

# Investigating the role of noradrenaline in behavioral switching using optogenetics

MS Project Report

in

*Bachelors of Science - Masters of Science  
Biological Sciences*

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# Declaration

I, Leafy Behera, 18MS123, a student of the Department of Biological Sciences of the Bachelor of Science – Master of Science (BS-MS) Program of IISER Kolkata, hereby declare that this MS project entitled “Investigating the role of noradrenaline in behavioral switching using optogenetics” is my own work and, to the best of my knowledge, it neither contains materials previously published or written by any other person, nor it has been submitted for any degree or diploma or any other academic award anywhere before. I have used the originality checking service to prevent inappropriate copying. I also declare that all copyrighted material incorporated into this thesis is in compliance with the Indian Copyright Act 1957 (amended in 2012) and that I have received written permission from the copyright owners for my use of their work. I hereby grant permission to IISER Kolkata to store the thesis in a database which can be accessed by anybody as permitted by the institute guidelines of IISER Kolkata.

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A handwritten signature in black ink, appearing to read "Leafy Behera".

# Certificate

**Place: Kolkata**

This is to certify that the MS project entitled “Investigating the role of noradrenaline in behavioral switching using optogenetics” submitted by Ms Leafy Behera has been carried out under my supervision. This is submitted in partial fulfilment of the requirements for the award of Bachelor of Science – Master of Science (BS-MS) degree by the Indian Institute of Science Education and Research Kolkata, and this work has not been submitted elsewhere for a degree.



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# 1 List of abbreviations

**fps:** Frames per second

**Hz:** Hertz

**mW:** milliwatts

**nm:** nanometer

**LC-NE:** Locus Coeruleus-nor-adrenergic

**FMM:** Fentanyl, Midazolam and Medetomidine

**A.P:** Anterior Posterior

**M.L:** Medial Lateral

**D.V:** Dorsal Ventral

**VBA:** Virtual Burrow Assay

**OFT:** Open Field Test

**OTPG:** Optogenetics TTL Pulse Generator

**FLIR:** FLIR systems

**TTL:** Transistor-transistor logic

**ms:** Milliseconds

**SN:** Substantia Nigra

**VTA:** Ventral Tegmental Area

**Na:** Nucleus Accumbens

**ANOVA:** Analysis of Variance

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### 3 Abstract

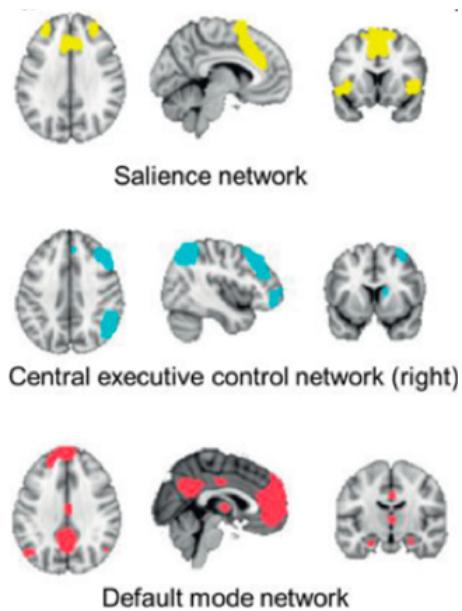
Dynamic environmental conditions often require switches in ongoing behaviors in response to new challenges. This ability is a crucial aspect of survival because understanding how animals switch between different behaviors can give us insights about how the brain functions and orchestrates behavior. Most studies investigating behavioral flexibility use task-structured designs, in which behavior is driven by external cues whereas the neural mechanisms underlying uninstructed, spontaneous behaviors remain largely unknown [1].

Studies investigating the role of neuromodulators in behavioral processes indicate that higher levels of noradrenaline increases arousal, promoting switches in behavior. Building upon this hypothesis, we targeted the Locus Coeruleus (LC) (a brainstem nuclei and the primary source of noradrenaline in the brain). Since high levels of noradrenaline can induce anxiety, we first established a frequency that could be used to stimulate the LC without inducing anxiety in the mice. We conducted experiments in the open field, where the LC was stimulated at 4Hz while monitoring the movement of the mice. In the open field test the stimulation of noradrenergic cells at 4hz did not induce anxiety but decreased overall locomotion. Since 4Hz did not induce anxiety, we used the frequency to stimulate the LC while the mice were head-fixed and exhibiting different spontaneous behaviors. Closed-loop stimulation of the LC at 4Hz in the VBA setup did not affect the behavior. Interestingly, we observed that only stimulation of the noradrenergic cells did not promote egress, suggesting that the activity of the LC alone does not promote the egress behavior. On the running wheel, we observed that stimulating the LC increased running after the stimulation period. Together, our findings indicate that stimulating the LC at 4Hz does not induce anxiety neither does it promote behavior but 4Hz stimulation does decrease locomotion as observed in the open field and running wheel experiments.

## 4 Introduction

### 4.1 Behavioral Flexibility

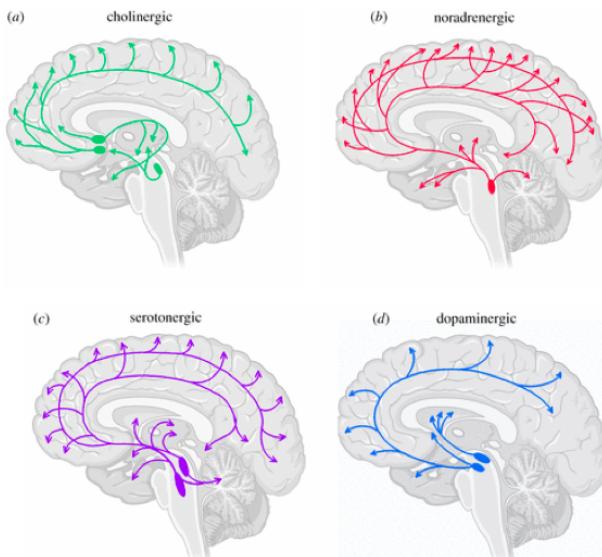
To adapt to the changing environmental conditions and optimize their survival, animals often need to switch between behaviors. During foraging, for example, animals need to take multiple factors into account to gain sufficient nutrition while escaping predators. To balance the trade-off between exploration and exploitation while foraging, animals need to switch to an appropriate behavior as per the situational demands. This flexibility in behavior in response to changing environmental conditions and situational demands is the basis of behavioral switching. Over the years, various theories have been proposed explaining the underlying neural mechanisms regulating switches in behavior. One of these theories comprise of the neuroimaging studies which proposes that the brain is organised into large scale interconnected networks of brain regions that are responsible for different functions<sup>[19]</sup>.



**Figure 1:** Image of three brain networks primarily involved in modulating various behavioral and cognitive processes (Elise Lesage et.al,2016)

Among these networks, there are three well studied brain networks which are involved in various cognitive and behavioral processes, namely the Salience Network (SN), Default Mode Network (DMN) and Central Executive Network (CEN).

The Default Mode Network is a network of brain regions that is active when an individual is not engaged in any specific task, and is instead focused on internal mental processes such as daydreaming, self-reflection and planning for the future<sup>[22,23,24]</sup>. In contrast, the Central Executive network (CEN) is a network of brain regions involved in a task that requires cognitive control, such as planning, decision-making or problem-solving<sup>[25]</sup>. The Salience Network (SN) is involved in detecting and responding to salient stimuli from both internal and external environments. It plays a critical role in directing attention and determining whether to engage in a particular task or switch to a new one. It is involved in a wide range of processes including decision-making, emotional processing and autonomic responses.



**Figure 2:** Major neuromodulatory system in mammalian brain (Claire O'Callaghan, 2020)

Studies have shown that these networks constantly interact with each other while modulating various cognitive and behavioral processes<sup>[15]</sup> while the salience network is known to be active during the periods of switching between the DMN and CEN, rather than during sustained periods of either network, suggesting that it plays a critical role in promoting switching behavior. <sup>[21]</sup> On the other hand, studies from the rodent experiments suggest that the release of specific neurotransmitters, such as dopamine, noradrenaline and serotonin, play a crucial role in modulating neural activity and regulating behavioral flexibility <sup>[20]</sup>. These neurotransmitters are believed to act on multiple brain regions, altering the processing of sensory information and the integration of cognitive and emotional signals<sup>[26]</sup>. This modulation of neural activity facilitates the adaptation of behavior to changing environmental demands by influencing attention, motivation and decision-making processes. For instance, a study conducted in 2005 demonstrated the role of the neurotransmitter noradrenaline in attention and arousal<sup>[5]</sup>. The authors found that psychostimulant medications that increase norepinephrine activity in the brain can improve attention and cognitive performance with the increase in arousal levels gradually promoting flexibility in ongoing behaviors <sup>[34]</sup>. However, both theories provide complementary perspectives on the neural mechanisms underlying behavioral flexibility and are supported by empirical evidence highlighting the importance of interactions between different brain regions.

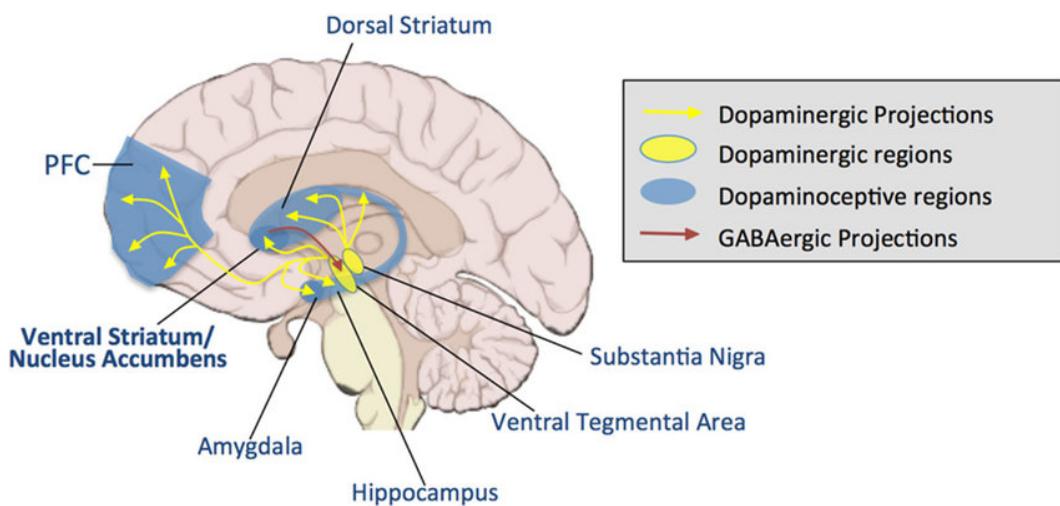
## **4.2 Neuromodulatory systems**

Neuromodulators are chemicals in the brain that regulate the activity of neurons by modifying their excitability or synaptic efficacy. They act as signaling molecules that can affect many neurons and brain regions, which is essential in shaping neural circuits and behavior. There are several neuromodulators in the mammalian system, of which dopamine, noradrenaline, serotonin, and acetylcholine are the significant systems innervating most of the brain regions<sup>[27]</sup>. Studies have shown that when a new behavior is associated with a high reward, an increase in dopamine release facilitates the transitions to the new behavior<sup>[6]</sup>.

