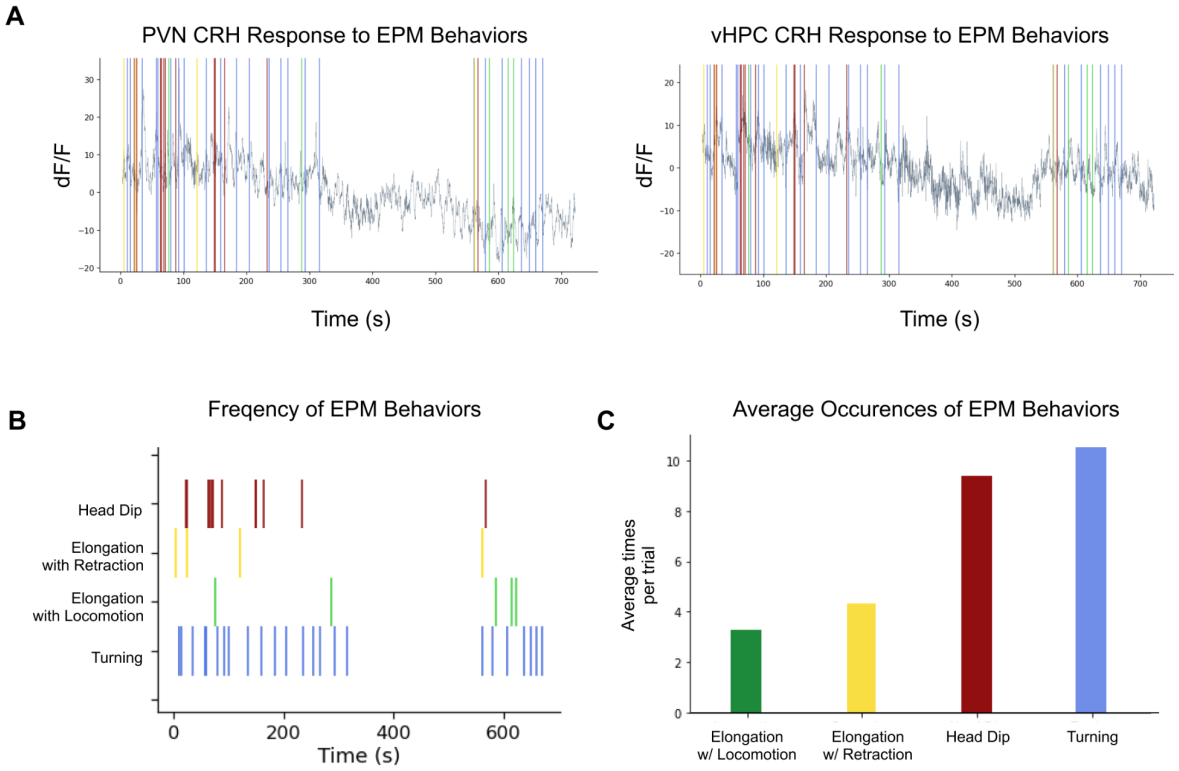
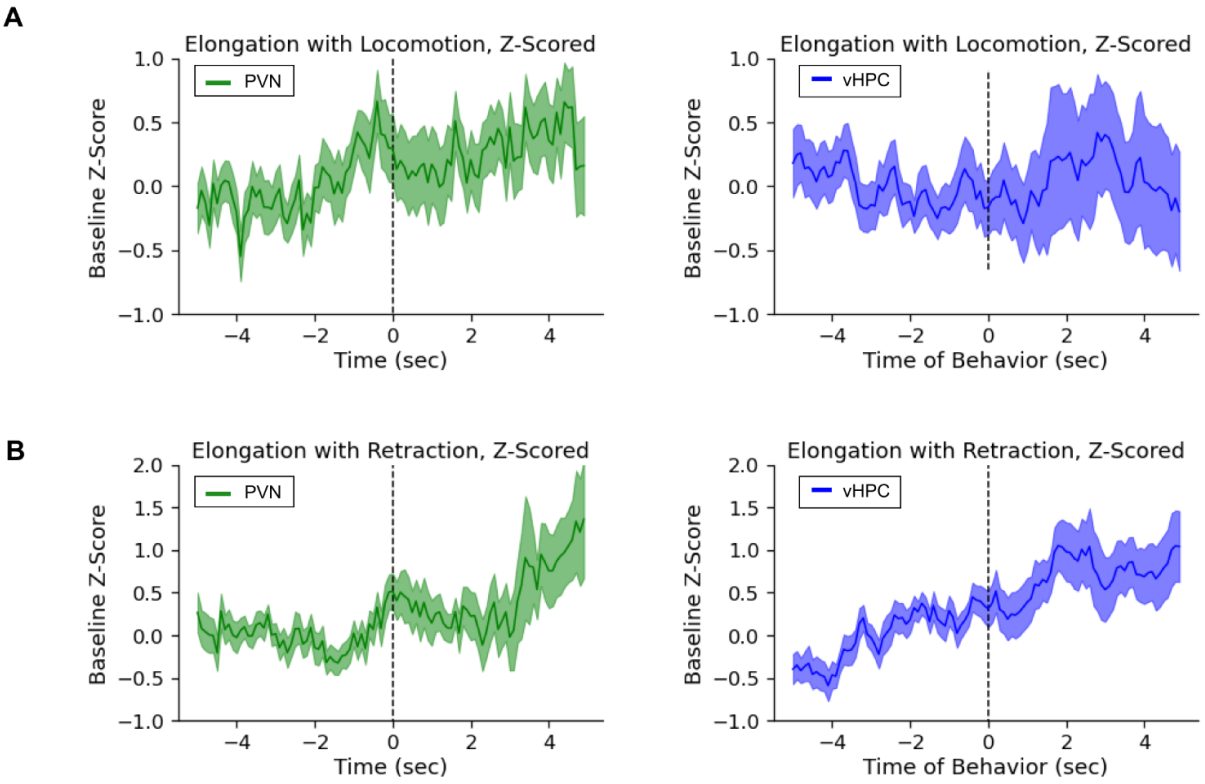