Norepinephrine, another important neuromodulator, is involved in attention and arousal and can promote the switch to a new behavior by increasing vigilance and readiness to respond to environmental changes. Moreover, neuromodulatory systems heavily influence brain regions like the prefrontal cortex, which is involved in controlling executive functions such as decision-making and working memory, affecting its ability to initiate or inhibit behaviors and to switch between different cognitive processes<sup>[27]</sup>.

#### 4.2.1 Dopaminergic system

The dopaminergic system is a group of neurons in the brain that produce and use the neurotransmitter dopamine. These neurons are located in several areas of the brain, including the midbrain, hypothalamus and basal ganglia. Dopamine is involved in a wide range of functions in the brain, including motor control, reward processing and motivation. The dopaminergic system plays a crucial role in regulating these functions by modulating the activity of other neurons in the brain<sup>[28]</sup>.



**Figure 3:** Diagrammatic representation of the dopaminergic system in human brain (Eva H Telzer, 2015)

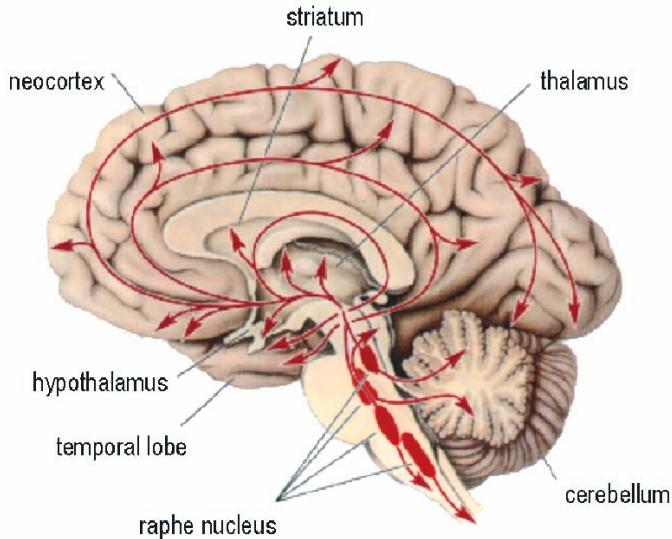
In the motor system, dopamine is involved in the control of movement and coordination<sup>[7]</sup>. Neurons in the substantia nigra (SN) region of the midbrain produce dopamine and project to the basal ganglia, where they modulate the activity of neurons involved in motor planning and execution<sup>[29]</sup>.

Dysfunction of the dopaminergic system in the midbrain can lead to movement disorders such as Parkinson's disease. In the reward system, dopamine is involved in the processing of rewarding stimuli, such as food, sex and drugs of abuse<sup>[8]</sup>.

Dopamine neurons in the ventral tegmental area (VTA) of the midbrain project to several areas of the brain, including the prefrontal cortex and nucleus accumbens (NA), where they modulate the activity of neurons involved in reward processing and addiction. In addition to motor control and reward processing, the dopaminergic system is also involved in several other functions, including cognitive control, attention and mood regulation. Dysfunction of the dopaminergic system has been implicated in a variety of psychiatric and neurological disorders, including schizophrenia, depression, addiction and attention deficit hyperactivity disorder (ADHD)<sup>[30,31]</sup>.

#### **4.2.2 Serotonergic system**

The serotonergic system is a group of neurons in the brain that produce and use the neurotransmitter serotonin. Serotonin is involved in a wide range of functions in the brain, including the regulation of mood, appetite and sleep. The serotonergic system is comprised of several different neural pathways that project to different areas of the brain. The most well-known pathway is the raphe nuclei in the brainstem, which sends serotonin to various regions of the brain<sup>[32]</sup>. Overall, the dopaminergic system plays a crucial role in regulating many aspects of brain function and behavior and its dysfunction can lead to a wide range of neurological and psychiatric disorders. One of the primary functions of the serotonergic system is the regulation of mood. Serotonin has been implicated in the pathophysiology of depression and anxiety and many antidepressant medications work by increasing serotonin levels in the brain<sup>[33]</sup>.



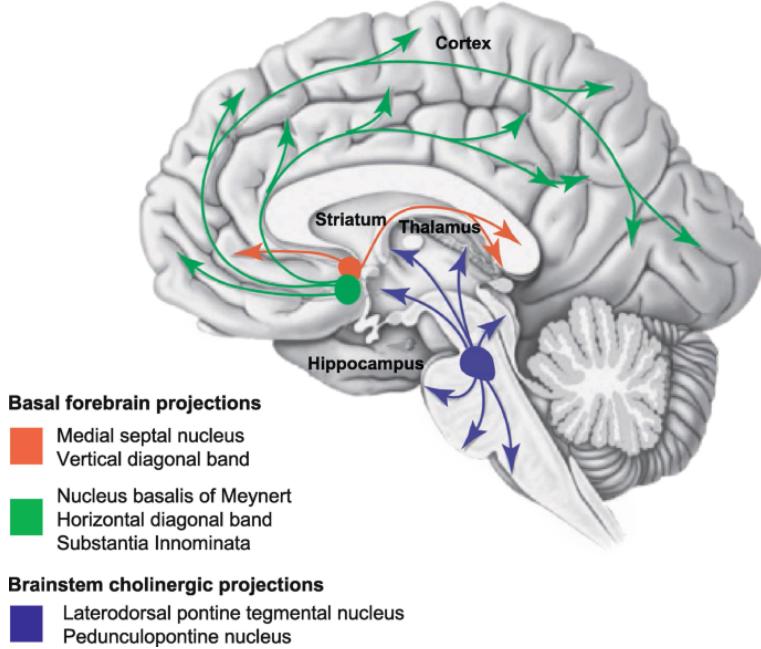
**Figure 4: Diagrammatic representation of the serotonergic system in human brain (J Borg, 2007)**

The serotonergic system is also involved in the regulation of appetite and digestion. Serotonin is released by specialized cells in the gut in response to food, and can affect feelings of satiety and fullness. In addition to these functions, the serotonergic system is involved in several other processes, including the regulation of sleep, pain and cognitive function<sup>[35]</sup>. Dysfunction of the serotonergic system has been implicated in a variety of psychiatric and neurological disorders, including depression, anxiety, migraine and irritable bowel syndrome (IBS)<sup>[9]</sup>. Overall, the serotonergic system plays a crucial role in regulating many aspects of brain function and behavior and its dysfunction can lead to a wide range of neurological and psychiatric disorders.

#### 4.2.3 Cholinergic system

The cholinergic system is a group of neurons in the brain that produce and use the neurotransmitter acetylcholine. Acetylcholine is involved in a wide range of functions in the brain, including learning, memory and attention<sup>[37]</sup>. The cholinergic system is comprised of several different neural pathways that project to different areas of the brain.

The most well-known pathway connects the basal forebrain, which sends acetylcholine to various regions of the cortex. One of the primary functions of the cholinergic system is the regulation of cognitive function, including attention, learning and memory<sup>[39,40]</sup>. The loss of cholinergic function has been implicated in the pathophysiology of Alzheimer's disease<sup>[10,36]</sup> and other forms of dementia.

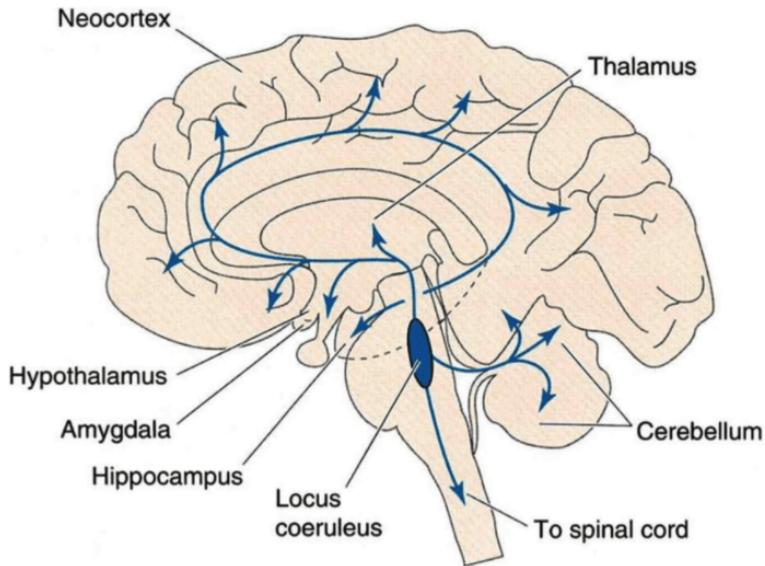


**Figure 5:** Diagrammatic representation of cholinergic system in human brain (Daniel Bertrand, 2020).

Cholinergic function has been implicated in the pathophysiology of Alzheimer's disease<sup>[10,36]</sup> and other forms of dementia. The cholinergic system is also involved in the regulation of movement and sensory processing. Acetylcholine is released by motor neurons in the spinal cord and at the neuromuscular junction, where it helps to activate muscle contractions<sup>[38]</sup>. In the sensory system, acetylcholine is involved in the processing of visual information in the retina<sup>[11]</sup>. In addition to these functions, the cholinergic system is involved in the regulation of autonomic function, including heart rate and digestion. Dysfunction of the cholinergic system has been implicated in a variety of neurological and psychiatric disorders, including Alzheimer's disease, Parkinson's disease, Schizophrenia, and attention deficit hyperactivity disorder (ADHD)<sup>[41,42,43]</sup>.

Overall, the cholinergic system plays a crucial role in regulating many aspects of brain function and behavior, and its dysfunction can lead to a wide range of neurological and psychiatric disorders.