Figure 3: Occurrences of each behavior matched to the PVN and vHPC recordings. **A)** Each bar represents an instance of each behavior. The blue lines represent the turning behavior, the green lines represent the elongation with walking behavior, the yellow lines represent the elongation with retraction, and the red lines represent the head dip behavior. These behaviors have been matched to the PVN and vHPC photometry recordings. There is a break of behaviors between 300s and 550s for this specific observation. **B)** The EPM behavior raster plot depicts the occurences of each behavior altogether in the same temporal relation. For this specific

mouse, the turning behavior has the largest amount of occurrences, whereas the elongation with retraction has the least amount of occurrences. **C)** The average number of occurrences of each EPM behavior. Elongation with locomotion happened the least amount of times, whereas the turning around behavior occurred the most amount of times. On average, both types of elongation (locomotion and retraction) happened significantly less than head dip or turning.



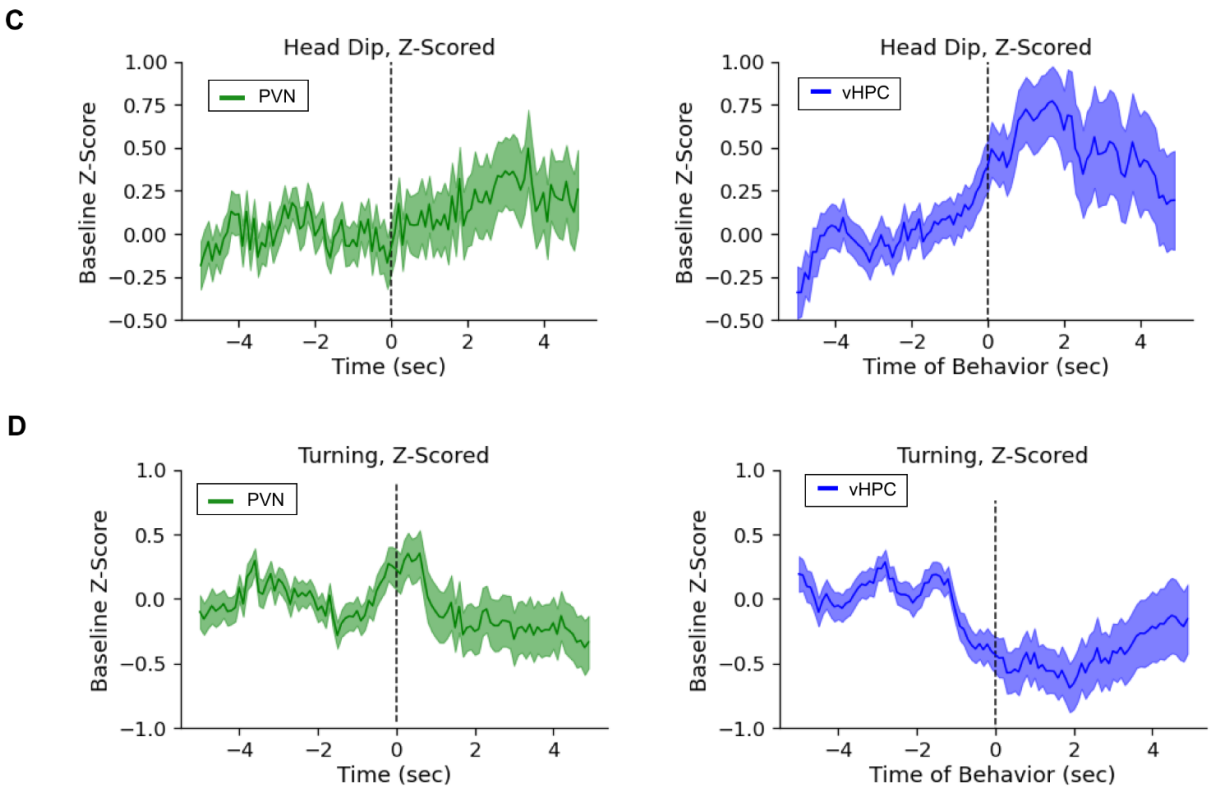


Figure 4: A - D) The PVN and vHPC activity of each behavior (elongation with locomotion, elongation with retraction, head dip, and turning) is observed at a pre-period of 4 seconds before the behavior occurs and at a post-period of 4 seconds after the behavior has occurred, assuming that the behavior itself is occurring for 1 second (the during-period). The dotted line indicates the time that the behavior occurs (taken from the average across all animals). The df/f values have been baseline z-scored. There was an increase in PVN activity after the head dip and turning around behaviors and a decrease in PVN activity after the elongation with locomotion behavior. The PVN activity reached a local peak at the time of the behavior; the activity then decreased for the turning around, elongation with retraction, and elongation with locomotion behaviors. There was an increase in vHPC activity after the head dip and elongation behaviors and a decrease in the vHPC activity after the turning around behavior. The peaks of vHPC activation were reached in the post-period (after the behavior occurred) for the head dip, elongation with retraction, and elongation with locomotion behaviors. In contrast, the peak of vHPC activity was observed in the pre-period (before the behavior occurred) for the turning around behavior.

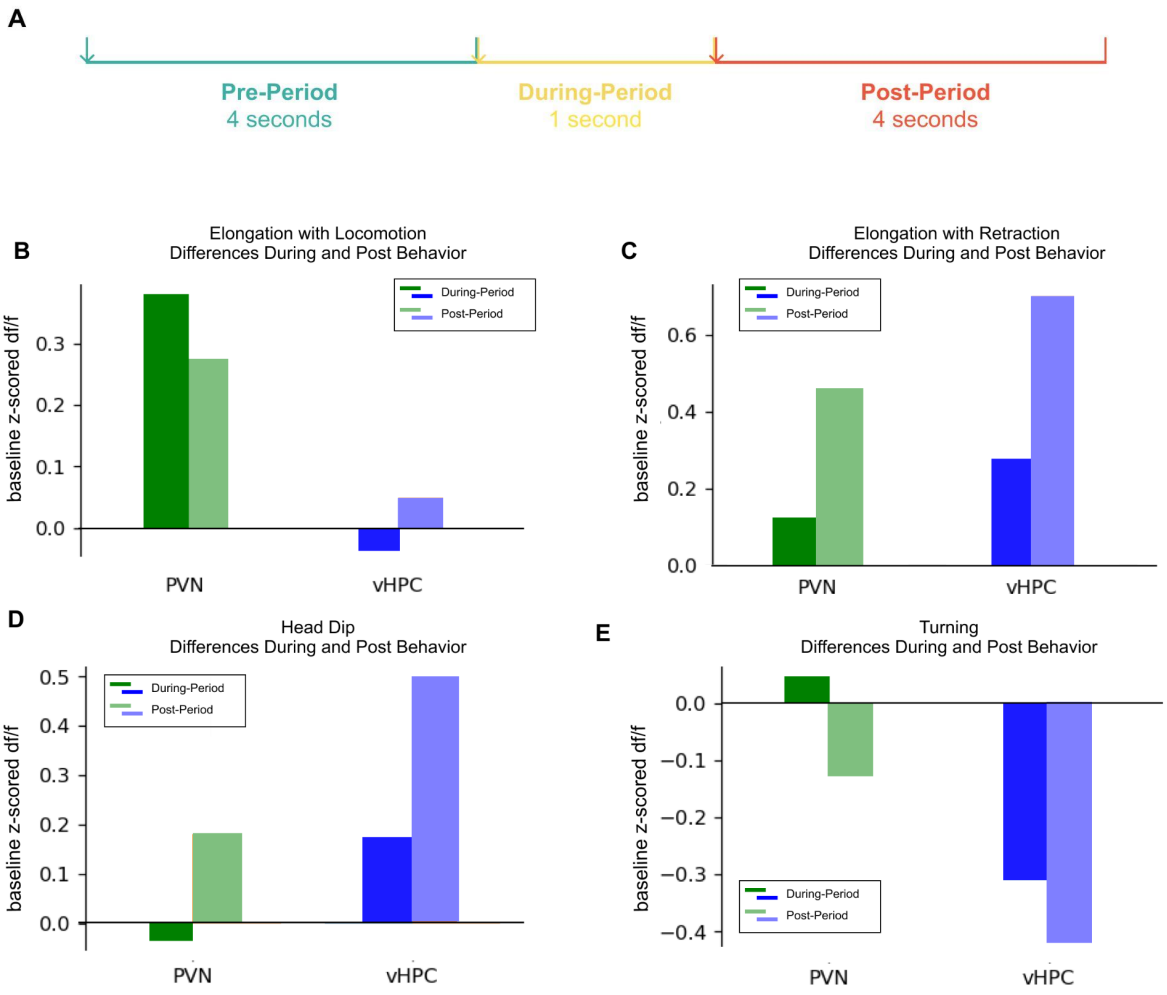


Figure 5: A) A diagram depicting the time windows for each period. The pre-period (before the behavior occurs) is 4 seconds long, the during-period (when the behavior is occurring) is 1 second long, and the post-period (after the behavior occurs). **B-E)** The graphs depict the differences in df/f values in the during-period and post-period of each behavior. These differences are displayed for PVN and vHPC separately. PVN activity increased from during to post periods for the head dip and elongation with retraction behaviors and it decreased from during to post periods for the elongation with locomotion and turning around behaviors. The differences in activity were significantly larger for the elongation with retraction behaviors. vHPC activity increased from the during to post periods in all of the behaviors (elongation with locomotion, elongation with retraction, head dip, and turning around behaviors). The differences in activity were significantly larger for the head dip and elongation with retraction behaviors.

Behaviors and Responses

Behaviors. Over the course of the trials, there was a greater frequency of EPM behaviors towards the beginning of the recording (0-300 seconds), followed by an occasional break of no behaviors for 100-200 seconds. After the break, the EPM behaviors would return, although at a lower frequency than the beginning period. The turning around behavior occurred the most, at an average of 10.5 times per observation. The head dip behavior followed at an average of 9.3 times per observation. There was a significant decrease in average occurrences for the elongation behaviors (elongation with retraction occurred at an average of 4.25 times and elongation with locomotion occurred at an average of 3.25 times. The turning around behaviors occurred at a constant rate throughout the trials. The elongation with locomotion behaviors would occur alongside the turning around behaviors. The head dip behaviors clustered together; the animal would head dip consecutively in a short period of time.

PVN and vHPC Responses. Between the during-period (1 second of behavior occurring) and post-period (4 seconds after the behavior occurred), the vHPC activity (baseline z-scored df/f) had increased for the elongation with locomotion, elongation with retraction, and head dip behaviors and decreased for the turning around behavior. The PVN activity had increased for the elongation with retraction and head dip behaviors and decreased for elongation with locomotion and turning around behavior. vHPC and PVN activity similarly increased in the post-periods for elongation with retraction and head dip behaviors and similarly decreased in the post-periods for the turning around behavior.