#### 4.2.4 Noradrenergic system

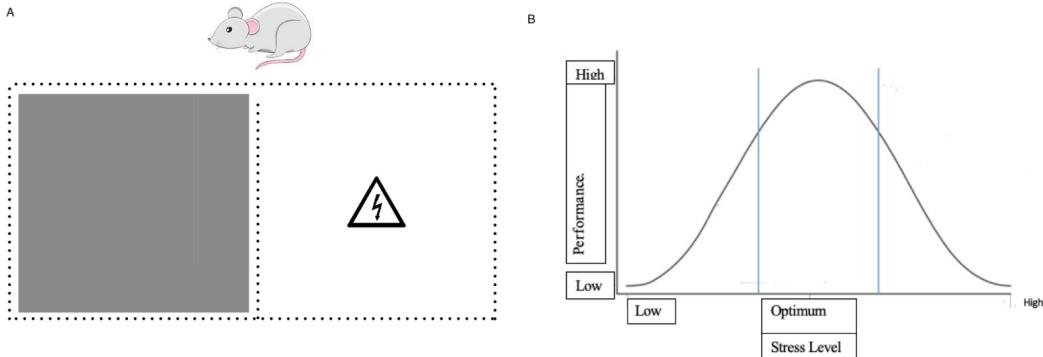


**Figure 6: Diagrammatic representation of nor-adrenergic system in human brain (Aston and Jones, 2005)**

The noradrenergic system is a group of neurons in the brain that produce and use the neurotransmitter norepinephrine (also known as noradrenaline). Norepinephrine is involved in a wide range of functions in the brain, including attention, arousal and stress response<sup>[44,45]</sup>. The noradrenergic system is comprised of several different neural pathways that project to different areas of the brain. Most of the pathways originate in the Locus Coeruleus in the brain stem which controls the release of noradrenaline in various regions of the brain. One of the primary functions of the noradrenergic system is the regulation of attention and arousal which can be seen in the form of physiological responses like pupil dilations<sup>[5]</sup>. Norepinephrine is released in response to novel or salient stimuli and can increase alertness and vigilance. Dysfunction of the noradrenergic system has been implicated in attention deficit hyperactivity disorder (ADHD) and other attention disorders<sup>[46]</sup>.

The noradrenergic system is involved in the regulation of mood and stress response. Norepinephrine has been implicated in the pathophysiology of depression and anxiety and many antidepressant and anxiolytic medications work by altering norepinephrine levels in the brain<sup>[47,48]</sup>. In addition to these functions, the noradrenergic system is involved in the regulation of autonomic function, including heart rate and blood pressure<sup>[49]</sup>. Dysfunction of the noradrenergic system has been implicated in a variety of neurological and psychiatric disorders, including PTSD, addiction and sleep disorders. Overall, the noradrenergic system plays a crucial role in regulating many aspects of brain function and behavior and its dysfunction can lead to a wide range of neurological and psychiatric disorders.

## 5 Yerkes Dodson Law



**Figure 7:** A)Yerkes Dodson experiment on Japanese dancing mouse(Yerkes and Dodson, 1908).B)The Yerkes Dodson Law (Aston and Jones, 2005)

Neuromodulators have been extensively studied to understand the underlying mechanisms of behaviors, of which noradrenaline is one of the most commonly studied neuromodulators because of its role in arousal and stress<sup>[50,51]</sup>. Over the years, studies investigating the role of the locus coeruleus (LC) and noradrenaline have hypothesized that increased arousal levels facilitate switching in behavior<sup>[5,51]</sup>. Through a classic study conducted by two American psychologists, Robert Yerkes and John Dodson showed the role of stress and arousal on performance<sup>[15]</sup>. In one of the experiments, the scientists used electric shocks to stimulate the mice while they moved between the experimental chambers[*Figure(6(A))*] and recorded their performance (i.e how quickly they learnt to avoid the shock chamber) while increasing the intensity of the shock. The results of these experiments were later theorised into what is today known as the Yerkes-Dodson Law[*Figure6(B)*]. The experiment showed that increase in the stress levels which leads to an increase in noradrenaline, increased the motivation and attention of the animals but upto a certain limit, beyond which the animals did not perform well. This study showed that the focus on a task is linked to the arousal levels.

Other more recent studies have found that the firing frequency for optimal task engagement is 3Hz while stimulation of the LC at 5Hz causes stress and anxiety.<sup>[52,53]</sup> Based on these findings we hypothesised that a stimulation of noradrenergic cells at 4Hz would increase arousal levels without causing anxiety and potentially promote switches in behavior.

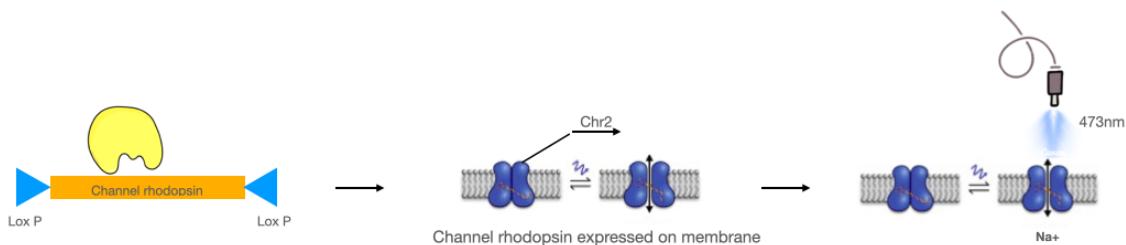
## 6 Technique

To study the role of noradrenaline in behavioral switching we expressed channelrhodopsin in the LC neurons to optogenetically activate them. Since channelrhodopsin is blue light activated membrane channel protein, we used laser of wavelength 473nm and varying stimulation protocols (according to experimental paradigm).

### 6.1 Optogenetics

Optogenetics is a field of neuroscience that involves the use of light to control the activity of specific neurons in the brain. This technique uses genetically modified cells that are made sensitive to light by expression of light sensitive proteins called opsins allowing to manipulate the neural activity in highly precise and targetted manner. The optogenetic approach typically involves introducing a gene encoding an opsin into specific neurons in the brain using viral vectors or transgenic mice. The opsin protein is then expressed in these neurons and can be activated by shining light of a specific wavelength onto the cells<sup>[54,55]</sup>. When the opsin protein is activated by light, it increases the open state probability of the channel leading to the changes in membrane potential and generation of an action potential [Figure8].

Optogenetics has revolutionized the field of neuroscience by enabling



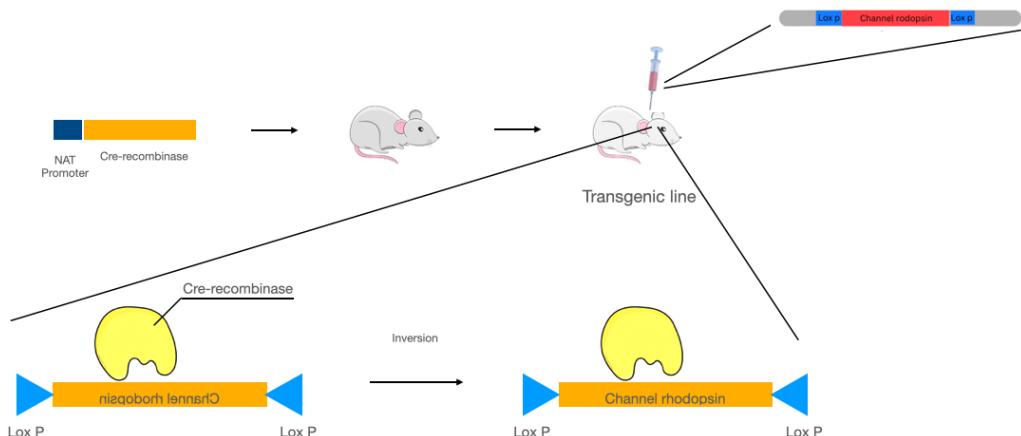
**Figure 8:** Illustration showing activation of channelrhodopsin on the membrane of neurons by shining light of a particular wavelength on it. (molecular devices.com)

researchers to manipulate and study the function of specific neural circuits in a highly precise and controlled manner.

It has been used to study a wide range of brain functions, including sensory processing, motor control, learning and memory and emotional regulation. It has also shown promise as a tool for developing new treatments for neurological and psychiatric disorders such as Parkinson's disease, depression, and epilepsy<sup>[56,57,58]</sup>.

## 6.2 Cre-Lox-P system

To study the effect of noradrenaline in behavioral switching, we targeted the brain stem nucleus called LC. To achieve this, we used the Nat-cre mouse line which was established through gene targeting technique involving the introduction of a DNA construct containing a Cre recombinase gene under the control of a tissue-specific promoter (NAT) into mouse embryonic stem cells. The presence of the tissue specific promoter guarantees the targeting of the noradrenergic cells in the brain. Cre recombinase is an enzyme derived from bacteria whose main func-



**Figure 9:** Illustration showing the interaction between cre-recombinase enzyme and lox sites.

tion is to recognise and cut specific DNA sequences, allowing for precise gene manipulation in cells that express Cre. To deliver the gene of interest i.e Channelrhodopsin (chr2) we used viral vectors with DNA constructs encoding for the channelrhodopsin 2 in our region of interest. We used AAV with DNA constructs encoding for channelrhodopsin 2 and locally injected it into the LC by stereotaxic surgeries.

The gene encoding for chr2 is initially flanked between two lox p sites in an inverted fashion and it acted upon by the cre-recombinase enzyme to make it functional<sup>[59]</sup>. This arrangement allows researchers to selectively activate or inhibit the activity of specific neurons in the brain in response to light stimuli<sup>[Figure 9]</sup>.

## 7 Methods

### 7.1 Experimental model and the subject details

All animal procedures were carried out with permission by and in accordance with the regulations of German Animal Welfare rules. In this study we used 8 males and 7 females (8 weeks old). To surgically target and stimulate the nor-adrenergic neurons Nat-Cre mice were used. AAV5-DIO-Chr2-YFP virus was injected bilaterally into the LC which was conditionally expressed upon the presence of Cre. The experiments were conducted 3 weeks after the expression of the channelrhodopsin in the targeted cells.

### 7.2 Method details

#### 7.2.1 Stereotaxic Surgery

Mice were anesthetized using a cocktail of Fentanyl, Midazolam and Medetomidine (FMM; 0.05/5/0.5 mg/kg; subcutaneously) and placed in a stereotaxic frame (Stoelting Co.). Mice were injected with ~450 nl/min of AAV5-DIO-cHR2-YFP bilaterally into the LC (coordinates from bregma : -5.4 anterior-posterior [A.P], ± 0.9 medial-lateral [M.L], -3.7 dorsal-ventral [D.V]). The viral injections were delivered using a 5  $\mu$ l hamilton syringe attached to the microsyringe and pump. The needles were left in place for 10 minute after infusion to minimize backflow of the virus upon withdrawal of the needle. Followed by the injection, the mice were then implanted with fibre optic implants (coordinates from bregma: -5.4 anterior posterior [A.P], ± 0.9 medial-lateral [ML], -3.5 dorsal-ventral [DV]). After surgery the mice were given a mixture of Flumazenil and Atipamezol (0.5/2.5 mg/kg) for recovery from anesthesia. To minimize the postoperative pain, all mice received buprenorphine (0.2ml/20 g mice, subcutaneously). The mice were given a recovery period of 3 weeks to allow sufficient time for viral expression before the experiments were performed.