Comparing Elongation Behaviors

The elongation with locomotion and elongation with retraction were both elongation behaviors distinguished by a specific modifier. Locomotion indicates that the mouse continued moving forward after extending the body and retraction indicates that the mouse returned to its default position that was maintained prior to the mouse's extension of the body. The elongation behavior occurs during risk assessment, when the animal is unsure about the presence or degree of danger associated with the potential threat. Elongation of the body allows for the animal to maintain a safe distance as the risk assessment is performed. In terms of brain activity, we expect the animal to have different activation patterns for retraction vs. locomotion after elongation. In elongation with retraction, the animal makes the choice to return back to its original position, after risk assessment of the threat. This choice to return is characterized as a change of decision, one that was most likely made after assessing that the potential threat is dangerous. However, with the locomotion behavior, the mouse continues onwards, towards or away from the threat after elongation risk assessment. In this experiment, regarding elongation

with retraction, as seen in Figure 5, the during-periods (1 second of behavior occurring) is significantly smaller, followed by a sharp increase of df/f value in the post-period for both the PVN (0.1 to 0.45) and vHPC (0.25 to 0.65) activity. In elongation with locomotion, the PVN df/f value decreases from the during-period to the post-period (0.4 to 0.28), and the vHPC df/f value has a small increase from -0.05 to 0.2. In the elongation with retraction behavior, we can assume that the increase in PVN and vHPC activation is attributable to the post risk assessment retraction of the animal back to its original position. In the elongation with locomotion behavior, there is a contradictory change in df/f value between the during-period and post-period where PVN df/f values decrease and vHPC df/f values increase.

Discussion

This project aimed to compare the activation of the vHPC and PVN during specific behaviors that were observed in the EPM. Specifically, we set out to examine, quantify, and compare the activity of the vHPC and PVN neurons during the elongation with locomotion, elongation with retraction, head dip, and turning around EPM behaviors. The previous model correlating the vHPC and PVN suggests that vHPC cells exert inhibition on PVN neuron activity. The vHPC has been found to suppress different stress responses by downregulating the HPA axis[21]. Therefore, we would expect the PVN to be inhibited by the vHPC after the stress response has occurred. We found that PVN and vHPC both increased after the elongation with retraction and head dip behaviors occur in the EPM and both decreased after the turning around behavior occurred. However, similar to the model, the PVN activity had decreased and vHPC activity had increased after the elongation with locomotion behavior was observed. The PVN and vHPC activation in the pre-period, during-period, and post-period is based on an average across all animals (as seen in Figure 4). In comparison to the model that previous studies used to determine that vHPC inhibits PVN in stressful situations, the observations from this project had a different timescale (an average activation during 4 seconds before the behavior, 1 second of behavior occurrence, and 4 seconds after the behavior). It is possible that the contradicting results (similar increase of both PVN and vHPC after the elongation with retraction and head dip behaviors and similar decrease of both PVN and vHPC after the turning around behavior) could be attributed to this difference in timescales. Perhaps, future studies could look at the activation with different lengths of pre-periods and post-periods in order to differentiate or reiterate these results.

The effects depicted by this project have been limited by the small sample size and type of behavioral avoidance model used. Only the elevated plus maze test was used to assess anxiety-related behaviors in mice. This type of experiment only works to produce self-controlled minor stressors that increase heart rate as the mouse enters the open arm of the maze.

However, the EPM is not as influential in generating large amounts of stress compared to other behavioral models, namely where the animal would be restrained on the full body. It would be informative to assess other kinds of fear and anxiety related behaviors and compare the results from other types of models as well. Specifically, the freezing response behavior is observed in the foot shock assay, which is a form of cued fear conditioning where a light is shone as an unconditioned stimulus (an electric shock) is administered to the mouse. Examining the frequency of the *freezing* response behavior has also been established as a reliable index of fear and could be a helpful comparison alongside the EPM behaviors[22]. Prior research has primarily focused on the identification of the behaviors in response to stressful situations, but only recently has it been possible to study the underlying neural processes involved in actually inducing those responses. Future studies that compare the PVN and vHPC roles in different experiments, models, and behaviors would aid in understanding how different fear and anxiety behavior correlating neural processes are interconnected.

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