### **7.2.2 Videography and movement analysis**

FLIR Blackfly camera was used to record the videos during experiments and MATLAB 2020a was used to analyse the data obtained. In the open field setup, the animals were tracked using Mouse Activity 5 and the facial and pupillary movements were determined using Facemap. In the VBA setup the VBA software as used to determine the distance of the mouse from the burrow and the pupil size was extracted using facemap. In the running wheel, to get the motion of the wheel, we used motion SVD calculator in Facemap.

## **7.3 Analysis and statistics**

All data was tested for normality by plotting the distributions. For normally distributed datasets we employed the paired ttest(ttest in MATLAB). For multiple measures from the same animal we used repeated measures analysis of variance(AnovaRM in python). Information about the sample size can be found on the figure legends.

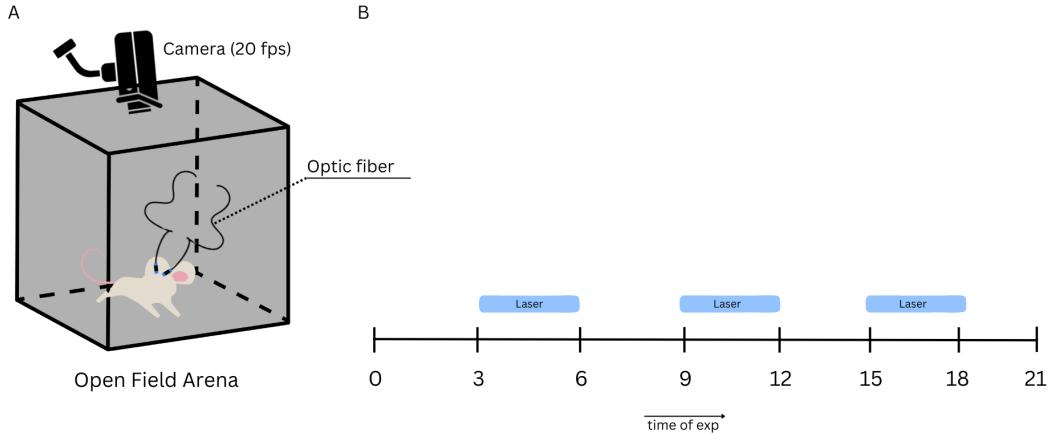
## 8 Behavioral Experiments

### 8.1 Optogenetic activation of the LC-NE neurons in the OFT

The day before testing, all mice were acclimated to the open field arena (40 cm by 40 cm) for 10 minutes. To determine the optimal stimulation frequency that could promote behavioral switching without causing anxiety in the mice, they were placed in the centre of the arena while receiving photostimulation at 4 Hz. The behavior was constantly video recorded throughout the session. The centre of the arena was defined as 55 % of the total arena area. During the 21-minute recording, the mice were allowed to roam freely in the arena while the LC neurons were stimulated in 3-minute time bins, alternating between stimulation and non-stimulation trials<sup>[12]</sup>.

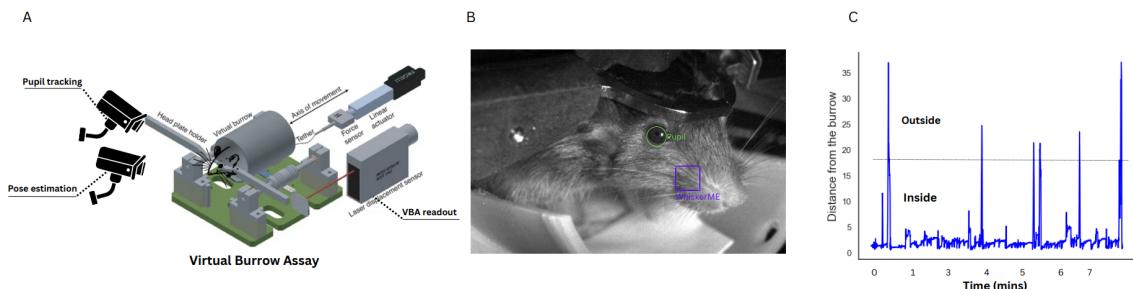
#### TONIC STIMULATION

**4Hz Stimulation:** The fibres on the head of the mice were connected to the optic cables which were connected to the laser and the animals were individually put at the centre of the arena. To trigger the camera and laser Doric Neuroscience Studio software V was used. The animals were stimulated with a frequency of 4Hz ( $n = 8$ ) in 3 minute time bins with alternating stimulation and non stimulation periods. The videos obtained from every animal were analysed using MATLAB scripts(modified **Mouse Activity 5 code**<sup>[13]</sup>). The analysis involved tracking the animals while they were exploring the arena and determining the distance covered along with speed of the animals during the stimulation and non-stimulation trials. Since we want to determine an optimal frequency that would promote behavioral switching without making the animals anxious, we also determined the time spent by the animals at the centre of the arena.



**Figure 10:** (A)Depiction of experimental setup<sup>[15]</sup>.The mouse is freely moving and imaged with a camera operating at 20fps.(B)The experimental paradigm with 3 min time bins. The blue shades depict the 3 min trials during which the mice were stimulated using the laser( $\lambda=473\text{nm}$ )

## 8.2 Optogenetic Activation of LC-NE neurons in VBA setup

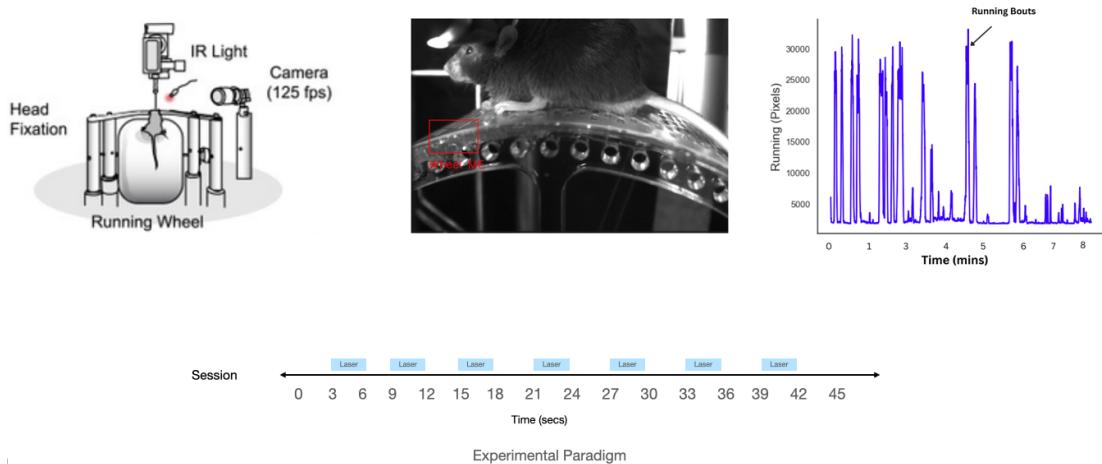


**Figure 11:** (A)Depiction of Virtual Burrow Assay with a head fixed mouse.(B)Image of the mouse's face from the video stream.(C)Example plot of VBA trace when the mouse moves in a Virtual Burrow Assay<sup>[5]</sup>

In the VBA setuo the mice were head fixed and placed in a tube simulating a burrow on an airfloating platform. The mice could show exploratory bouts by moving out of the burrow (egress). All mice were habituated in the VBA setup<sup>[Figure11(A)]</sup> for 10 minutes on the first day of the experiment.

After the habituation the first set of experiments were conducted in the VBA setup where the noradrenergic cells were stimulated at 10 Hz for 2 secs to observe the pupil dilation and hence confirm the expression of the virus. [Figure 13(C)]. The VBA setup was also used to see the effect of 4 Hz stimulation of LC in a closed loop fashion. The optic cables were connected to the fibres on the head of the mouse and the laser was controlled in a closed loop with the egress behavior. When the mouse exits the burrow a feedback is generated and sent to the computer which triggers the laser ( $\lambda=473\text{nm}, \nu=4\text{Hz}$ ) for 5 seconds if the mouse crossed a certain threshold. In this way the closed loop paradigm allowed us to study the effect of optogenetic stimulation on behavior when coupled to egress. The sessions were video recorded using Flir Camera(fps: 20Hz) and analysed using MATLAB 2020a.

### 8.3 Optogenetic Activation of LC-NE neurons in running wheel setup



**Figure 12:** (A) Depiction of Running wheel setup with a head fixed mouse.(B) Image of the mouse on the running wheel(Wheel ME, Wheel motion energy).The red box indicates the region used to measure the motion energy.(C)Example plot of running trace when the mouse runs on the wheel.(D)Experimental paradigm with 3 min long stimulation periods. The blue shades depict the 3 min trials in which the mice were stimulated using the laser( $\lambda= 473\text{nm}$ )

The mice were head-fixed in the setup and placed on a cylindrical running wheel [*Figure 12(A)*] and habituated for 5 consecutive days (starting with 10 minutes on day 1 and gradually increasing the habituation time every day upto 60 mins on the last day of habituation).

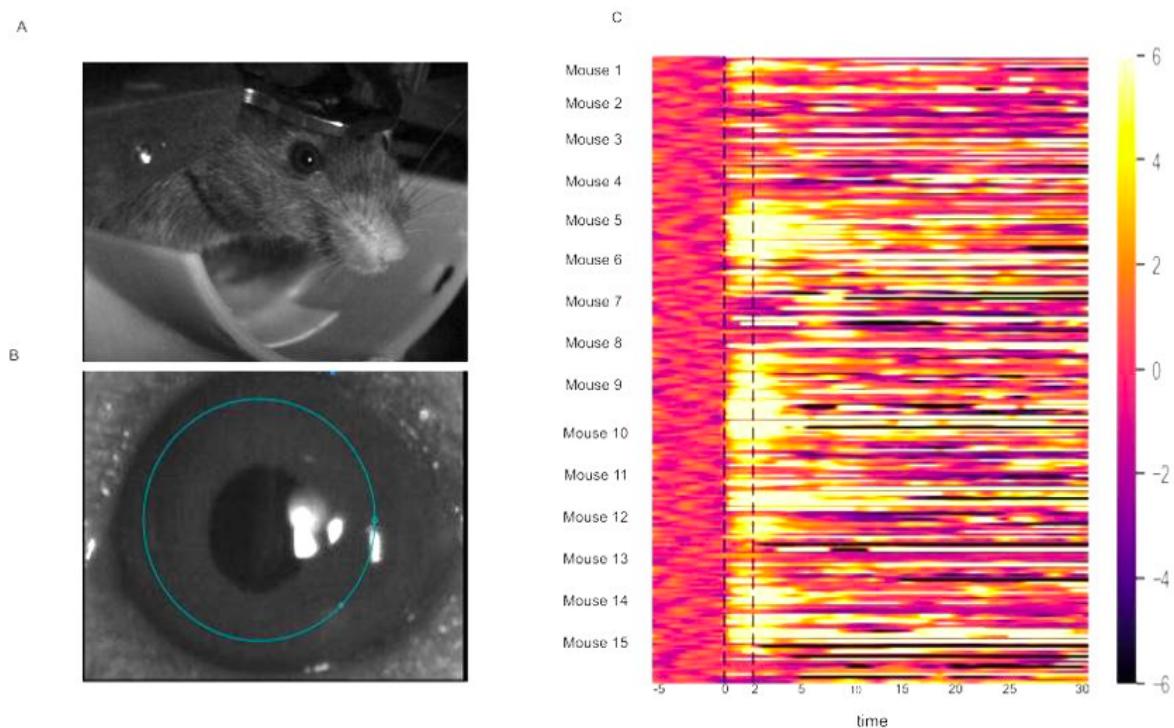
Once the habituation was over the experimental sessions were conducted, where the mice were placed on the running wheel with optic cables attached to the fibres on their heads. The LC neurons were stimulated with a frequency of 4Hz in 3 min time bins alternating between stimulation and non stimulation trials. The videos were obtained from every animal and analysed using customised MATLAB scripts in which the running and pupil traces were extracted for every trial to see the effect of stimulation [*Figure 21*].

## 9 Results

To conduct the study, first we had to check for the expression of channelrhodopsin in the mice. It is well established that an increase in LC activity leads to an increase in arousal and is seen as an increase in the pupil diameter. Based on this knowledge, we expected to see a pupil dilation as a result of stimulating the nor-adrenergic cells.

### 9.1 Screening mice for the study

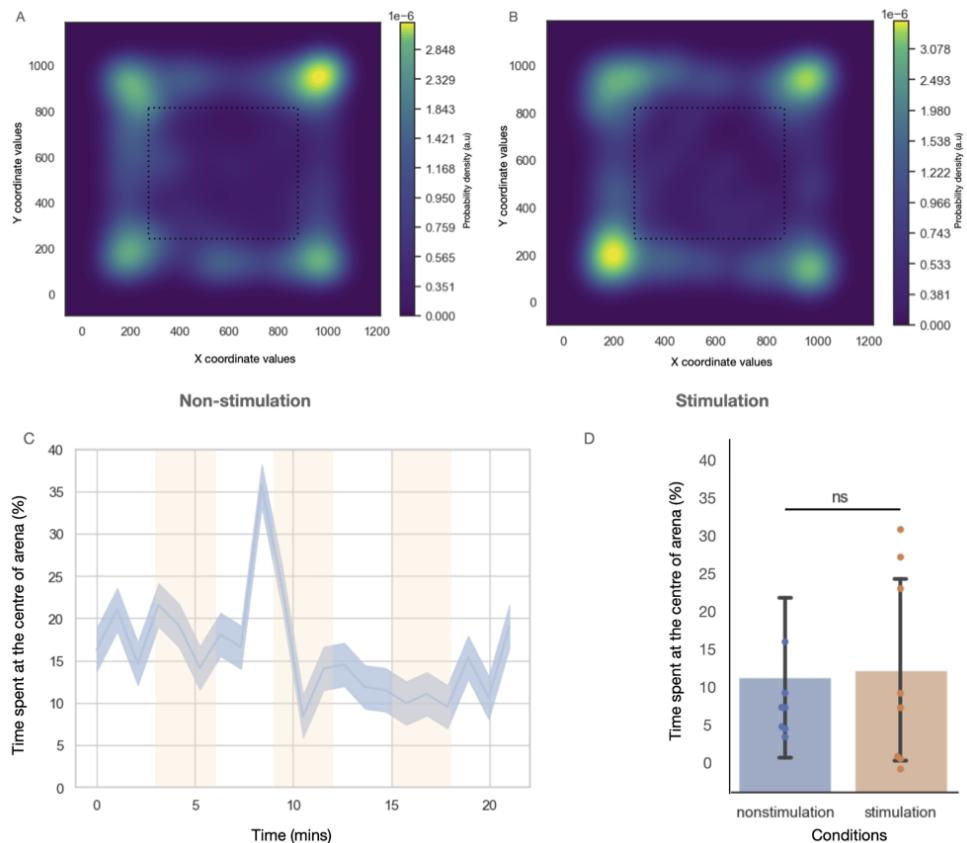
I did stereotaxic surgeries<sup>[Supl.Fig[1]]</sup> targeting the LC in 15 mice( $M = 8, F = 7$ ). To check the expression of the virus in the mice, we used the VBA setup, where the mice were placed inside the burrow and stimulated at 10Hz for 2 secs in 30 sec trials.



**Figure 13:** (A) Virtual Burrow Assay setup: the mice were placed in the burrow and their pupil was recorded while being stimulated at 10Hz. (B) The pupil size extracted using facemap. (C) Heatmap showing pupil dilation during the 2 sec stimulation of the LC.

Analysis of the change in pupil size during the stimulation period resulted in 8 out of 15 mice showing strong dilations. Consequently these mice were used in further experiments[*Figure 13(C)*].

## 9.2 Stimulating the nor-adrenergic cells at 4Hz did not induce anxiety



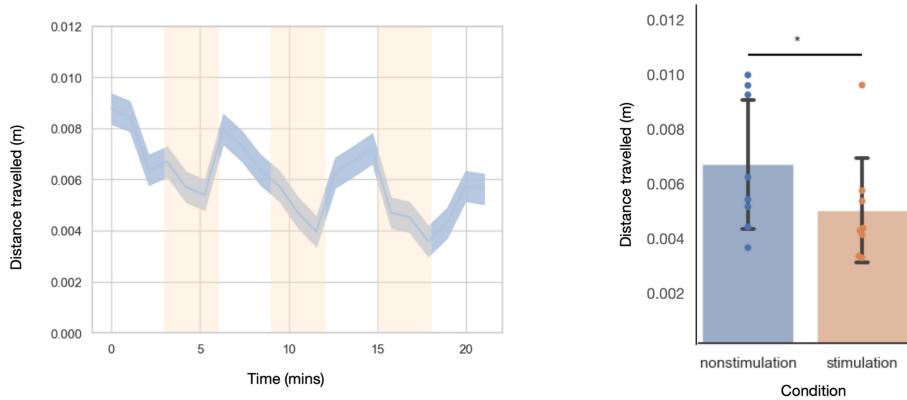
**Figure 14:** A)Probability heatmap showing the amount of time spent by the animals in the arena during non-stimulation trials.B)Probability heatmap showing the amount of time spent by the animals in the arena during stimulation trials.Line plot(C) and Bar plot(D) showing the time spent at the centre of the arena during stimulation and non-stimulation periods.The orange shades depict the stimulation periods

To study the effect of 4Hz tonic stimulation of noradrenergic cells on anxiety levels, the mice were placed in an open field and recorded for 21 minutes [*Figure10(B)*]. It is well known that increase in anxiety makes mice avoid the centre of an OFT arena . Therefore, we quantified the time spent by the mice in centre of the arena during 4 Hz stimulation of LC was not significantly different during the stimulation and non-stimulation periods[*Figure14(A,B)*] indicating that the LC stimulation at 4Hz did not induce anxiety in the OFT. To quantify the findings we did a student's t test on the amount of time spent during the stimulation and non stimulation conditions ( $n= 8, p= 0.25$ ).[*Figure14(D)*]

### **9.3 Tonic stimulation of the LC decreased locomotion in the open field**

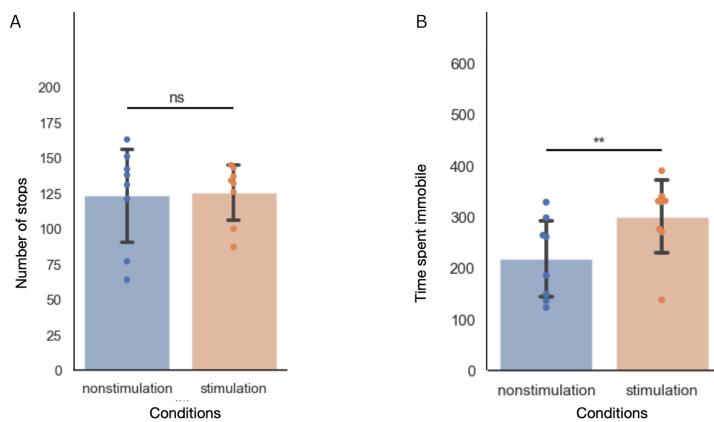
To study the effect of 4Hz stimulation of the nor-adrenergic cells on locomotion we analysed the movement of the mice in the arena during the stimulation and non-stimulation periods. We found that the mice travelled significantly less during the stimulation periods compared to the non-stimulation periods.[*Figure15*] To quantify the findings, we used a student's t test on the distance travelled the stimulation and non stimulation conditions. ( $n=8, p=0.024$ ) [*Figure15(B)*]

To further quantify different aspects of freely moving we analysed how often and for how long the mice were stopping in the arena during the stimulation and non stimulation periods. The data showed that there was no significant difference between the number of stops[*Figure16(A)*] made by the mice during stimulation of noradrenergic cells but the length of the stops made by the mice was significantly longer during the stimulation trials compared to non-stimulation trials[*Figure16(B)*]. Indicating that the mice stayed immobile for longer once they stopped moving.



**Figure 15:** A) Line plot showing the distance travelled in the arena during stimulation and non stimulation periods. The orange shades represent the stimulation trials. B) Bar plot representing the significance distance travelled during stimulation and non stimulation conditions.

In the next step, we introduced a food pellet and a novel object in the open field arena to study whether the animals spent more or less time in the area around the object and food. Clearly, a decrease in the time spent around the areas during the stimulation periods would indicate that the mice were more prone to switch their behaviors. These set of experiments were performed at 2Hz, 4Hz and 5Hz to compare the effect of LC stimulation with different frequencies.



**Figure 16:** A) Bar plot showing the number of stops made by the mice in the open field arena during stimulation and non stimulation periods. B) Bar plot representing the time spent immobile in the open field arena during the stimulation and non stimulation periods.

We did not observe any significant difference in the time spent at the centre in either of the frequencies. To study the effect of different stimulation frequencies on the movement and locomotion, we analysed the distance travelled by the mice in the arena. We concluded that the mice travelled less when the LC neurons were stimulated at higher frequencies (4hz, 5hz) [*Suppl.Fig(29,30)*] observed in previous experiments.

#### **9.4 Tonic stimulation at 4Hz had a no effect on the egress behavior and did not induce egress behavior**

We used calibrated closed loop optogenetics to study the effect of tonic stimulation of the nor-adrenergic cells during the egress behavior. Closed loop optogenetics allows targeted control of neural activity coupled to an animal's behavior.

In this part of the study we investigated three main conditions:

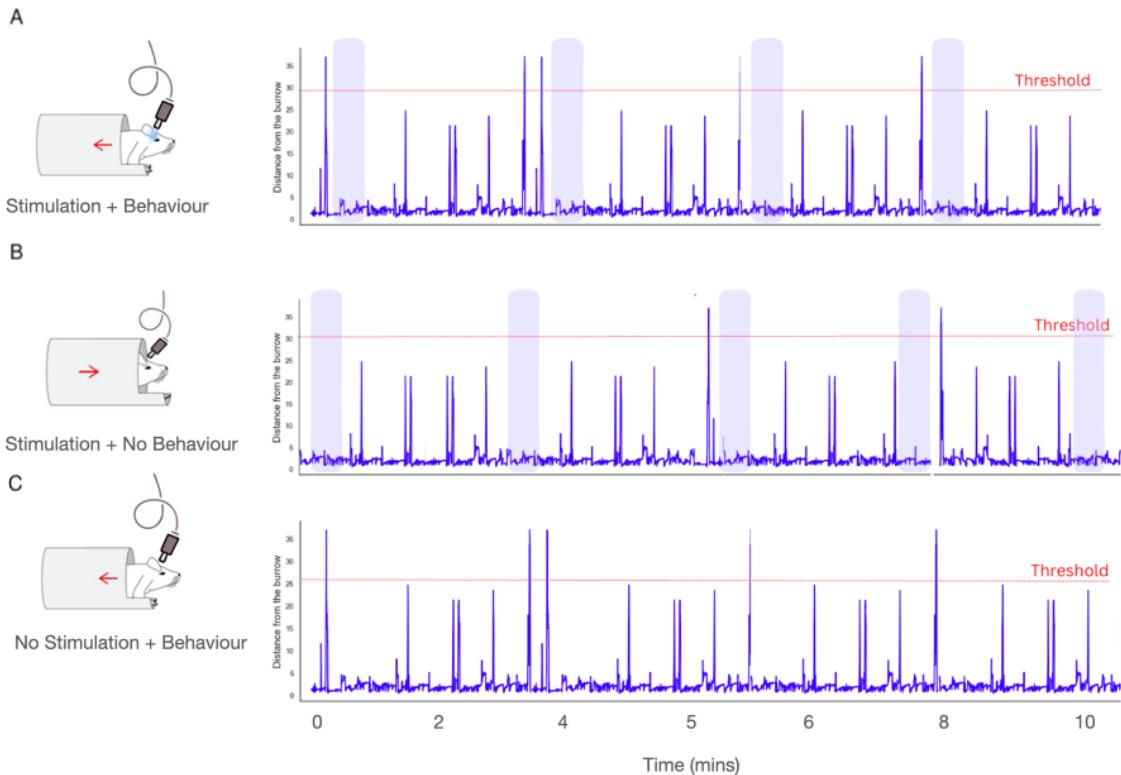
**1) Behavior + Stimulation:** The laser was triggered when the mice egressed. This suggests that when the distance of the mouse from the burrow crossed a particular threshold (pre-defined) and egress behavior was detected and the laser was triggered and consequently the LC-NE neurons were activated. The aim of this condition was to see if stimulation of the LC neurons while the mice egressed has an effect on the behavior i.e if it prolonged or shortened the behavior itself[*Figure17(A)*].

**2) No Behavior + Stimulation:** The nor-adrenergic cells were stimulated in the second experiment at the exact same time points when the mice had egressed previously in the first experiment. The aim of this condition was to see if stimulation of the LC neurons induced a behavioral response independent from the behavioral state of the animal[*Figure17(B)*].

**3) Behavior + No Stimulation:** There was no stimulation in this condition, however the timepoints recorded in egress was detected using the same threshold as above[*Figure17(C)*]. This condition serves as a control egress in which there is no stimulation even though the mice showed behavior.

This condition would allow us to compare the previous condition in which the laser was activated along with behavior to egress events without stimulation.

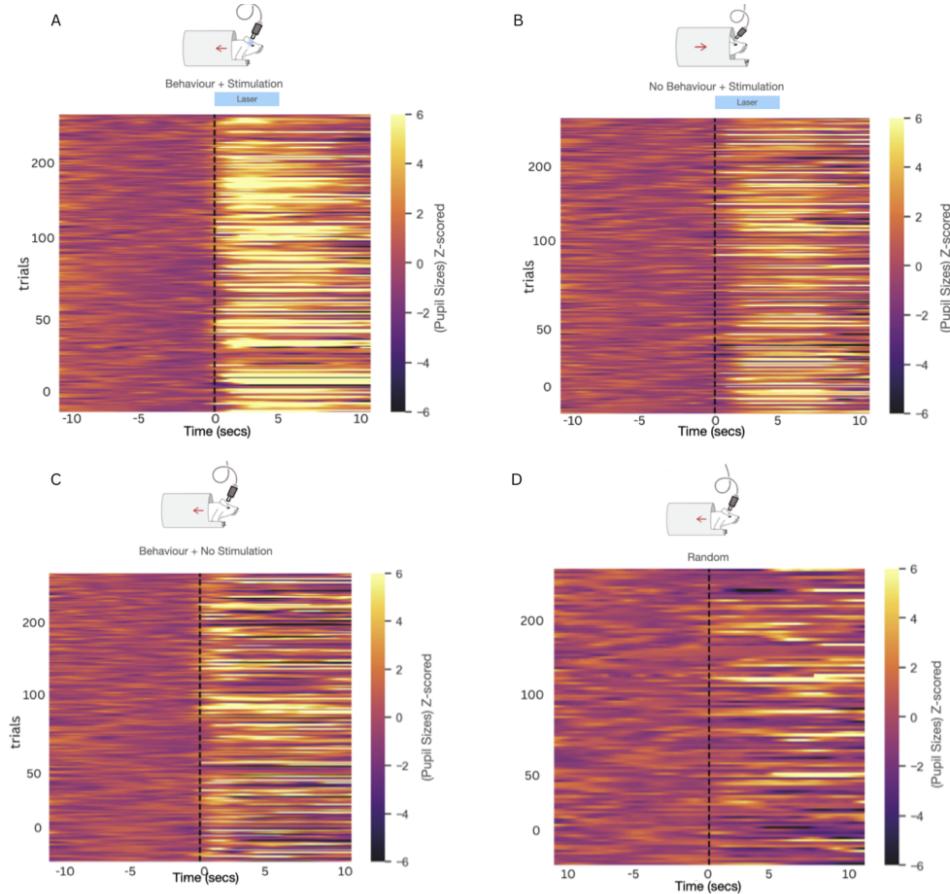
We also included another control condition in which random time points were picked from the recordings to see if there was a general trend in the behavior.



**Figure 17:** A) Behavior + stimulation: Condition in which the laser was on when egress was detected. B) No Behavior + Stimulation: Condition in which the laser was on irrespective of behavior. C) No Stimulation + Behavior: Condition in which the laser was off even though the egress behavior was detected.

To confirm that the nor-adrenergic cells were successfully stimulated in this part of the experiment, we extracted the pupil sizes for 10 secs before and after laser onset.

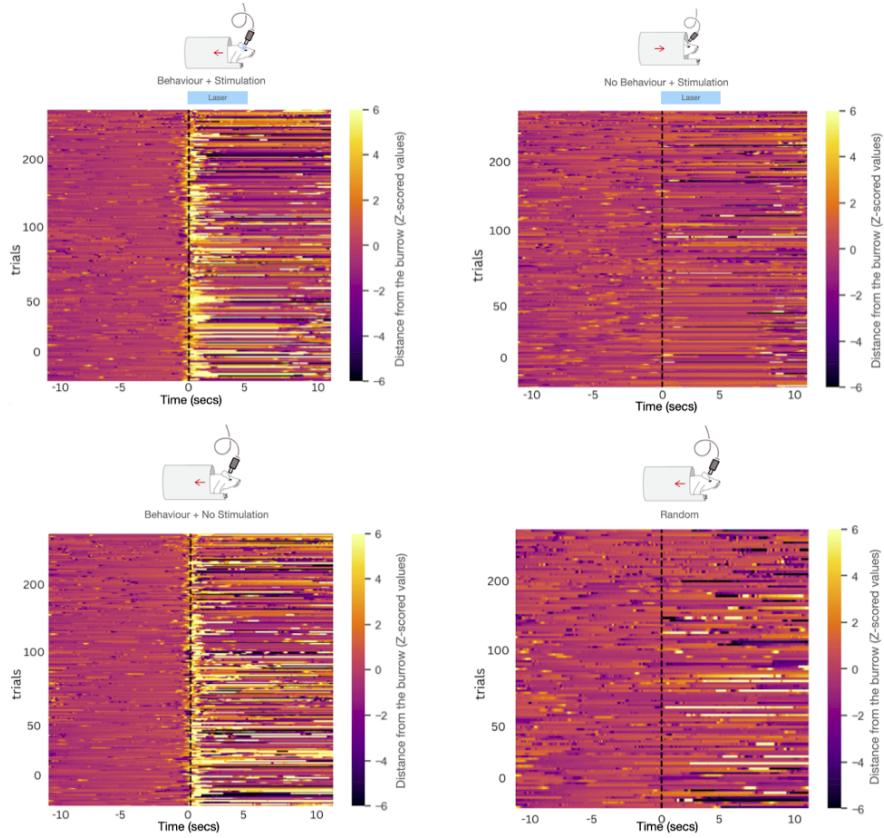
We observed a very strong dilation of pupil during the **Behavior + Stimulation** and **No Behavior + Stimulation** conditions which was expected, since the stimulation of nor-adrenergic cells leads to pupil dilations.<sup>[5]</sup> Dilation was also observed when the mice egressed and the



**Figure 18:** (A)Behaviour + Stimulation: Heat map of pupil dilation for all trials in which the laser was triggered when the mice made an egress bout.(B)No Behaviour + Stimulation: Heat map of pupil dilation for all trials in which the laser was triggered at the same time points when the mouse had made an egress bout previously.(C)Behaviour + No Stimulation: Heat map of pupil dilation for all trials in which the mouse made an egress bout but there was no stimulation.(D)Random: Heat map of pupil dilation from randomly picked trials.

laser was off (**No stimulation + Behavior**) because physical movement stimulates the sympathetic nervous system leading to increase in the arousal levels as seen by an increase in pupil size<sup>[5,12]</sup>.

From the plots we concluded that pupil dilation was higher in the condition where egress and stimulation were coupled, compared to the condition in which there was no stimulation even though the mice egressed. This suggests that increase in arousal resulting from stimulation of the noradrenergic cells increases the dilation of pupil.

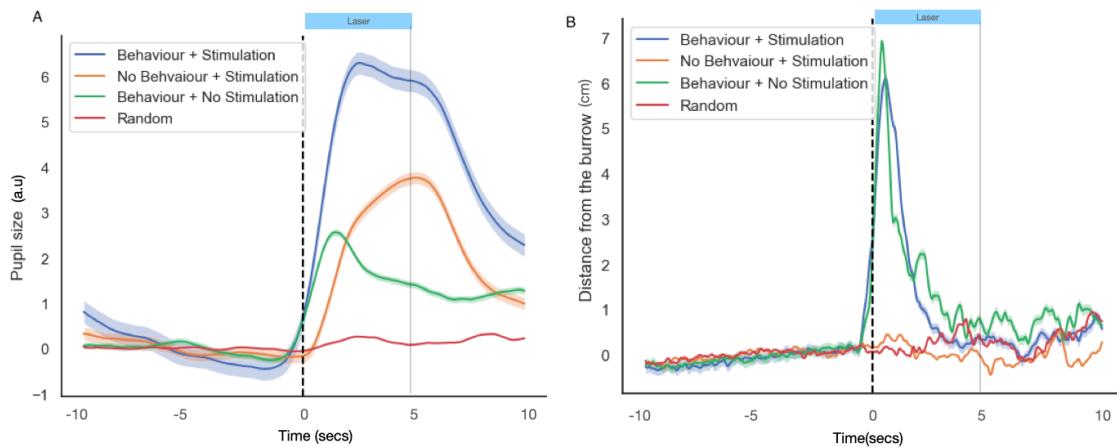


**Figure 19:** (A) Behavior + Stimulation: From the heatmap we can see that the behavior was slightly prolonged when the mice were stimulated during the behavior.(B) No Behavior + Stimulation: No behavior was induced as a result of LC stimulation suggesting that activation of nor-adrenergic cells do not promote a behavior.(C) Behavior + No Stimulation: The heatmap show that the mice egressed but there.(D) Random time points were selected from the session to check if there was a general trend in the egress behavior as a control measure.

To study the effect of tonic stimulation of the LC (4Hz) on egress behavior we extracted the distance of the mouse from the burrow, 10 secs before and after the onset of stimulation.

We observed that the stimulation of the nor-adrenergic cells did not have an effect on the egress [Figure 19(A)]. Interestingly, there was no egress when we only stimulated the nor-adrenergic cells (No Behavior + Stimulation) [Figure 19(B)]. This suggests that tonic stimulation of the nor-adrenergic cells do not promote behavior. To study the effect of the stimulation of the noradrenergic cells we compared the condition 1 (Behavior + Stimulation) in which the LC was stimulated when the mice egressed with the second condition (Behavior + No Stimulation) where the LC was not stimulated even though the mice egressed [Figure 20(A)].

To quantify the effect of stimulation on egress we calculated the areas



**Figure 20: (A)** Mean pupil size (animals= 5,sessions = 6) representing all four conditions.**(B)** Mean of the distance of the mouse from the burrow (animals= 5,sessions = 6)

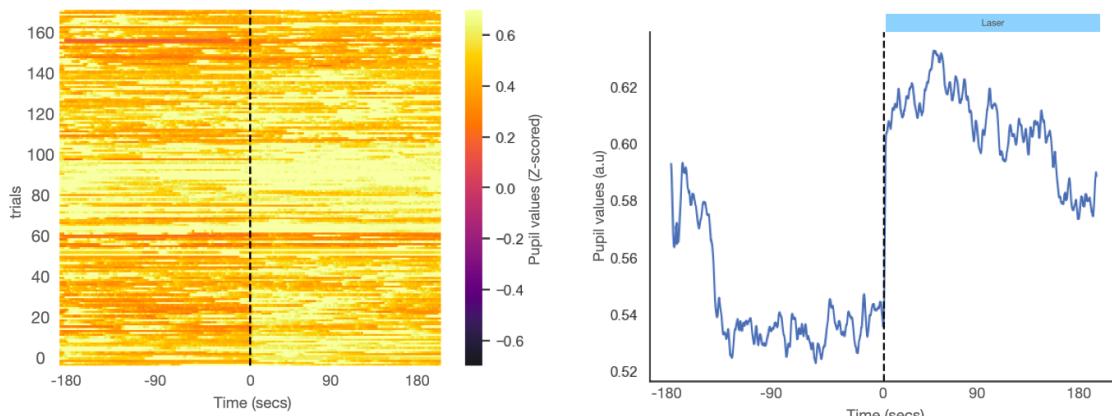
under the curves for two conditions. One, in which the LC neurons were stimulated when the mice egressed and the second condition in which there was no stimulation even though the mice egressed [Suppl. Fig 27].

Since data from both conditions were normally distributed, we first used a student's t-test to see if the difference in the areas was significant and this was followed by a repeated measures analysis of variance (rmANOVA). From the statistical analysis we confirmed that there was a significant difference between the areas under the curves representing pupil size [Figure 20(A)] for conditions 1 and 2 ( $F=17.03, p = 0.0145$ ).

The areas under the curve for the egress behavior were not significantly different ( $F=0.81, p=0.418$ ) [Figure 20(B)] suggesting that the stimulation of the noradrenergic cells at 4Hz was not enough to prolong the egress bouts. Only stimulating the noradrenergic cells in the absence of the behavior did not promote the egress indicating that LC doesn't have a direct influence on the egress behavior which falls in line with other studies<sup>[18]</sup>.

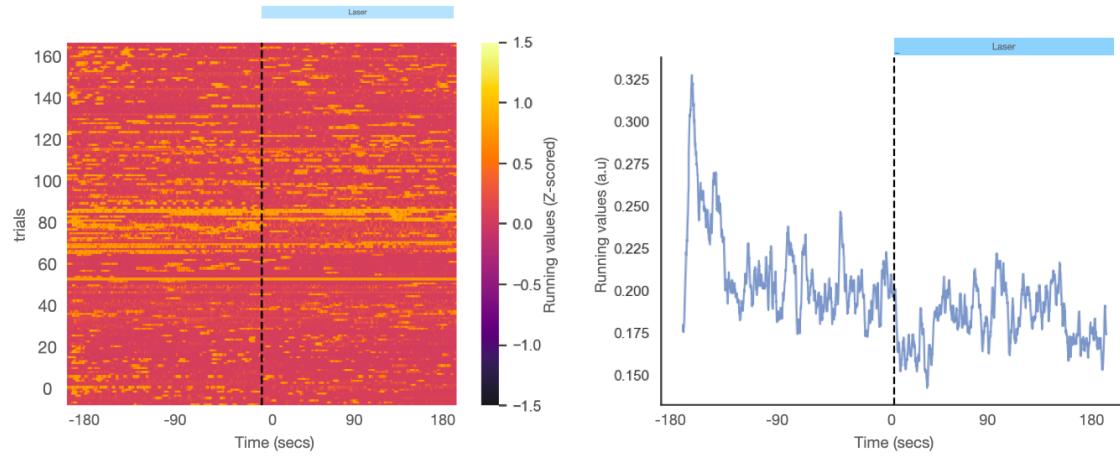
### 9.5 LC activation at 4Hz induced running after stimulation

To broaden our dataset of spontaneous behaviors we included another setup in which we could study the running behavior in head-fixed mice. To confirm that LC was being stimulated we checked the change in the pupil size at the onset of the stimulation. As we have already observed in previous experiments, the pupil size increased significantly when the LC was stimulated in the running wheel setup confirming that we were targeting the right place [Figure 21(B)].



**Figure 21:** (A) Combined heat map of all trials from all animals comparing the pupil size between the stimulation and non stimulation periods. (B) Line plot showing the change in pupil size after the onset of the stimulus.

However, we observed that there was no increase in running at the onset of stimulation but there was a significant increase in the running when the laser was turned off indicating an OFF response. [Figure 22(A)]



**Figure 22:** (A) Combined heat map showing the running behavior before and after the laser onset. The black boxes show the difference in the run traces at the beginning of the off periods and on periods. (B) Running trace showing the difference in the running behavior before and after the laser onset.

## 10 Discussion

To investigate the role of the neuromodulator noradrenaline in the modulation of spontaneous behaviors, I conducted localized targeting of noradrenergic neurons through viral injections. Given the established knowledge that increased activity of noradrenergic neurons leads to noradrenaline release and subsequently increases arousal, resulting in pupil dilation, I applied high-frequency stimulation to the locus coeruleus (LC) neurons to observe pupil dilation. The hypothesis was that successful targeting of LC neurons in mice would lead to a significant increase in pupil size following stimulation. Mice exhibiting pronounced pupil dilation were selected for further experiments. Previous studies have demonstrated that heightened LC activity induces anxiety and increases arousal, while some have proposed that it also facilitates behavioral switching. Building upon this hypothesis, we determined a frequency that could stimulate LC neurons without inducing anxiety. We monitored the mice in an open field arena while stimulating them at 4Hz. Our findings from the open field tests suggest that 4Hz stimulation did not induce anxiety in the mice, but it significantly reduced their movement within the arena. Compared to periods without stimulation, the mice traveled less during the stimulation periods and displayed longer periods of immobility. In the subsequent experiments, we introduced food and a novel object into the open field arena while the mice explored it, and stimulated them at different frequencies (2Hz, 4Hz, and 5Hz). We did not observe any significant increase in approach behavior towards the food or object, but the mice exhibited significantly reduced movement during high-frequency stimulation (i.e., 4Hz and 5Hz). Furthermore, employing closed-loop stimulation of the LC neurons in the virtual burrow assay (VBA) setup at 4Hz did not yield any significant effects on egress behavior. This indicates that stimulating noradrenergic neurons neither prolonged nor reduced the duration of an egress bout. Merely stimulating the noradrenergic neurons, regardless of the ongoing behavior, did not promote the behavior, suggesting that the LC does not directly control egress behavior.

However, a limitation of the VBA setup was that the mice experienced increasing stress as the number of sessions progressed, which hindered longer recordings. Additionally, stimulation of the LC neurons in the running wheel did not result in an immediate increase in running activity upon stimulation onset. However, an increase in running activity was observed when the laser was turned off. Based on the results obtained from the open field and running wheel experiments, it is clear that stimulating noradrenergic neurons at 4Hz decreases overall movement in the arena. The mice exhibited reduced travel distance and longer periods of immobility within the arena. However, as a potential avenue for future research, it would be fascinating to implement a closed-loop system in the open field, enabling us to observe the mice's behavior while simultaneously stimulating the LC neurons during their approach towards food and novel objects. In the running wheel setup, we could administer LC neuron stimulation when the mice initiate running and closely monitor their subsequent behavior. This approach would provide valuable insights into the impact of LC neuron activity on specific behavioral responses.

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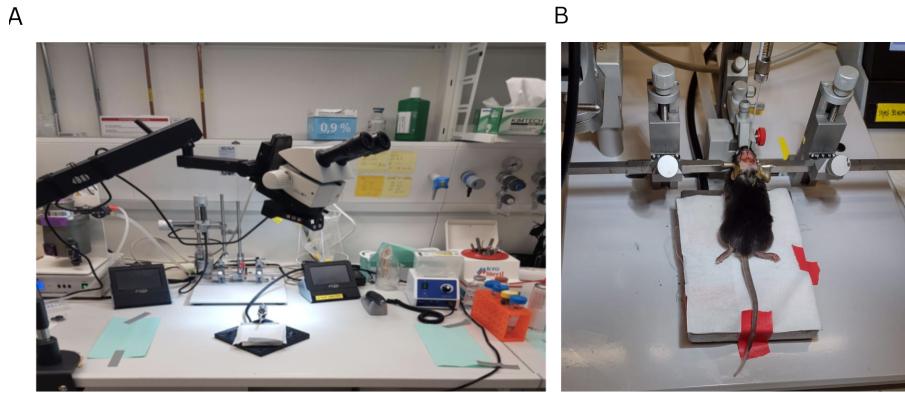
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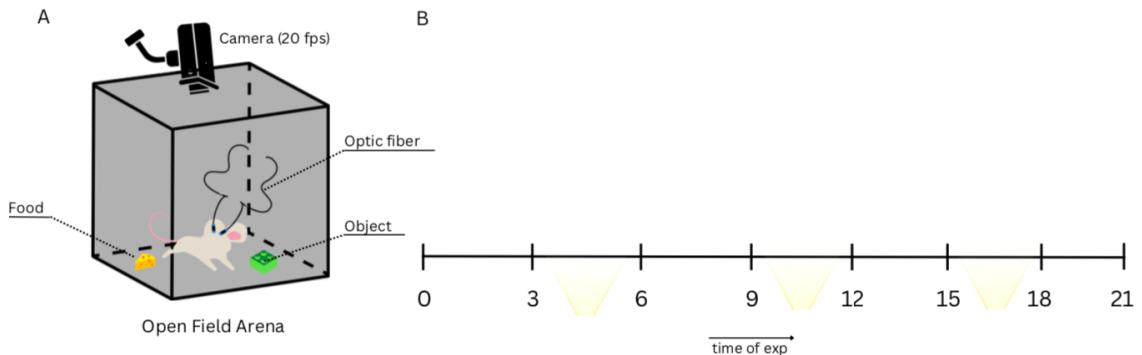
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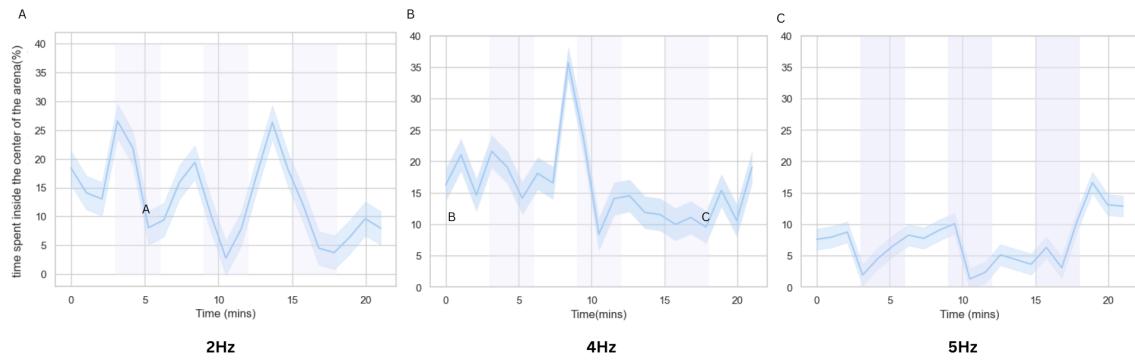
## 12 Supplementary



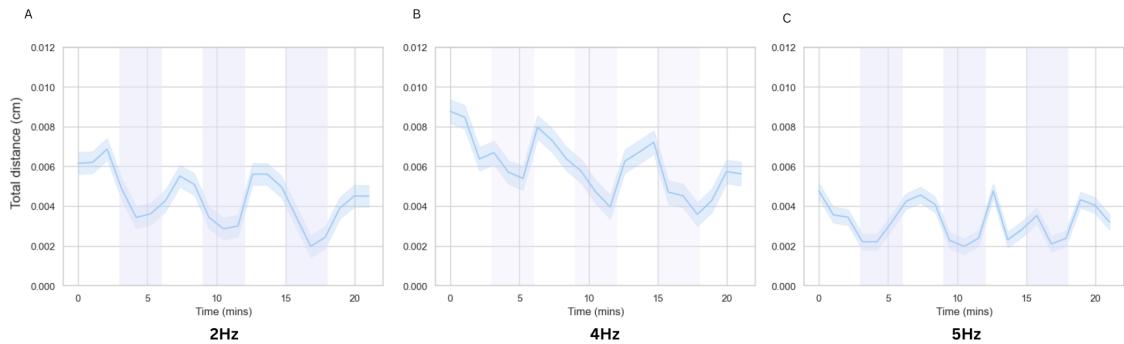
**Figure 23:** (A)Stereotaxic surgery setup used for targeting the LC.(B)Mouse placed on the surgery setup and fixed using the head-bars.



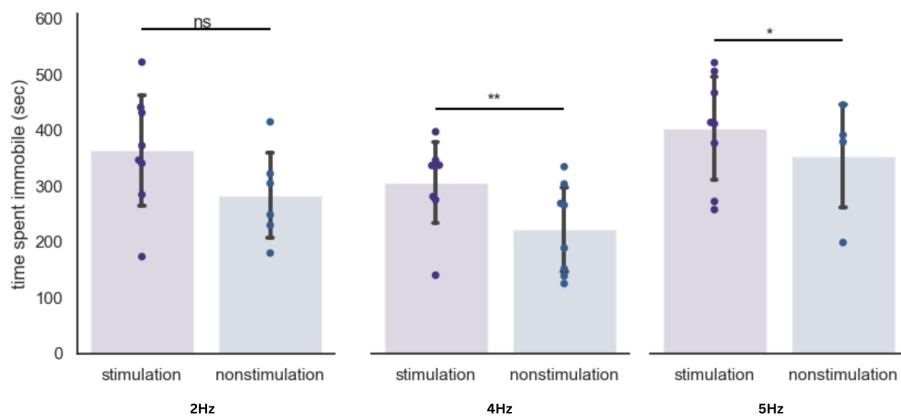
**Figure 24:** (A)Depiction of experimental setup.The mouse is freely moving in the arena with food and object placed at the corners and imaged by a camera operating at 20fps.(B)Experimental paradigm with 3 min time bins.The yellow shades depict the 3 min trials in which the mice were stimulated using the laser(473nm)



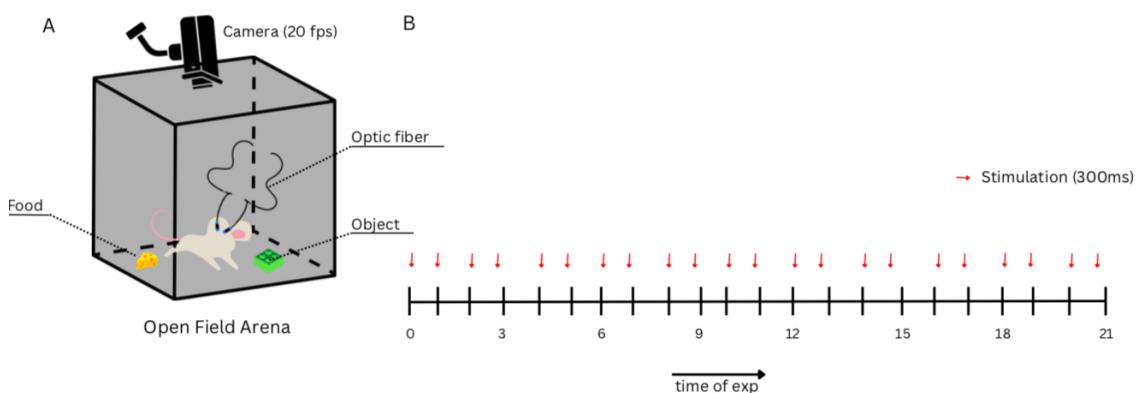
**Figure 25:** %Time spent at the centre of the arena during the whole experimental period at different frequencies(2Hz(A),4Hz(B) and 5Hz(C))



**Figure 26:** Plots showing the distance travelled during the whole experimental time at different frequencies(2Hz(A),4Hz(B),5Hz(C)).



**Figure 27:** Plots showing length of the stop-bouts made by the mice during stimulation at different frequencies(2Hz(A),4Hz(B),5Hz(C)).



**Figure 28:** (A)Depiction of experimental setup.The mouse is freely moving in the arena while being stimulated at 10Hz for the first 300ms every minute.(B)The experimental paradigm 21 minutes long with the red arrows indicating stimulations

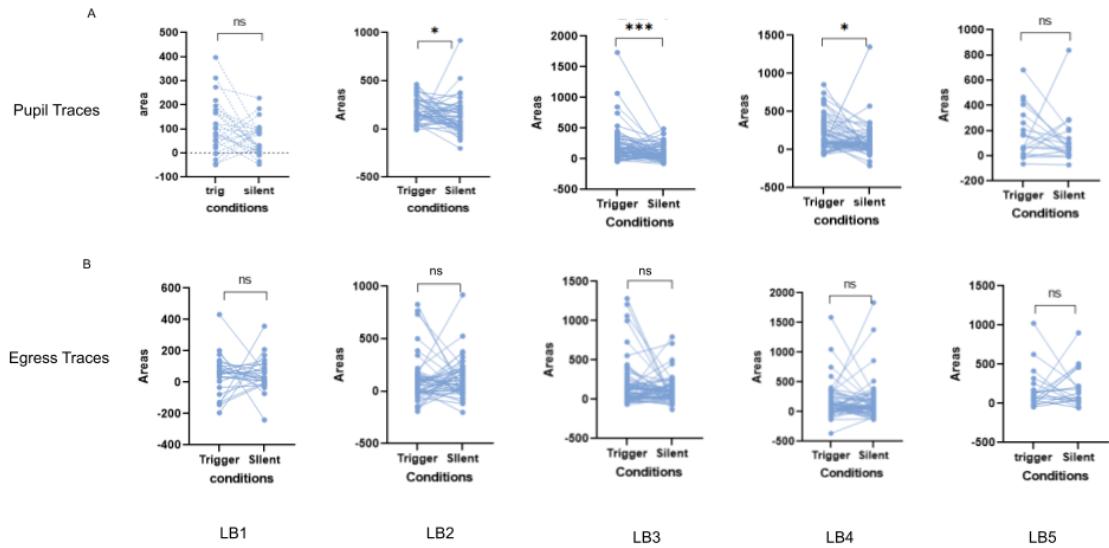


Figure 29: (A)Area under the curve plots (during trigger and silent conditions) for pupil traces.(B)Area under the curve plots (during trigger and silent conditions) for egress traces.